Plant Growth Regul (2017) 83:397–408
DOI 10.1007/s10725-017-0306-z

ORIGINAL PAPER

Tools to evaluate *Herbaspirillum seropedicae* abundance and *nifH* and *rpoC* expression in inoculated maize seedlings grown in vitro and in soil

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Received: 6 October 2016 / Accepted: 29 July 2017 / Published online: 2 August 2017

Abstract The plant growth promoting bacteria *Herbaspirillum seropedicae* is an important model to study biological nitrogen fixation and it is proposed as crop inoculants for grasses. In this study we developed new tools to evaluate the abundance of *H. seropedicae* SmR1 and its expression in planta and investigated the association of the plant growth promoting bacteria *H. seropedicae* with maize grown in sterile and nonsterile conditions. Maize seedlings (P30F53) were inoculated with *H. seropedicae* SmR1 and grown in vitro and in soil. The plants were sampled at 4, 7 and 10 (in vitro) or 14, 21 and 28 days after inoculation (soil). Using qPCR we quantified *H. seropedicae* DNA and measured *nifH, rpoC* and *hrcN* levels of bacterial transcripts. In vitro assay inoculated plants presented highest amount of DNA and transcript contents, not detected in control plants. *nifH* and *rpoC* gene expression were detected on roots of inoculated maize cultivated in both growth conditions. However, it was not possible to detect *hrcN* gene expression in maize roots cultivated in soil. TaqMan assay is species-specific for *H. seropedicae* DNA and *nifH* and *rpoC* transcript levels could be used to monitor *H. seropedicae* gene expression in planta in sterile and nonsterile growth conditions. We developed specific, reliable and efficient tools to monitor *H. seropedicae* DNA abundance and *H. seropedicae nifH* and *rpoC* expression in planta.

Keywords *Herbaspirillum seropedicae* · Plant–bacteria interaction · Maize · qPCR, RT-qPCR · Diazotroph · Plant-growth promoting bacteria

Introduction

Diazotrophic bacteria are able to fix atmospheric nitrogen by biological nitrogen fixation (BNF) process (Bhattacharjee et al. 2008). Some diazothrophs live in close association with plants and have the capacity to transfer the fixed nitrogen to the host (Chubatsu et al. 2012; Carvalho et al. 2014). Furthermore, they can be used to improve crop yield and quality (Chebotar et al. 2015; Berg 2009). Future biotechnological applications, such as an alternative for chemical fertilizers (Babalola 2010), increasing crop production or even conserving biodiversity (Germida et al. 1998; Stets et al. 2013), are processes that can be driven based on the advance of plant-diazotrophic bacteria association's knowledge. Plant response to inoculation depends on the plant genotype and the plant growth bacterial inoculant (Amaral et al. 2016; Alves et al. 2015). During plant–beneficial bacteria interaction, microorganisms modulate plant molecular responses (Vargas et al. 2012, 2014; Brusamarello-Santos et al. 2012; Berg 2009), and both partners are altered in response to interaction (Cordeiro et al. 2013; Tadra-Sfeir et al. 2015; Balsanelli et al. 2015).

Herbaspirillum seropedicae is an endophytic diazotrophic bacterium, which is able to colonize internal tissues of plants (Chubatsu et al. 2012). It belongs to β-*proteobacteria* class and colonizes mainly roots, stems and leaves of Poacea (Olivares et al. 1996; James

Electronic supplementary material The online version of this article (doi:10.1007/s10725-017-0306-z) contains supplementary material, which is available to authorized users.

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et al. 1997; Baldani et al. 1986). Assessing 21 strains of *Herbaspirillum* in two maize varieties, Alves et al. (2015) observed that *H. seropedicae* ZAE94 contributed to plant growth in greenhouse assay and its application as field inoculant increased maize yield up to 34% and can provide 37% of nitrogen plant demand by BNF. One of the best studied strain, *H. seropedicae* SmR1 (Monteiro et al. 2012) has its complete genome sequenced (Pedrosa et al. 2011). Recent work using *Setaria viridis* co-inoculated with two nitrogen-fixing bacteria, *H. seropedicae* SmR1 and *Azospirillum brasilense* FP2 revealed that the plant could incorporate the fixed nitrogen by BNF in its proteins. Additionally, the nitrogen fixed by the bacteria directly affects overall plant metabolism (Pankievicz et al. 2015). BNF process is mediated by nitrogenase enzymatic complex (Rees and Howard 2000), which is highly regulated at transcriptional and post-translational levels in *H. seropedicae* (Chubatsu et al. 2012). Nitrogenase metalloenzime is composed by two structural components and the smaller one, Fe-protein, is encoded by *nifH* gene (Rees and Howard 2000; Dixon and Kahn 2004; Peters et al. 1995; Machado et al. 1996). It was showed that *H. seropedicae nif* expression occur on and inside roots, leaves and stems of maize, rice, wheat and sorghum plants and *nif* gene expression in colonized plants suggests that infected tissues provide a suitable environment for BNF (Roncato-Maccari et al. 2003).

In bacteria that interact with eukaryotic hosts, the T3SS is the most common structural apparatus used to translocate effector proteins into the host cytoplasm (He et al. 2004). Analyses of the H. seropedicae SmR1 genome have indicated the presence of genes homologous to the T3SS, suggesting that it is present in this bacterium, and raising the possibility that it may be involved in its interaction with host plants (Monteiro et al. 2012). T3SS is one of the pathways by which some secreted proteins are exported across the inner and outer membranes in Gram-negative bacteria and its machinery is termed injectiosome. Therefore, injectiosome function is to deliver effectors across the bacteria and host membranes into the cytosol of host cells, where they may modulate a large variety of host cell functions, including immune and defense responses (Tseng et al. 2009; Desvaux et al. 2004; He et al. 2004). In a previous study, *H. seropedicae* dinitrogenase reductase (*nif*) and T3SS ATP synthase were identified by peptide mass fingerprint among 18 differentially accumulated proteins on proteome of inoculated maize roots 7 days after inoculation (DAI) (Ferrari et al. 2014). DNA-directed RNA polymerase subunit beta' (*rpoC*) is a constitutive gene that has been used as a reference gene for normalization in relative gene expression by RT-qPCR in *H. seropedicae* pure culture in the presence of sugarcane extract in the bacterial culture medium (Cordeiro et al. 2013).

A major problem related to inoculants is the survival of inoculated bacteria in the rhizosphere and the effective plant colonization (Stets et al. 2015). Regarding bacterial DNA presence in planta, quantitative PCR (qPCR) has been the method of choice to quantify plant growth promoting bacteria because it is fast and specific. PCR and qPCR have been used to identify *Azospirillum* isolates from rhizosphere and soil (Shime-Hattori et al. 2011; Baudoin et al. 2010) and to monitor *Azospirillum* colonization in wheat and maize (Stets et al. 2015; Couillerot et al. 2010, 2013). qPCR assay was developed using SYBR Green and HERBAS1 species-specific primers to quantify *H. seropedicae* DNA in inoculated maize roots (Pereira et al. 2014), however a hydrolysis probe assay present higher specificity comparing to a SYBR Green assay.

Herein we developed a hydrolysis probe qPCR tool to quantify *H. seropedicae* DNA in planta and RT-qPCR assays to measure *H. seropedicae* transcript levels in plant tissues to evaluate gene expression of a constitutive gene, DNA-directed RNA polymerase subunit beta' (*rpoC*), and two regulated genes, related to injectiosome apparatus T3SS (*hrcN*) and nitrogen fixation (*nifH*). Our aim was to develop new tools to address the abundance of *H. seropedicae* SmR1 and its expression (*nifH, rpoC* and *hrcN*) in planta in order to monitor the effective plant colonization by this bacterium in two maize growth conditions.

Materials and methods

Bacterial growth and experimental conditions

Herbaspirillum seropedicae strain SmR1, a spontaneous streptomycin resistant mutant of strain Z78 (ATCC 35893), was routinely grown in orbital shaker (120 rpm) at 30 °C in 30 mL NFbHPN medium supplemented with 5 mg L^{-1} malic acid (Klassen et al. 1997). The bacterial strains used for qPCR probe specificity assay were grown as previously described (Pereira et al. 2014). The optical density (OD) of bacterial cell cultures was measured at 600 nm using Hitachi U2910 Spectrophotometer (Tokyo, Japan). *H. seropedicae* SmR1 was inoculated in maize seedlings (Pioneer 30F53) cultivated in two distinct conditions:in vitro and in soil. Maize seeds were surface-sterilized in laminar flow by immersion in 70% ethanol for 5 min, followed by submersion in 2% sodium hypochlorite plus 2.5% Tween-20 solution for 30 min. Seeds were then rinsed 3 times with sterile distilled water, germinated on plates containing water-agar 0.8% (in vitro) or water moist filter paper (in soil) and stored at 25 °C in a dark chamber for 3 days. Three days old seedlings were inoculated with washed *H. seropedicae* SmR1 culture ($OD_{600}=1$; 10^8 cells of *H. seropedicae* SmR1 mL^{-1}) after dilution to 10^5 cells mL^{-1} (in vitro)

or 10^7 cells mL^{-1} (in soil) in sterile NFb malate medium without nitrogen source, for 30 min with constant agitation (80 rpm) (Balsanelli et al. 2010). For seedlings grown in vitro, control seedlings were mock-inoculated under the same conditions as described above. For cultivation, seedlings were washed in autoclaved 0.9% saline buffer for 1 min and placed in the glass tubes containing plant medium solution. The seedlings were placed side-by-side in a controlled-environmental chamber with 16 h photoperiod, photosynthetic active radiation of 150 µmol m^{-2} s^{-1}, 25 °C light/23 °C dark and 40% humidity. The experiment was conducted in completed randomized blocks in three biological replicates. Plants were randomly collected 4, 7 and 10 DAI. For seedling grown in soil, the mock inoculum was prepared in the same conditions as described, but using a bacterial culture boiled at 100 °C for 15 min in water bath until bacteria complete inactivation (Pankievicz et al. 2015). After inoculation, the seedlings were placed in 2 L pots containing 1.5 kg soil (Cerro Negro, Santa Catarina, Brazil, 27°47′43″S, 50°52′33″W) previously supplemented with 100 mL nutrient solution (Rodriguez-Salazar et al. 2009) with reduced nitrogen (0.5 mM KNO_3). Plants were grown in greenhouse under natural light without temperature control (average temperature about 29 °C), watered daily and every 2 days was added 100 mL of nutrient solution per pot. The experiment was conducted in completed randomized blocks in three biological replicates. Plants were randomly collected 14, 21 and 28 DAI. Plant tissues were immediately frozen in liquid nitrogen and stored at −80 °C until analysis.

Herbaspirillum seropedicae DNA quantification

Total DNA was isolated from maize tissue using DNeasy Plant Mini Kit (Qiagen) according to the manufacturer's protocol. Each sample was a pool of three roots or leaves, obtained by manual crushing with mortar and pestle in the presence of liquid nitrogen. DNA extraction from bacterial cultures was performed using Wizard® Genomic DNA purification kit (Promega™, Madison, WI, USA) with modifications (Pereira et al. 2014). DNA concentration was estimated from measurements at 260 and 280 nm on a Thermo Scientific NanoDrop 2000 spectrophotometer (Willmington, DE, USA). *H. seropedicae* DNA quantification in maize plants grown in vitro and in soil was performed by qPCR using HERBAS1 species-specific primers, targeting Bacteriophage Tail Fiber Protein (GenBank accession CP002039.1| 216,307–217,431), and SYBR Green (Pereira et al. 2014). Additionally, a TaqMan probe (HERBAS1) was designed using Primer Express 3.0 (Applied Biosystems) (Table S1). The HERBAS1 probe specificity test was performed using bacterial DNA isolated from *H. seropedicae* SmR1, *Herbaspirillum hiltneri, Herbaspirillum huttiense, Herbaspirillum lusitanum, Herbaspirillum rubrisubalbicans, Herbaspirillum frisingense, Azospirillum brasilense, Bacillus cereus, Bacillus subtilis, Escherichia coli, Rhizobium* sp., *Microbacterium* sp., and *Pseudomonas* sp. The amplification reactions contained 12.5 µL of 2× SYBR Green Master Mix or 2× TaqMan Universal PCR Master Mix (Applied Biosystems, Foster City, CA, USA), 100 nM of HERBAS1 forward and reverse primers, 100 nM HERBAS1 probe (TaqMan assay), water and 20 ng of template DNA in a final volume of 25 µL. All control samples reactions were carried out in duplicate and the inoculated samples in triplicate following the cycling conditions: an initial incubation step at 50 °C for 2 min, 95 °C incubation for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. For SYBR Green reactions, an additional dissociation curve with linear increase from 60 to 95 °C was performed immediately after each run. All real-time PCR runs were analyzed using automatic software settings. The reactions were performed in ABI PRISM 7500 Detection System (Applied Biosystems, Foster City, CA, USA). In order to quantify the genomic DNA amount in a pure culture of *H. seropedicae* SmR1, standard curves were prepared by serial dilution. Genomic DNA was 10-fold serially diluted in ultra-pure water to final concentrations ranging from 10^6 to 10^0 DNA copies, equivalent to concentrations of 6.03 ng to 6.03 fg. The number of genome copies (m) was calculated based on *H. seropedicae* SmR1 genome size (5.51 Mbp) (Pedrosa et al. 2011) as described previously (Pereira et al. 2014). A standard curve was designed for each growth condition as well as plant tissue (roots and leaves) for both SYBR Green and TaqMan assays. The qPCR reactions were carried out separately, in triplicate. Amplification efficiency values were calculated from the equation $E=(10^{-1/S}-1)\times 100$, where E is the efficiency (percent) and s is the slope obtained from the standard curve.

Bacterial transcript content quantification

Total RNA was isolated from approximately 100 mg of a pool of three maize roots using TRI Reagent Protocol (Sigma-Aldrich, St. Louis, USA). RNA samples were quantified by Nanodrop 2000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA) and treated with DNase I Amplification Grade (Sigma-Aldrich) to eliminate DNA contamination. cDNA synthesis was obtained using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems), with 2 µg of purified RNA and random primer. For absolute quantification of bacterial transcripts in roots of maize plants grown in vitro and in soil, *H. seropedicae* SmR1 DNA was used as standard and three standard curves were performed using three distinct *H. seropedicae* SmR1 DNA extracts for each bacterial target gene *nifH,*

hrcN and *rpoC* (Table S2), in triplicate. Primer Express 3.0 (Applied Biosystems) software was used to design nifH and hrcN primers. Reactions for in vitro and in soil samples were performed in triplicate, with 40 ng of cDNA template and *nifH, hrcN* and *rpoC* primer pairs separately. Primer concentrations were described in Table S2. The qRT-PCR reactions were carried out in ABI PRISM 7500 Detection System (Applied Biosystems), following the amplifications conditions as described above using 2× SYBR Green Master Mix (Applied Biosystems) in a final volume of 20 µL. Standard curves (Ct versus log DNA copy number) were used to estimate *nifH, rpoC* and *hrcN* gene expression in roots of control and inoculated plants. Gene expression was expressed as *nifH, rpoC* and *hrcN* cDNA copy number per 40 ng of total cDNA template. In order to verify the interference of the maize DNA presence in amplification efficiency values, standard curves were constructed for each gene using cDNA from an inoculated maize root sample grown in vitro. The cDNA was fivefold serially diluted in ultra-pure water, with concentrations ranging from 80 to 0.3125 ng of cDNA template, in duplicate and the amplification efficiencies were determined as mentioned above.

Results

qPCR parameters and probe specificity test

Standard curves were prepared for each experimental condition (in vitro and in soil) and plant tissue (maize roots and leaves), using SYBR Green or TaqMan reagents to obtain qPCR parameters such as efficiency, slope and correlation coefficient (R^2). SYBR Green assay (Table 1) showed efficiency values varying from 89 to 100%. The qPCR parameters obtained through the standard curves using TaqMan probe (Table 2) showed efficiency values ranging from 84 and 99%. Using six *H. seropedicae* SmR1 DNA standard curves constructed for the TaqMan assay (six different runs), the general efficiency value obtained was 91% and slope value −3.55. The limit of detection (LOD) using TaqMan assay was established as 10^1 copies, which corresponds to mean Ct = 32.8 (Fig. S1). Probe specificity test was conducted for TaqMan assay, which was able to detect selectively *H. seropedicae* SmR1 DNA (Table 3). Even though it was detected late Ct value for different bacteria species but same genera (*H. hiltneri, H. huttiense, H. lusitanum, H. rubrisubalbicans* and *H. frisingense*), *H. seropedicae* SmR1 presented early Ct value (10.21) related to the others (Ct > 32.30).

Table 1 Parameters of SYBR green qPCR standard curves for *H. seropedicae* SmR1 quantification using HERBAS1 primers (100 nM) and bacterial DNA serial dilution

Experimental condition	Efficiency (%)	Slope	R^2
In vitro[a]			
Root	95	−3.44	0.998
Leaf	92	−3.53	0.989
Soil[a]			
Root	89	−3.61	0.999
Leaf	100	−3.32	0.993

[a]Each standard curve was used for the respective experimental condition

Table 2 Parameters of TaqMan qPCR standard curves for *H. seropedicae* SmR1 quantification using HERBAS1 primers (100 nM), HERBAS1 probe (100 nM) and bacterial DNA serial dilution

Experimental condition	Efficiency (%)	Slope	R^2
In vitro[a]			
Root	84	−3.77	0.999
Leaf	84	−3.79	0.999
Soil[a]			
Root	99	−3.34	0.999
Leaf	99	−3.35	0.989

[a]Each standard curve was used for the respective experimental condition

Table 3 Positive amplifications and Ct values generated by TaqMan qPCR specificity assay using primers HERBAS1, HERBAS1 Probe and template DNA extracted from *H. seropedicae* SmR1 or other bacteria

Template DNA	Positive/total	Mean Ct
H. seropedicae SmR1	3/3	10.21
H. hiltneri	1/3	36.97
H. huttiense	3/3	35.64
H. lusitanum	1/3	36.49
H. rubrisubalbicans	3/3	32.68
H. frisingense	3/3	32.30
A. brasilense	0/3	–
Burkholderia tropica	0/3	–
Bacillus cereus	0/3	–
Bacillus subtilis	0/3	–
E. coli	0/3	–
Rhizobium sp.	0/3	–
Microbacterium sp.	0/3	–
Pseudomonas sp.	0/3	–

All samples were analyzed at a final concentration of 6 ng DNA per reaction (n = 3)

Quantification of *H. seropedicae* DNA in maize cultivated in vitro and in soil

Total DNA isolated from roots and leaves of maize plants (control and inoculated) grown in two conditions were used as template to qPCR in order to quantify *H. seropedicae* DNA. Considering plants grown in vitro (Table 4), inoculated samples presented around 10^9 *H. seropedicae* DNA copy number/g of fresh root tissue (4, 7 and 10 DAI) and ranged from 10^7 (4 DAI) to 10^9 (7 and 10 DAI) in leaf tissues using SYBR Green. *H. seropedicae* DNA contents determined by TaqMan assay were similar to SYBR Green results. As expected, bacterial DNA was not detected in control samples by both qPCR assays, either roots or leaves of seedlings grown in vitro. Considering plants grown in soil (Table 5), *H. seropedicae* DNA was detected using SYBR Green in 9 out of 9 inoculated root samples 14 DAI, 6 out of 9 samples 21 DAI and 3 out of 9 samples 28 DAI. On the other hand, *H. seropedicae* DNA was detected in only 2 out of 6 control root samples (14 and 21 DAI). *H. seropedicae* DNA was not detected in leaf samples. Similarly, few control root samples showed *H. seropedicae* DNA presence comparing to inoculated in TaqMan assay. *H. seropedicae* DNA was not detected in control leaf samples, while two inoculated leaf samples (14 DAI) showed positive values for DNA presence.

Transcript quantification of *H. seropedicae* in maize cultivated in two conditions

In this study, the cDNA quantity of bacterial transcripts *nifH, hrcN* and *rpoC* in maize root samples were determined by RT-qPCR using serial dilutions of DNA from *H. seropedicae* SmR1 pure culture as standard. Therefore,

Table 4 Bacterial DNA copy number/g root or leaf (fresh weight) of P30F53 *Zea mays* cultivated in vitro, inoculated and control samples after inoculation with *H. seropedicae* SmR1

qPCR assay	DAI	>LOD/n	Bacterial DNA copy number/g root	>LOD/n	Bacterial DNA copy number/g root	>LOD/n	Bacterial DNA copy number/g leaf	>LOD/n	Bacterial DNA copy number/g leaf
		Root				Leaf			
		Control		Inoculated		Control		Inoculated	
SYBR Green	4	0/6	–	9/9	$1.29 \pm 0.86 \times 10^9$	0/6	–	9/9	$1.27 \pm 1.07 \times 10^7$
	7	0/6	–	9/9	$2.03 \pm 0.36 \times 10^9$	0/6	–	9/9	$1.03 \pm 0.84 \times 10^9$
	10	0/6	–	9/9	$3.52 \pm 0.15 \times 10^9$	0/6	–	9/9	$1.69 \pm 2.37 \times 10^9$
TaqMan	4	0/6	–	9/9	$5.62 \pm 2.55 \times 10^8$	0/6	–	8/9	$9.37 \pm 8.86 \times 10^6$
	7	0/6	–	9/9	$1.23 \pm 0.22 \times 10^9$	0/6	–	9/9	$6.76 \pm 5.71 \times 10^8$
	10	0/6	–	9/9	$1.94 \pm 0.85 \times 10^9$	0/6	–	9/9	$0.99 \pm 1.35 \times 10^9$

The SYBR Green qPCR assays were realized with 100 nM HERBAS1 primers and 20 ng of sample DNA. The TaqMan qPCR assays were realized with 100 nM HERBAS1 probe. Values presented as means ± standard deviation. Means represents only the values detected over the limit of detection (> LOD) of 10 copies (n = 6 for control, n = 9 for inoculated samples)

Table 5 Bacterial DNA copy number/g root or leaf (fresh weight) of P30F53 *Zea mays* cultivated in soil, inoculated and control samples after inoculation with *H. seropedicae* SmR1

qPCR assay	DAI	>LOD/n	Bacterial DNA copy number/g root	>LOD/n	Bacterial DNA copy number/g root	>LOD/n	Bacterial DNA copy number/g leaf	>LOD/n	Bacterial DNA copy number/g leaf
		Root				Leaf			
		Control		Inoculated		Control		Inoculated	
SYBR Green	14	2/6	$2.57 \pm 0.06 \times 10^5$	9/9	$1.32 \pm 0.92 \times 10^5$	0/6	–	0/9	–
	21	2/6	$5.13 \pm 0.99 \times 10^4$	6/9	$3.09 \pm 0.48 \times 10^5$	0/6	–	0/9	–
	28	0/6	–	3/9	$7.43 \pm 1.97 \times 10^4$	0/6	–	0/9	–
TaqMan	14	2/6	$4.24 \pm 0.02 \times 10^5$	9/9	$3.02 \pm 2.22 \times 10^5$	0/6	–	2/9	$16.1 \pm 0.07 \times 10^3$
	21	2/6	$8.21 \pm 2.20 \times 10^4$	7/9	$6.06 \pm 3.12 \times 10^5$	0/6	–	0/9	–
	28	0/6	–	3/9	$2.07 \pm 0.09 \times 10^5$	0/6	–	0/9	–

The SYBR Green qPCR assays were realized with 100 nM HERBAS1 primers and 20 ng of sample DNA. The TaqMan qPCR assays were realized with 100 nM HERBAS1 probe. Values presented as means ± standard deviation. Means represents only the values detected over the limit of detection (> LOD) of ten copies (n = 6 for control, n = 9 for inoculated samples)

the standard curves were constructed by plotting Ct versus log DNA copy number to obtain efficiency and slope values (Fig. 1) and to estimate *nifH, rpoC and hrcN* gene expression in roots of control and inoculated plants. The bacterial gene expression (*nifH, rpoC* and *hrcN*) was quantified in maize root samples grown in vitro (Fig. 2) and in soil (Fig. 3) by RT-qPCR. In vitro, gene expression for *nifH, rpoC* and *hcrN* was not observed on control roots, whereas on inoculated roots, these genes were expressed 4, 7 and 10 DAI, in different patterns (Fig. 2). The *nifH* gene expression decreased from 4 to 10 DAI in inoculated samples. *rpoC* gene expression in inoculated samples slightly decreased from 4 to 10 DAI. Concerning plants grown in soil (Fig. 3), the cDNA bacterial quantification showed highest *nifH* bacterial gene expression in inoculated samples comparing to control. *rpoC* gene expression decreased

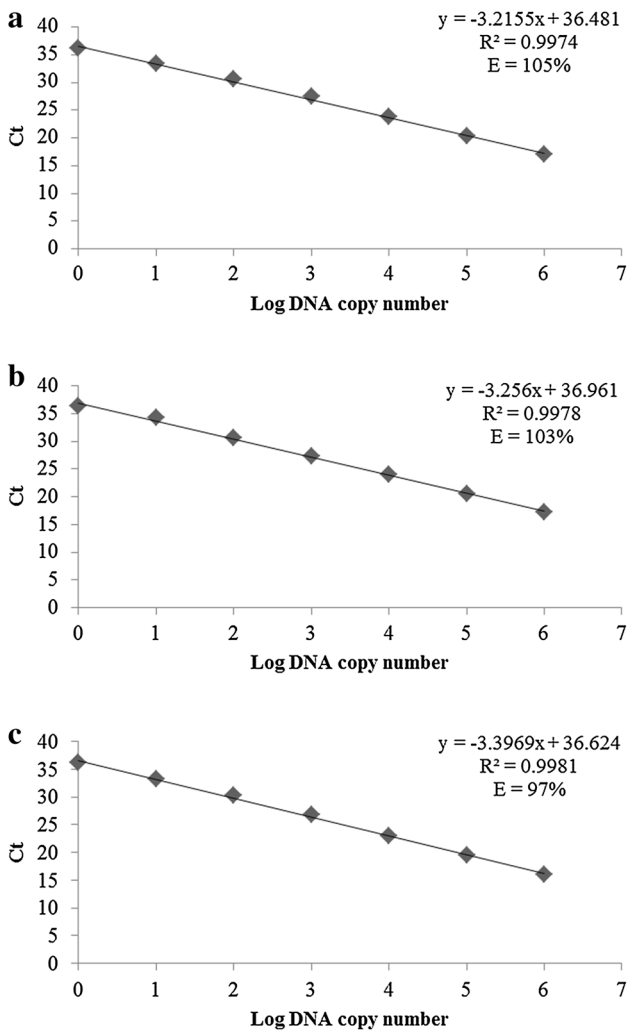

Fig. 1 qPCR standard curves for *H. seropedicae* transcript quantification generated using three DNA extractions from *H. seropedicae* strain SmR1 as template and **a** nifH, **b** rpoC and **c** hrcN primers (n = 9)

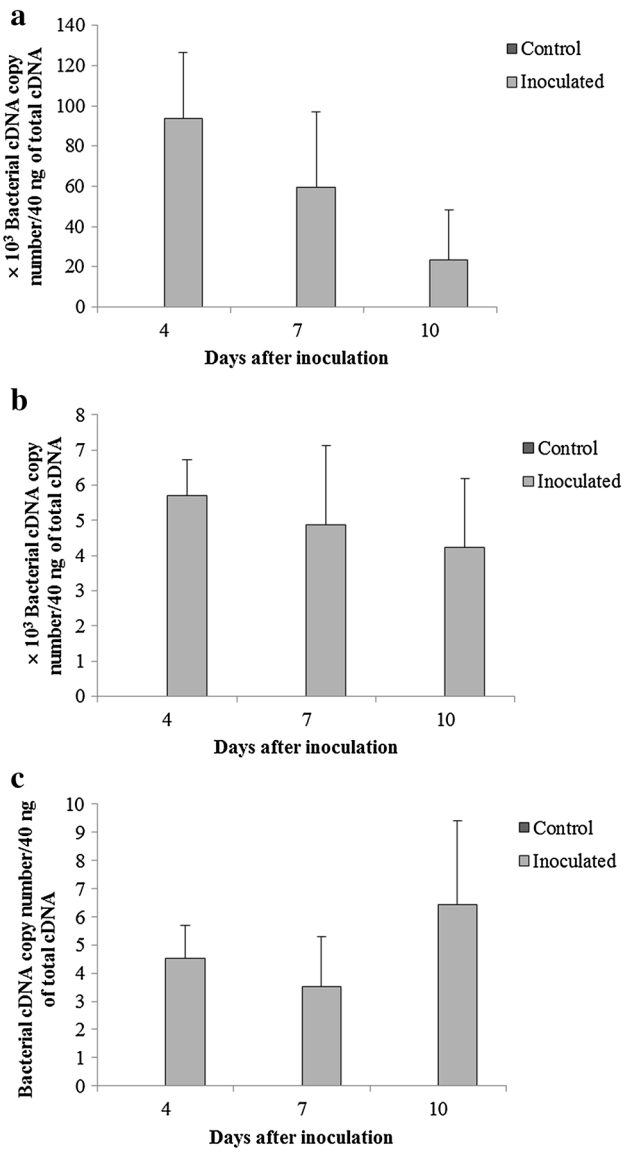

Fig. 2 Quantification of bacterial cDNA copy number per 40 ng of total cDNA, maize (P30F53 variety) grown in vitro after inoculation with *H. seropedicae* strain SmR1. **a** *nifH* gene expression, **b** *rpoC* gene expression, **c** *hrcN* gene expression. Data are presented as mean ± SD (n = 9)

in inoculated samples from 14 to 28 DAI. It was not possible to quantify *hrcN* gene in control or inoculated samples even 14, 21 or 28 DAI. Standard curves were also constructed for each gene using cDNA from inoculated root as template, which generate curve equations determining qPCR parameters such as efficiency and R^2 (Table S3).

Discussion

Considering that there are few studies involving *H. seropedicae* response inside the plant, and assuming that different

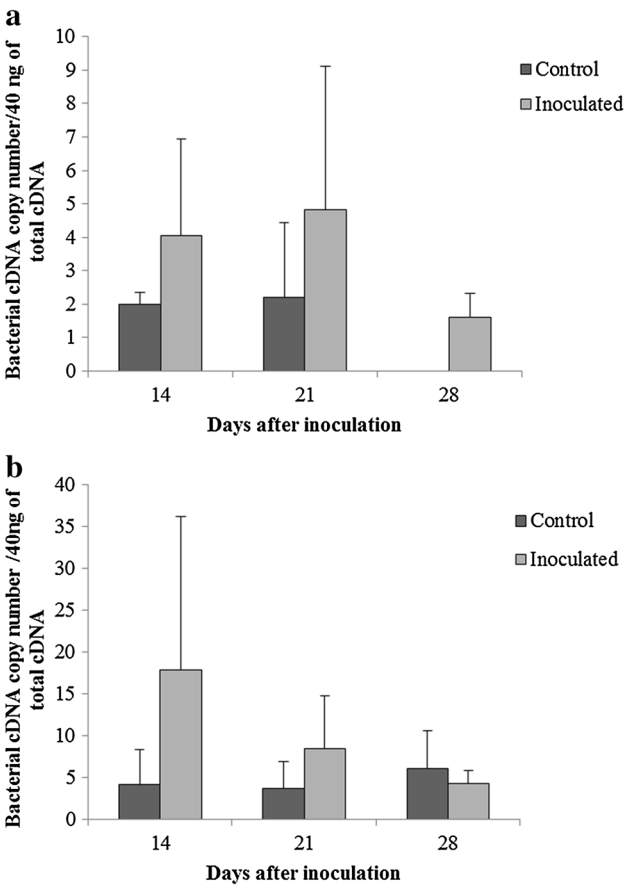

Fig. 3 Quantification of bacterial cDNA copy number per 40 ng of total cDNA, maize (P30F53 variety) grown in soil after inoculation with *H. seropedicae* strain SmR1. **a** *nifH* gene expression and **b** *rpoC* gene expression. Data are presented as mean ± SD (n = 9)

experimental settings would be required for a better comprehension about the plant–endophyte interaction (Hardoim et al. 2015), we developed qPCR assays to evaluate *Zea mays* colonization by *H. seropedicae* SmR1 in two growth conditions, in vitro and in soil. Herein we quantified *H. seropedicae* DNA in maize roots and leaves using HERBAS1 species-specific primer set previously designed for SYBR Green qPCR assay (Pereira et al. 2014). Besides, we aimed to validate a more specific tool for *H. seropedicae* DNA quantification, designing TaqMan HERBAS1 probe. TaqMan assay is the choice for qPCR targeting specific phytopathogens (Oliveira et al. 2002; Li et al. 2013). The labeled fluorogenic hybridization probe (TaqMan) is cleaved by 5′ nuclease activity of *Taq* DNA polymerase during the extension phase of PCR, yielding a real time measurable fluorescence emission directly proportional to the concentration of the target sequence (Heid et al. 1996). TaqMan probe with conjugated minor-groove-binding (MGB) groups form extremely stable duplexes with single-stranded DNA targets, allowing the design of shorter probes with high specificity of the hybridization (Kutyavin et al. 2000).

In order to establish a reliable DNA quantification, qPCR parameters for SYBR Green and TaqMan assays were defined using standard curves, once the use of a substantial standard curve based on a known DNA concentration makes it theoretically possible to quantify DNA from any source (Timmusk et al. 2009). Considering that a consistent standard curve should have a R^2 value of more than 0.95 and a slope between −3.0 and −3.9 (corresponding to PCR efficiencies of 80 and 115%) (Zhang and Fang 2006), all curves presented reliable qPCR parameters (Tables 1, 2). Using a specific standard curve for each plate run, *H. seropedicae* DNA quantity on roots and leaves of maize grown in vitro and in soil could be properly estimated using SYBR Green and TaqMan probe assay. Aiming to verify if the TaqMan probe assay could differentiate *H. seropedicae* from other bacteria (even other bacteria from the same genera), we used DNA samples isolated from pure cultures of *H. seropedicae* and other bacteria species. Our results confirm the specificity of the HERBAS1 probe (Table 3) based on the Ct values presented by *H. seropedicae* (Ct = 10.21). In contrast, other species belonging to *Herbaspirillum* genera presented positive signals, but with late Ct values (>32.30). Furthermore, other bacteria species did not shown amplification signals. The difference of Ct values between the target and other bacteria species (at least 22.9) is more than enough to validate the use of this experimental condition, since the unspecific amplification will not influence the analysis (Martin-Sanchez et al. 2013). In a previous work using SYBR Green chemistry to detect and quantify *H. seropedicae* DNA in maize plants (Pereira et al. 2014), HERBAS1 primers were tested for *H. seropedicae* specificity, proving that this pair of primers is very specific for *H. seropedicae* DNA detection and quantification in planta. Comparing the unspecific Ct values using SYBR Green (25.59 for *H. rubrisubalbicans* and 29.62 for *H. frisingense*) with the unspecific Ct values using TaqMan probe (32.68 for *H. rubrisubalbicans* and 32.3 for *H. frisingense*), the unspecific amplification was reduced using TaqMan assay as expected. Taken together, these results showed that *H. seropedicae* DNA can be properly detected and quantified by SYBR Green assay or alternatively using more specific TaqMan assay.

The sensitivity of qPCR quantification experiments is given by the LOD, which is defined as the lowest DNA concentration in a sample that can be reliable detected. In qPCR assays, it is accepted that the LOD is the DNA amount at which 95% of the positive samples are detected (or no more than 5% failed reactions should occur) (Bustin et al. 2009). For the SYBR Green and TaqMan assays, the amplifications below the LOD were discarded, while those above the LOD were accounted and Ct values were

used to calculate the mean value of the bacterial DNA copy number/g fresh tissue (Tables 4, 5). For *H. seropedicae* DNA quantification in maize roots and leaves using SYBR Green assay, the LOD was previously established as 10^1 genome copies, corresponding to a mean Ct value of 32.15 (Pereira et al. 2014). Using TaqMan probe assay, LOD also corresponded to 10^1 genome copies (Ct 32.8), permitting a reliable DNA quantification from 10^6 to 10^1 genome copies, similar to SYBR Green assay. Despite the difficulties in comparing LOD values between studies due to the differences in experimental designs, such as number of replicates, differences in master mix constituents, or conditions of DNA quantification (Price et al. 2012), the LOD found in the present study (10^1 genome copy number, corresponding to 60.3 fg) is in accordance with other studies using TaqMan PCR assays to detect and quantify microbial genomic DNA. For example, the genomic DNA of the pathogenic fungus *Magnaporthe oryzae* in rice, could be detected as low as 1 pg (Su'udi et al. 2013), as well as to detect *Paenibacillus polymyxa* DNA in wild barley (Timmusk et al. 2009). Also, for *Bacillus subtilis* and *Pseudomonas fluorescens* detection in soil, it was set a LOD of 10 pg (Kim et al. 2010).

Using the established set of primers HERBAS1 and HERBAS1 probe, the present study quantified *H. seropedicae* presence on roots and leaves of maize (P30F53 variety) cultivated in vitro and in soil employing SYBR Green and TaqMan chemicals in qPCR assays. There is a single copy of the HERBAS1 target sequence in *H. seropedicae* genome (Pedrosa et al. 2011), which allows us to estimate the bacterial cell number in maize tissues (Pereira et al. 2014). *H. seropedicae* DNA quantification in plants cultivated in vitro, using both SYBR Green and TaqMan qPCR assays (Table 4), indicates an increase or at least maintenance of the genome copy number per gram of root in inoculated maize roots and leaves along the time course (4, 7 and 10 DAI). Our results are consistent with those found previously in maize root plants (DKB240 variety) grown in the same conditions from 1 to 10 DAI (Pereira et al. 2014). Under laboratory conditions, *H. seropedicae* invade and colonize very quickly maize tissues, and 24 h after inoculation, roots (including xylem vessels) are completely colonized by the bacteria. The plant vascular system colonization allows the bacteria distribution throughout the host, reaching aerial parts (Monteiro et al. 2008, 2012). Our results strengthen these findings, once *H. seropedicae* DNA was detected in both belowground and aboveground tissues. Likewise qPCR assays were performed to quantify *A. brasilense* FP2 on roots of maize (Faleiro et al. 2013) and wheat (Stets et al. 2015). For both maize P30F53 and DKB240 varieties, *A. brasilense* DNA copy number per gram of root increased from 10^6 to 10^9 (Faleiro et al. 2013), confirming the possibility to detect and quantify diazotrophic bacteria in maize tissue and the rapid colonization of the plant tissue by plant growth promoting bacteria. Regarding to *H. seropedicae* DNA quantification of plants grown in nonsterile soil (Table 5), inoculated plants usually presented higher rates of *H. seropedicae* presence, comparing to control plants. Inoculated root samples showed bacteria presence about 10^5 DNA copy number per gram of fresh root for SYBR Green and TaqMan assays at 14, 21 and 28 DAI. Otherwise, *H. seropedicae* DNA was only detected in 2 out of 27 leaf samples of inoculated plants. Even if it is not possible to directly compare different analyzed times after inoculation among the different substrates, it is important to note that under axenic conditions there is absence of competing bacteria and the introduction of competition may substantially change the pattern of the plant colonization observed. Regarding that bacteria (even benefic or pathogenic) penetrate the plant tissue by the same attachment points, competing bacteria may secrete anti-bacterial substances and the plant may elaborate a defense against the mixed bacterial population (Monteiro et al. 2008). *H. seropedicae* attach and colonize maize root surface progressively from 3 to 15 days after inoculation (Roncato-Maccari et al. 2003). In contrast, a longer time study, which examined the population dynamics of *H. seropedicae* strain HRC 54 associated with maize roots, showed a continuous decrease in number of bacterial cells per gram of root (fresh weight) from 7 to 14, 21 and 30 DAI (Silva et al. 2014). Similar to our soil experiment, in which *H. seropedicae* DNA presence was lower 28 DAI (3 positive/9 total) comparing to 14 and 21 DAI. The same study also detected *H. seropedicae* HRC 54 presence in control plants, but fewer than in inoculated, as our results from plants grown in soil.

It is noteworthy that *H. seropedicae* DNA quantification stands in the higher bacteria genome copy number found on roots comparing to leaves in maize plants in both growth conditions. This may be a common behavior in diazotrophic bacteria, once other studies also found a greater bacteria presence in rhizosphere of grasses comparing to aerial parts (Rodriguez-Blanco et al. 2015; Pariona-Llanos et al. 2010). In most plants, the endophytes number in belowground is higher comparing to aboveground tissues (Rosenblueth and Martinez-Romero 2006). Higher *Herbaspirillum* spp. amounts were also observed on roots than stems in sugarcane field experiment (Reis et al. 2000). Additionally, *Bacillus megaterium*, a Gram-positive nitrogen-fixing bacteria which has similar colonization pattern to some Gram-negative endophytic diazotrophs, such as *A. brasilense*, migrates slowly from roots to leaves (Liu et al. 2006). This might explain the lower amount of bacteria found in shoots in these studies comparing to roots.

Bacterial gene expression (*nifH, rpoC* and *hrcN*) was analyzed on maize roots by RT-qPCR absolute

quantification using standard curves performed with serial dilutions of *H. seropedicae* SmR1 DNA. Similar approach has already been employed in study of environmental diazotrophic diversity using *nifH* gene expression as a molecular marker (Martensson et al. 2009). The accuracy of the bacterial transcript quantification assay on roots of maize inoculated with *H. seropedicae* SmR1 in the present study was verified by qPCR standard curves parameters using bacterial DNA as template. According to Zhang and Fang (2006) the efficiencies between 80 and 115% are consistent with reliable standard curves. In this way, the efficiency values found in this study for *nifH* (105%), *rpoC* (103%) and *hrcN* (97%) bacterial genes (Fig. 1) are in accordance with the established parameters and hence the bacterial absolute transcript quantification could be properly measured. In this study, *H. seropedicae nifH, rpoC* and *hrcN* transcripts were quantified in maize roots grown in two growth conditions. We were able to detect *nifH* gene expression on inoculated maize roots, cultivated in two growth conditions. The highest amounts of *nifH* transcripts was found in inoculated samples grown in vitro, remaining about 10^3 bacterial cDNA copy number at 4, 7 and 10 DAI, although lower amounts were observed in soil plants. Our results are in accordance with previous studies showing that genera *Herbaspirillum* were able to express *nif* genes and presented nitrogenase activity when associated with other Poaceae, such as sorghum, rice and sugarcane (James et al. 1997, 2002; Olivares et al. 1997; Gyaneshwar et al. 2002; Elbeltagy et al. 2001).

Inoculated maize samples cultivated in axenic conditions (in vitro) had a relatively constant *rpoC* gene expression, between 5.7 and 4.2×10^3 cDNA copy number per 40 ng of total cDNA. Our results suggest that *rpoC* transcript level is a possible tool to monitor *H. seropedicae* activity in planta. *rpoC* gene expression was tested as a reference gene for *Gluconacetobacter diazotrophicus* grown in different carbon sources, however it was highly variable (Galisa et al. 2012). In contrast, *rpoC* presented constant expression for *H. seropedicae* culture in the presence of sugarcane extract, and it has been used as a reference gene for normalization of relative gene expression by RT-qPCR (Cordeiro et al. 2013). However our report is the first that successfully measure *rpoC* transcript levels in planta. Herein, transcripts of *hrcN* gene, coding for T3SS ATP synthase, was also measured. In vitro inoculated roots presented higher rates of *hrcN* transcripts at 10 DAI, comparing to 4 and 7 DAI. In contrast, *hrcN* transcript was not detected on roots in soil experiment at 14, 21 or 28 DAI. It was previously suggested that specific physiological conditions might be required for expression and activity of T3SS and synthesis of effector proteins in *H. seropedicae* (Pedrosa et al. 2011; Monteiro et al. 2012). It is possible that the necessary conditions were achieved in vitro plant growth condition 4, 7 and 10 DAI, but not in soil 14, 21 and 28 DAI. Despite transcriptomic analysis of in vitro *H. seropedicae* and maize interaction did not reveal T3SS gene expression (Balsanelli et al. 2015), T3SS was identified by mass spectrometry when investigating the root proteome of *H. seropedicae* and maize interaction 7 DAI (Ferrari et al. 2014). We observed that *H. seropedicae* DNA copy number slightly increased in roots and leaves of plants grown in vitro from 4 to 10 DAI, while *nifH* transcript level decreased over time in roots, but the same was not observed for *rpoC* or *hrcN* transcript levels, suggesting that *nifH* expression was regulated in the beginning of this plant–bacteria interaction.

In conclusion, we quantified *H. seropedicae* DNA presence on roots and leaves of maize plants (P30F53 variety) inoculated with diazotrophic bacteria *H. seropedicae*, and also measured the transcripts levels of bacterial genes on maize roots of plants grown in sterile and nonsterile conditions. We developed a specific, reliable and efficient TaqMan probe for qPCR assay, to quantify *H. seropedicae* DNA in plant tissues. *H. seropedicae nifH* and *rpoC* gene expression were detected on roots of inoculated maize grown in both growth conditions, however it was not possible to verify *hrcN* gene expression in plants grown in soil. Our results suggest that it is possible to use *nifH* and *rpoC* transcript levels to monitor *H. seropedicae* gene expression inside plant tissue.

Acknowledgements This work was financially supported by the National Institute of Science and Technology-Biological Nitrogen Fixation (INCT-FBN), National Counsel of Technological and Scientific Development (CNPq), Ministry of Science and Technology, Brazil. Pâmela Dall Asta, Tomás Pellizzaro Pereira and Fernanda Plucani Amaral were recipients of PhD fellowships from Coordination of Personnel Improvement of Higher Education (CAPES), Ministry of Education, Brazil and Ana Carolina Maisonnave Arisi is recipient of research fellowship (PQ2) from CNPq.

Compliance with ethical standards

Conflict of interest No conflict of interest declared.

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