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Vol. 8(31), pp. 2937-2946, 30 July, 2014
DOI: 10.5897/AJMR2014.6779
Article Number: F56A9A546423
ISSN 1996-0808

http://www.academicjournals.org/AJMR

African Journal of Microbiology Research

Full Length Research Paper

Development of a real-time PCR assay for the detection and quantification of *Gluconacetobacter diazotrophicus* in sugarcane grown under field conditions

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Received 14 March, 2014; Accepted 27 June, 2014

The objective of this study was to evaluate the viability of real-time polymerase chain reaction (PCR) to detect *Gluconacetobacter diazotrophicus* in sugarcane inoculated and non-inoculated with diazotrophs which are grown under field conditions. The primer pair PAL5F and PAL5R yielded a specific band of 189 bp using real time PCR with SYBER Green I. This primer pair was the most sensitive one to detect endophytic bacteria in sugarcane plants grown under field conditions and inoculated or not with bacterium. The lower limit of detection was 5 fg of template DNA, which corresponds to 12 bacterial cells. In contrast, a cultivation-dependent approach was not capable of detecting the bacteria in the same sample. The quantification of *G. diazotrophicus* from field grown plants using real-time PCR and a set of specific primers can be used to determine the number of bacterial cells that colonize endophytically the plant after inoculation. A highly sensitive and specific assay was developed to quantify *G. diazotrophicus* in sugarcane plants grown under field conditions. This assay can be used to evaluate the occurrence of the bacterium in different sample types.

Key words: Inoculant, diazotrophs, endophyte, colonization, quantitative polymerase chain reaction (qPCR).

INTRODUCTION

Gluconacetobacter diazotrophicus is a Gram negative bacteria originally isolated from roots and stems of sugarcane grown in different regions of Brazil (Cavalcante and Dobereiner, 1988) and later from

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sugarcane plants grown in Argentina, Uruguay, Mexico, Cuba, United States, India and Canada (Baldani et al., 1997; Dong et al., 1994; Loganathan and Nair, 2003). This diazotrophic (N_2-fixing) bacterium has also been found to be associated with other plants that are rich in sucrose such as sweet potato, banana, pineapple, coffee, carrot and beet root (Jimenez-Salgado et al., 1997; Perin et al., 2004; Saravanan et al., 2008), as well as with other plants of the *Poaceae* family, including rice (Muthukumarasamy et al., 2005) and elephant grass (Videira et al., 2012). *G. diazotrophicus* is an endophytic bacterium that colonizes the internal tissues of the plant without causing apparent phytopathogenic symptoms (Baldani et al., 1997) and can adhere to the roots to establish colonies at the base of the stems (James et al., 1994) as well as in the apoplastic fluid (Dong et al., 1994) and xylem vessels (James et al., 2001). This colonization likely involves exopolysaccharide (EPS) produced by *G. diazotrophicus*, as was demonstrated in a study using rice roots (Meneses et al., 2011).

Besides fixing nitrogen, *G. diazotrophicus* can provide additional benefits to the host plant, including the production of phytohormones and biocontrol substances, as well as phosphate (Dobbelaere et al., 2003; Fuentes-Ramirez et al., 1993) and zinc solubilization. Several sugarcane field experiments involving co-inoculation of *G. diazotrophicus* with strains from *Burkholderia tropica*, *Azospirillum amazonense, Herbaspirillum seropedicae* and *Herbaspirillum rubrisubalbicans* showed a positive effect on the plants in terms of biomass and yield increases as well as accumulation of N derived from biological nitrogen fixation (Oliveira et al., 2006). Based on these and other field inoculation experiments, biofertilizers carrying these different strains were developed by Embrapa Agrobiology (Seropédica, RJ - Brazil) and made available to the inoculant industry (Reis et al., 2009).

An important aspect to examine during field inoculation experiments is the establishment of target inoculated bacteria within plant tissues (Ruppel et al., 2006), which would indicate whether the observed effects on plants are indirectly related to bacterial colonization. Many techniques have been applied with success to monitor the presence (Oliveira et al., 2009; Silva-Froufe et al., 2009) or even quantify (Gomes et al., 2005; Munoz-Rojas and Caballero-Mellado, 2003) the inoculated bacterial strains within plants.

Among these tools, real time PCR (qPCR) was shown to be very useful for quantifying bacteria due to its highly sensitive detection levels, which are around 1 copy DNA μl^{-1} (Boeckman et al., 2000). QPCR has been applied to quantify bacteria directly in pure culture, soil samples (Sellek et al., 2008; Trabelsi et al., 2009) and plant tissues (Lacava et al., 2006; Ruppel et al., 2006; Trabelsi et al., 2009) using either 16S rRNA (Jung et al., 2010), *nif*H gene (Coelho et al., 2009) or species-specific sequences (Jung et al., 2010; Timmusk et al., 2009).

Although Couillerot et al. (2010) could quantify the presence of *Azospirillum lipoferum* in the rhizosphere of younger maize plants using qPCR with primers specific for this specie, most species-specific primers used to monitor nitrogen-fixing bacteria asso-ciated with *Poaceae* family plants were designed for use with conventional PCR (Lin et al., 2011; Muthukumarasamy et al., 2005; Sevilla et al., 2001). As such, there are few studies that evaluate the establish-ment of inoculated nitrogen-fixing bacteria in plants.

In the present study, specific primers were designed based on the 16S rRNA variable region of *G. diazotrophicus* and used to monitor and quantify this endophytic nitrogen-fixing bacterium following co-inoculation with other diazotrophic strains in sugarcane plants grown in the southern region of Brazil.

MATERIALS AND METHODS

Plant material

Detection and quantification of G. diazotrophicus in sugarcane plant tissues

A sugarcane field experiment was installed near Jaguari, Rio Grande do Sul, latitude -29° 28' 1.88", longitude 54° 44' 13.28" in a red Ultisol soil type to evaluate the establishment and effect of bacterial inoculants on sugarcane yield. The soil layer of 0-20 cm had the following physical and chemical characteristics: 230 g kg^{-1} clay, pH = 5.3 in water, value = 6.1, 6.8 mg dm^{-3} P (Mehlich), 44 mg dm^{-3} K, 0.2 $cmol_c$ dm^{-3} of Al^{3+} and 1.3% organic matter (OM). The sugarcane variety RB867515 was planted in a randomized block design with 3 replicates. There were three treatments: a) control, b) inoculated, and c) N-fertilized using 120 kg N ha^{-1}. The phosphorus and potassium nutrients were provided in the amount of 480 kg ha^{-1} (formula 0-25-20) according to the soil analysis and 30 kg ha^{-1} of FTE BR 12 as a micronutrient source.

The Embrapa sugarcane inoculant contained five endophytic diazotrophic strains: *G. diazotrophicus* (strain PAL5, BR11281)*, H. seropedicae* (strain HRC54, BR11335)*, H. rubrisubalbicans* (strain HCC103, BR11504), *A. amazonense* (strain CBamC), BR11115, *B. tropica* (strain Ppe8, BR11366).

Each strain was grown separately in liquid Dyg's culture medium, inserted at a final concentration of 10^9 cells g^{-1} into five separate plastic bags containing 250 g peat and then incubated at 4°C for thirty days. At the time of planting, the bags containing the inoculated strains were dispersed and mixed in a tank with 200 L of water. The sugarcane plantlets with 3 germinating nodes were immersed into this bacterial suspension for one hour and then planted in the soil.

Quantification of G. diazotrophicus within plant tissues

The sugarcane plants were harvested 7 and 11 months after planting and *G. diazotrophicus* population in the stem internodes was then quantified. The internodes of base, intermediate and top of sugarcane plants stems were disinfected superficially with 70% ethanol to eliminate the epiphytic bacterial community that colonizes this region of the stems.

The internodes were macerated in liquid nitrogen generating a composite sample from 3 plant regions and 200 mg of powdered

Table 1. Species-specific primers designed for *G. diazotrophicus*.

Primer	Sequence 5′-3′	Amplicon (bp)
PAl5F	GGCTTAGCCCCTCAGTGTCG	189
PAl5R	GAAACAGCCATCTCTGACTGC	
PAL5F1	GATGTTGGGTGGCTTAGCCCC	
PAL5R1	ACAGCCATCTCTGACTGCAGC	
PAL5F2	GGCTGCAGTCAGAGATGGCTG	300
PAL5R2	CGATTCCACCTTCATGCACTC	

sample was used for total DNA extraction with CTAB protocol according to Doyle and Doyle (1987). The extracted DNA was quantified and the final concentration adjusted to 40 ng μL^{-1} using a Nanodrop 2000c at 260 nm. This DNA was used for the qPCR reactions as described below. The C_T values obtained from the plant samples amplified by qPCR were used to determine the number of bacterial cells present in the plant samples using the standard equation: y=-3.3917x+39.935, where "y" corresponds to the sample C_T and "x" is the Log_{10} bacterial number for the qPCR reaction. To estimate the number of bacteria per gram of fresh stalk, the methodology proposed by Ruppel et al. (2006) was applied. The number of bacteria estimated from qPCR reactions was multiplied by 5 because the DNA was extracted from 200 mg of tissue. These values were then multiplied by 50, which corresponds to dilution factor of the extracted DNA.

Primer design

The primers were designed based on the 16S rRNA sequences of *G. diazotrophicus* strains and other *Gluconacetobacter* species deposited in the NCBI GenBank (CP001189, NR027591, X75618, AY230814, AY958232, NR_028767, AF127412, AF127404, NR_026132, NR_024959 and AY230813). These sequences were aligned using ClustalW (Thompson et al., 1994) and the most polymorphic regions were used to design different sets of primers. The specificity of each primer pair was checked *in silico* using BlastN (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and the probeCheck database for 16S probes (http://131.130.66.200/cgi-bin/probecheck/probecheck.pl). After the *in silico* analysis, PCR reactions were carried out to establish the optimal reaction conditions and select the primer pair from those listed in Table 1 that has the best specificity for detecting *G. diazotrophicus.* The PCR reactions were then analyzed to assess the amplicon size produced by these primers at different concentrations of primer, $MgCl_2$ and DMSO, as well as various times and annealing temperatures.

Strains

A total of 27 strains (Table 2) belonging to different nitrogen-fixing species deposited in the Embrapa Agrobiology Culture Collection were used to confirm the specificity of the primers designed to specifically amplify genetic material from *G. diazotrophicus.*

Isolation of total DNA from pure bacterial cultures

The strains listed in Table 2 were grown in liquid DYGs medium (Rodrigues Neto et al., 1986) for 16 h at 30°C with shaking (150 rpm). Total DNA was extracted from 1 ml of culture using the CTAB method described by Doyle and Doyle (1987). The amount and purity of DNA was assessed at 260 nm and with a 260/280 nm ratio determined using a NanoDrop 2000c instrument (NanoDrop Products, Wilmington, DE, USA). The final DNA concentration of the sample was then adjusted to 80 ng μl^{-1}.

Primer-specific analysis using conventional PCR

Conventional PCR amplification was first carried out to validate the *in silico* analysis and establish the optimal reaction conditions for the best *G. diazotrophicus*-specific primer pair. Specific PCR reaction was performed in 25 µl containing 0.5 µl (80 ng) DNA template, 10 mM Tris-HCl (pH 9.0), 40 mM KCl, 250 µmol l^{-1} dNTPs, 1 U *Taq* DNA polymerase, l.5 mM $MgCl_2$ and 10 pmol of each primer (Table 1). A Mastercycler® gradient thermocycler (Eppendorf, Hamburg, Germany) was used for PCR reactions with the following protocol: The PCR program consisted of initial denaturation at 95°C for 5 min, followed by 40 cycles of 95°C for 1 min, annealing at 63°C for 15 s, extension at 72°C for 1 min and a final extension at 72°C for 7 min.

The amplified product was analyzed by electrophoresis on 2% agarose gels stained with ethidium bromide (10 mg ml^{-1} of agarose solution).

Amplification of *G. diazotrophicus* 16S rRNA gene using primers 27F and Amp2

Genomic DNA extracted from *G. diazotrophicus* (strain PAL5) was used as a template in PCR reactions with primers 27F (5'-AGA GTT TGA TCC TGG CTC AG-3') (Furushita et al., 2003) and Amp2 (5'-AAG GAG GTG ATC CAR CCG CA-3') (Wang et al., 1993) to amplify 16S rRNA. The reaction was performed in in 25 µl containing 0.5 µl (80 ng) DNA template, 10 mM Tris-HCl (pH 9.0), 40 mM KCl, 250 µmol l^{-1} dNTPs, 1 U *Taq* DNA polymerase, l.5 mM $MgCl_2$ and 10 pmol of each primer. The PCR program consisted of initial denaturation at 95°C for 5 min, followed by 35 cycles of 95°C for 1 min, annealing at 58°C for 1 min, extension at 72°C for 1 min and a final extension at 72°C for 7 min. The PCR products were analyzed by 2% gel agarose electrophoresis as described above. The resulting 1,512 bp PCR-amplified product was purified using the Wizard® SV Gel and PCR Clean-Up System kits (Promega®, USA), quantified, and serially diluted up to 10^{-10} ng μl^{-1}. This DNA was used to define the detection limit and used as a concentration standard.

Detection limit of the primer-specific species

Nested PCR was performed using 0.5 µl of the 1,512 bp diluted

product from the first PCR reaction and a pair of primers designed to amplify a 189 bp fragment of *G. diazotrophicus* 16S rRNA gene under the same reaction conditions used to test the different primer pairs.

Real time PCR (qPCR) standard curve

The copy number of 16S rRNA gene in the *G. diazotrophicus* genome and its molecular mass was used to convert the 1,512 bp 16S rDNA concentration into a bacterial cell number. The molecular mass was calculated using Avogadro's number (6.023 x 10^{23} DNA copy mol^{-1}) in the equation below as recommended by Applied Biosystems, and then converted to the number of gene copies. These values were used to calculate the number of the bacteria present in the plant tissues and also applied to determine the qPCR detection limit. The number of bacteria was calculated based on the copy number of the *G. diazotrophicus* 16S genome (Bertalan et al., 2009).

$$m = \left[\ n\ \right]\left[\frac{1\ mole}{6.023e23\ molecules\ (bp)}\right]\left[\frac{660\ g}{mole}\right] = \left[\ n\ \right]\left[\frac{1.096e\text{-}21\ g}{bp}\right]$$

$$m = \left[\frac{1512\ bp}{1}\right]\left[\frac{1.096e^{-21}g}{bp}\right]$$

$$m = 1.66e^{-18}g = 1.66e^{-9}\ ng$$

$$m = 1.66e^{-18}g = 1.66e^{-9}\ ng$$

$$\frac{1.66e^{-9}\ ng}{0.5\ ng} = \frac{1\ fragment\ 16S}{x}$$

$$X = 3.02e^{8}\ copies$$

$$X = 3.02e^{8}\ copy\ X\ 4 = 1.21e^{9}\ bacteria$$

Equation 1

Where: n = 16S DNA size (bp); m = mass; Avogadro's number = $6.023e^{23}$ molecules mol^{-1}; Average MW of a double-stranded DNA molecule = 660 g mol^{-1}. The *G. diazotrophicus* genome has four copies of the 16S rRNA gene

A standard curve was generated by plotting the number of bacteria (calculated as described above contained in a known concentration of 16S rDNA from *G. diazotrophicus* (strain PAL5) previously amplified with primers 27F and Amp2, against the C_T (Cycle Threshold) values obtained by qPCR, then serially diluted from 10^0 up to 10^{-6}, which corresponds to 0.5 to 5 $x10^{-7}$ ng of 16S DNA.

QPCR was performed using a 7500 Fast Real Timer PCR system (Applied Biosystems) with the SYBR Green PCR Master Mix (Applied Biosystems). Reactions were carried out in 96 well microtiter plates with a total volume of 15 µl containing 7.5 µl SYBR Green PCR Master Mix, 20 pmol of each primer and 5.0 µl DNA diluted 1:10. The protocol 7500 v2.0.5 with the following conditions was applied: 2 min at 95°C, followed by 40 cycles of 20 s at 95°C and 30 s at 63°C. The amplification efficiency was calculated using the formula E = 10^(-1/slope)$^{-1}$. The reaction was carried out with triplicate samples and the amplified 189 bp qPCR product was analyzed by gel agarose electrophoresis.

RESULTS

Sensitivity and specificity of primers

Six primers were designed to discriminate *G. diazotrophicus* from the other inoculated bacterial species. A BLAST analysis showed specificity for the target bacteria, and virtual PCR with the vector NTI program showed 100% annealing (data not shown). Evaluation with the program probeCheck (http://131.130.66.200/cgi-bin/probecheck/content.pl?id= home) confirmed primer specificity.

The best pair of primers, named PAL5F and PAL5R, were checked using agarose gel electrophoresis as shown in Figure 1.These primers amplified a 189 bp fragment in the absence of nonspecific products as shown (Figure 1) by the amplicon melting temperature and agarose gel electrophoresis.

For the other primer sets, non-specific and low qPCR amplification efficiency was observed for primer concentrations of 100 nmol. The optimal amplification efficiency for primers PAL5F and PAL5R was obtained using concentrations of 200 and 300 nmol, respectively. Thus, the subsequent PCR reactions were performed with 200 nmol of each primer, an annealing temperature at 63°C and an annealing time of 10 s.

The primer specificity was assessed using genomic DNA extracted from 27 wild type bacterial species (Table 2). The primers PAL5F and PAL5R showed strong specificity for the bacterium *G. diazotrophicus* and amplified a 189 bp rRNA gene fragment, which confirmed the *in silico* analysis. The other primer pairs produced amplified products of different sizes for other bacterial species (Table 1).

The sensitivity of conventional specific PCR was determined using serial dilution from the 16S rRNA gene amplified with genomic DNA of *G. diazotrophicus* (strain PAL5) as template and the 27F and Amp2 primers. A PCR product of approximately 1512 bp was purified, quantified and diluted at concentrations ranging from 100 to 10^{-10} ng.

A nested PCR reaction was then performed using the diluted product from the first reaction and the PAL5F and PAL5R primers. The 189 bp target fragment could be amplified using a minimum concentration of 5 x 10^{-9} ng template DNA, which was equivalent to approximately 12 bacterial cells (Figure 2), as calculated according to Equation 1.

Determination of a standard curve for qPCR quantification

The 1,512 bp fragment amplified by conventional PCR using *G. diazotrophicus*-specific primers 27F and AMP2 generate a standard curve for qPCR. Dilutions of the first PCR reaction product were used to estimate the number of bacteria and generate a curve ranging from 5 $x10^{-1}$ to 5 $x10^{-7}$ ng DNA (Table 3). The efficiency of the initiators was on the order of 97% and the standard curve generated by means of C_T and Log_{10} of the number of bacteria allowed calculation of the number of *G. diazotrophicus*

Table 2. Origin of different diazotrophic strains used to determine the specificity of primers designed to identify *G. diazotrophicus.*

Species	Strain	Strain origin
G. diazotrophicus	PAL5	Sugarcane - roots
	PAL3	Sugarcane - roots
	38F2	Sugarcane - stems
	AR20	Sugarcane - roots
	CFNE550	Sugarcane - Pink mealybug
	PRC1	Elephant grass - stems
	Ppe4	Sugarcane - roots
	3R2	Sugarcane - roots
	URU	Sugarcane - roots
	AF3	Sugarcane - stems
	PRJ50	Sugarcane - stems
Acetobacter hansensii	LMG1527	Vinegar
Acetobacter liquefaciens	LMG1381	dried fruits of *Diospyros kaki*
Herbaspirillum seropedicae	HRC54	Sugarcane - roots
H. seropedicae	ZAE94	Rice - roots
H. rubrisubalbicans	HCC103	Sugarcane - stems
H. frisingense	GSF30	*Miscanthus sachariflorus* - leaves
Azospirillum amazonense	CBAmC	Sugarcane - stems
	Y2	*Hyparrhenia rufa* - roots
A. lipoferum	SP59	Wheat - roots
A. brasilense	SP245	Wheat - roots
	SP7	*Digitaria decumbens* - roots
Burkholderia tropica	Ppe8	Sugarcane - stems
B. vietinamiensis	TVV75	Rice - rhizosphere
B. kururiensis	M130	Rice -roots
	KP23	-
B. silvatlantica	SRMrh-20	Maize-rhizosphere

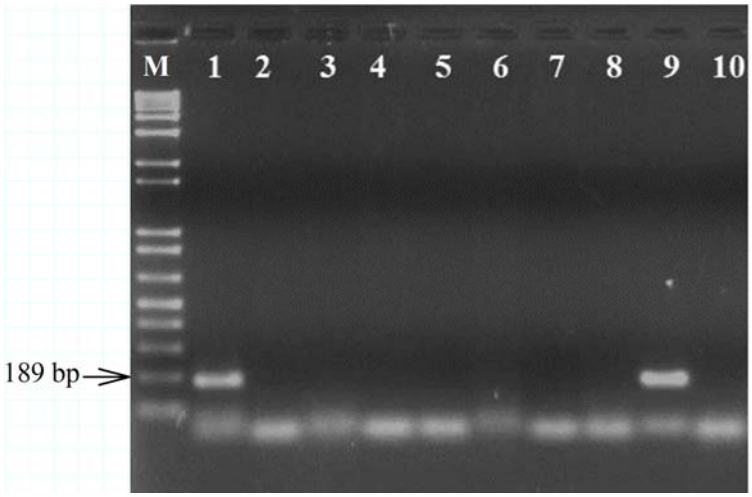

Figure 1. PCR amplification products of different nitrogen-fixing bacteria using species-specific primers for *G. diazotrophicus* (Gd). M - Molecular weight marker (1 kb Plus DNA ladder - Invitrogen®). Lanes 1-10 corresponded to template DNA from different diazotrophicus (see Table 2 for species identification): 1 - Gd (PAL5); 2 - Aa (CBamC); 3 - Hs (ZAE94); 4 - Hs (ZAE94); 5 - Hs (HRC54); 6 - Hs (HRC54); 7 - Bt (Ppe8); 8 - Hr (HCC103); 9 - Mixture of DNA from six bacterial strains; and the lane 10 was used as a control.

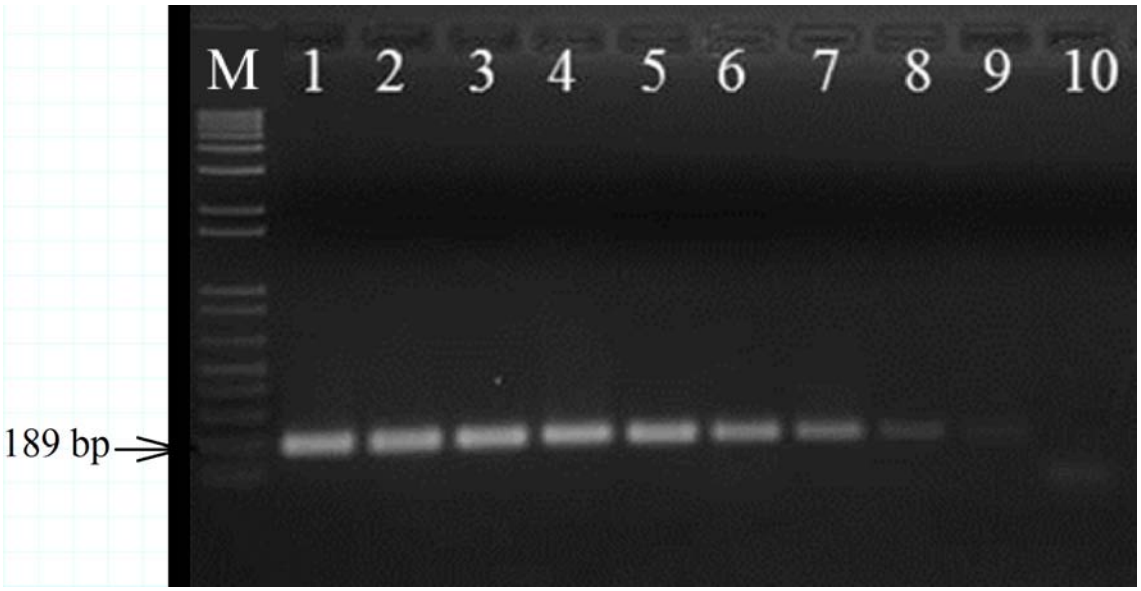

Figure 2. PCR products of *G. diazotrophicus* species amplified using species-specific primers. M - Molecular weight marker (1 kb Plus DNA ladder - Invitrogen®). Lane 1-10: DNA concentration in nanograms used as a template in the PCR reactions: (1) - 5 x10^{-1} ng; (2) - 5 x10^{-2} ng; (3) - 5 x10^{-3} ng; (4) - 5 x10^{-4} ng; (5) - 5 x10^{-5} ng; (6) - 5 x10^{-6} ng; (7) - 5 x10^{-7} ng; (8) - 5 x10^{-8} ng; (9) - 5 x10^{-9} ng; (10) - 5 x10^{-10} ng.

Table 3. Estimation of the number of bacteria according to the C_T average obtained for each DNA concentration.

[DNA] ng	No. of bacterial cells	Average C_T
5 x10^{-1}	1.21 x 10^{9}	8.49
5 x10^{-2}	1.21 x 10^{8}	12.77
5 x10^{-3}	1.21 x 10^{7}	16.31
5 x10^{-4}	1.21 x 10^{6}	19.66
5 x10^{-5}	1.21 x 10^{5}	22.71
5 x10^{-6}	1.21 x 10^{4}	25.98
5 x10^{-7}	1.21 x 10^{3}	29.20

cells present in the sugarcane samples (Figures 3 and 4). A linear regression between the number of bacteria and C_T yielded $R^2 = 0.99$ (Figure 3).

Detection and quantification of *G. diazotrophicus* in sugarcane plant tissues previously inoculated with a mixture of diazotrophic bacterial species

Total DNA was extracted from sugarcane plants grown under field conditions in the southern region of Brazil that were previously inoculated with a mixture of diazotrophic strains as well as from plants fertilized with nitrogen and non-inoculated control plants. A 189 bp amplified product was obtained using *G. diazotrophicus*-specific primers and confirmed by agarose gel electrophoresis. These results support the melting curve analysis of qPCR products.

The standard curve generated by the C_T average using different 16S rDNA concentrations permitted the calculation of the number of bacteria present in sugarcane plant tissues cultivated under field conditions by substituting the C_T values of the samples into the standard curve (Figure 3). The number of bacterial cells on sugarcane plant stalks of inoculated plants ranged from 2.3 to 2.6 log cells (6.5 x 10^4 - 1.2 x 10^5 bacteria per gram of fresh stalk) at 7 and 11 months, respectively (Figure 4). Although there were no significant statistical differences, for all treatments the number of cells varied with plant age. The control treatment showed a small

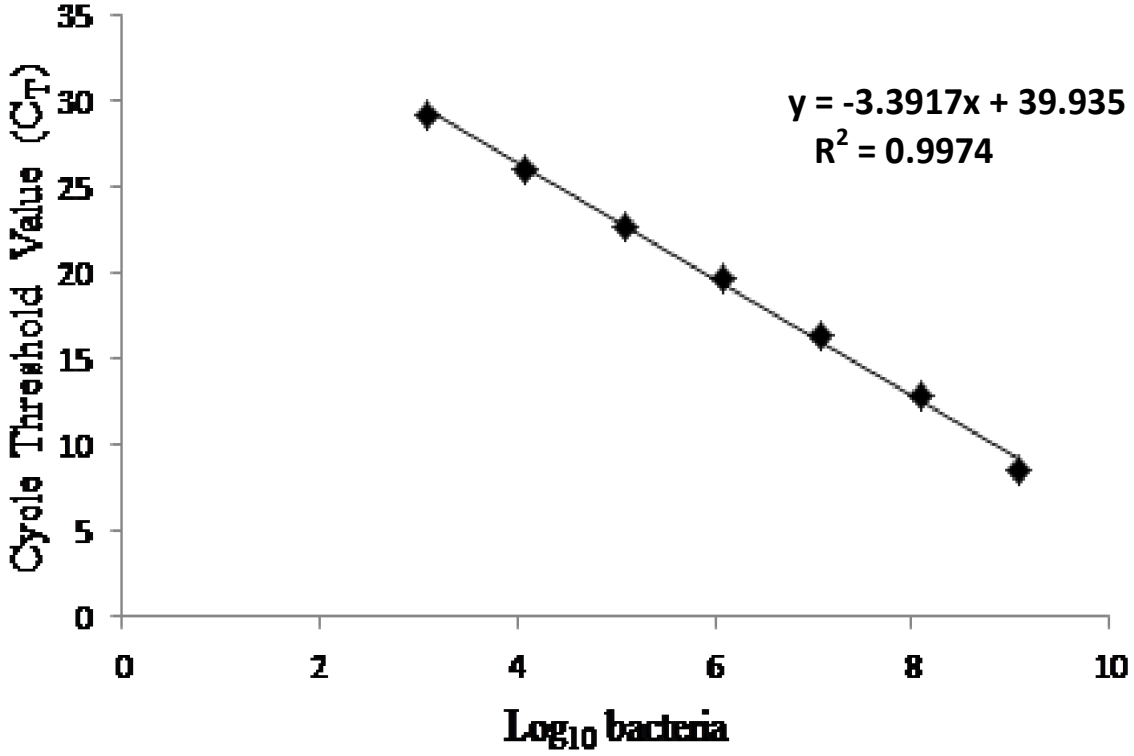

Figure 3. Standard curve dilutions of DNA versus C_T average. Three replicates were used for all dilutions.

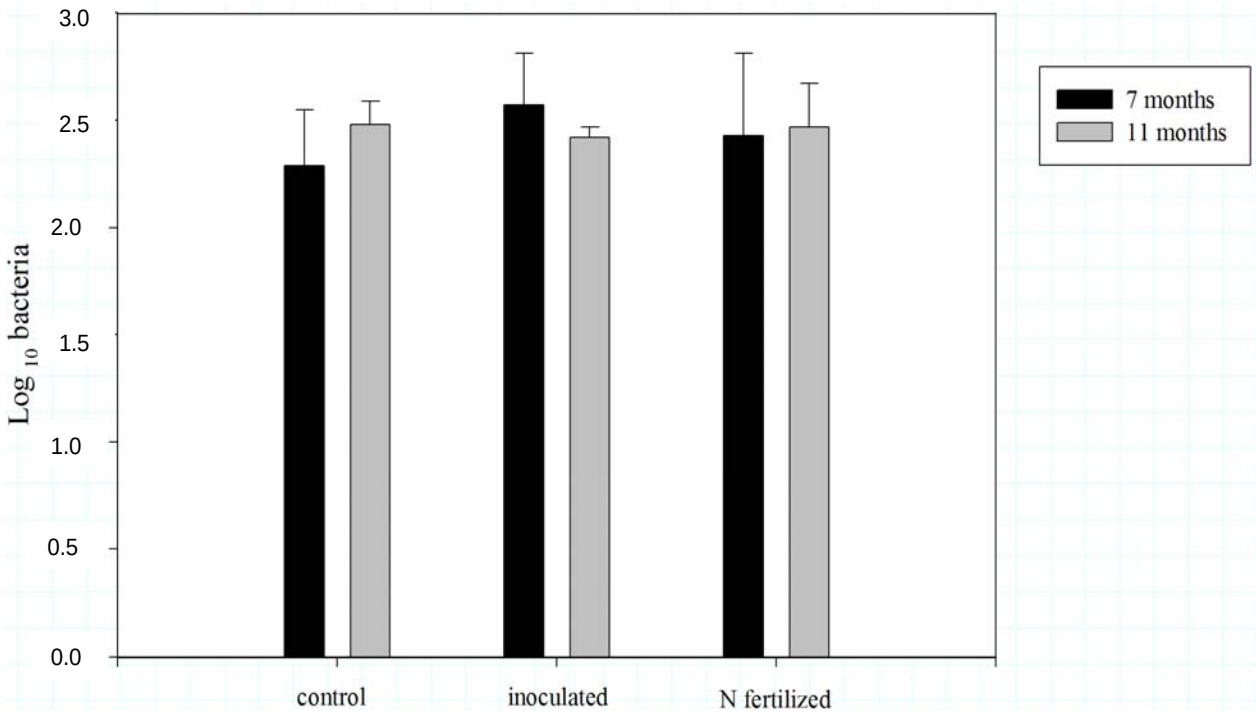

Figure 4. Quantification of *G. diazotrophicus* by real time PCR in inoculated (mixture of five diazotrophicus strains) and non-inoculated sugarcane plants harvested at two growth stages (7 and 11 months). The values did not differ statistically by LSD test at 5% significance.

increase from 2.3 to 2.5 log cells (6.5×10^4 - 8.0×10^4 bacteria per gram of fresh stalk) at 7 and 11 months, respectively, while inoculated plants showed a slight decrease from 2.6 to 2.4 log cells (1.2×10^5 - 6.7×10^4 bacteria per gram of fresh stalk). Plants fertilized with nitrogen had from 2.5 to 2.4 log cells (1.2×10^5 - 8.8×10^4 bacteria) per gram of fresh stalk at 7 and 11 months, respectively (Figure 4). The coefficient of variation generated by statistical analysis was 16%. It may be possible that the absence of statistical differences among the treatments was due to the presence of large number of bacterial cells even in the non-inoculated plants. Meanwhile, the most probable number bacterial counting method using semi-selective LGI-P medium did not show the presence of *G. diazotrophicus* within the same plant tissues (data not shown).

DISCUSSION

G. diazotrophicus has been isolated from roots and stems of sugarcane in different regions of the world (Baldani et al., 1997; Cavalcante and Dobereiner, 1988; Dong et al., 1994; Loganathan and Nair, 2003). In addition to fixing nitrogen, *G. diazotrophicus* benefits the host plant through the production of hormones and substances of biological control, as well as promoting phosphate and zinc solubilization (Dobbelaere et al., 2003; Fuentes-Ramirez et al., 1993; Natheer and Muthukkaruppan, 2012). However, to allow successful inoculation and manifest these benefits, the bacteria must first be able to establish viable colonies in the plant tissue amongst the other indigenous bacteria that are present.

Quantification of *G. diazotrophicus* is usually performed using the culture medium LGI-P (Reis et al., 1994) where bacterial growth is confirmed by the presence of characteristic pellicles formed on the surface of a vial containing semisolid LGI-P medium and the number of bacteria is estimated using the McCrady table (Reis et al., 1994). However, as discussed by Silva-Froufe et al. (2009) this method presents several limitations, such as the presence of other microorganisms that can inhibit *G. diazotrophicus* growth, which can lead to an under-estimation of the actual population. Furthermore, the method assume that all bacteria present in plant are isolated and not form aggregates, so the maceration extracts contain all of the bacteria present in the plant tissue in a homogeneous suspension of individual bacterial cells. These premises could be not real, but are important for dilutions based method (Silva-Froufe et al., 2009). Moreover, bacterial cells may be present in a viable but non-culturable form that may also lead to an underestimation of bacterial numbers, as was observed for the bacteria of the genus *Herbaspirillum* (Olivares et al., 1996).

On the other hand, qPCR has been used to detect and quantify various bacteria species present in different types of samples (Coelho et al., 2009; Jung et al., 2010; Lacava et al., 2006; Ruppel et al., 2006; Sellek et al., 2008; Timmusk et al., 2009; Trabelsi et al., 2009) and therefore may represent an alternative means for quantifying *G. diazotrophicus*.

The primers PAL5F and PAL5R showed high sensitivity and specificity for *G. diazotrophicus* even with the conventional PCR reaction and allowed detection of as little as 5 fg of template DNA, which is equivalent to 12 bacterial cells (Figure 2). This value was similar to results from a study using qPCR with species-specific primers for 16S rDNA to detect *Lactococcus garvieae* in food and environmental samples that found up to 32 fg of genomic DNA, which was equivalent to ten target bacterial cells (Jung et al., 2010).

Total DNA extracted from sugarcane tissues allowed us to quantify by SYBR Green I detection real-time PCR up to 2.3 log bacteria (6.5×10^4 bacteria per gram of fresh stalk) in non-inoculated plants (control) grown for 7 months in field conditions. Similar results were observed for the endophytic bacterium *Methylobacterium mesophilicum* during the colonization of *Catharanthus roseus* (Lacava et al., 2006) wherein a population of 4.6 $\log_{10}$ cells was detected when the plants were inoculated with only this bacterium and $\log_{10}$ 2.6 when the plants were inoculated with a mixture of *M. methylobacterium* and *Xylella fastidiosa*.

Plant growth-promoting rhizobacteria were also detected and quantified using qPCR and species-specific primers with Taqman probes for *Enterobacter radicincitans* (Ruppel et al., 2006). The population of this bacterium associated with *Brassica oleracea* ranged from 10^7-10^9 cells per gram of fresh leaf tissue, and around 10^8 cells per gram of fresh root (Ruppel et al., 2006). These studies showed a significant colonization of the leaves with a population in the range of 3.9×10^4 to 1.8×10^7 cells per gram leaf fresh weight depending on the concentration of the initial inoculum. In root tissue, this value was around 10^7 cells per gram of fresh roots 14 days after inoculation. The number of copies of *E. radicincitan* 16S rDNA represented approximately 10-16% of the total bacterial community.

Previous studies have used conventional PCR to detect endophytic bacteria in plant tissues of the family *Poaceae* (Muthukumarasamy et al., 2005; Sevilla et al., 2001). Although conventional PCR is very effective to detect endophytic bacteria, this method does not permit precise microorganism quantification. The real-time PCR enable the detection and quantification of endophytic bacteria, and can detect very low numbers of these microorganisms. In the present study, primers were designed to *G. diazotrophicus* able to detect up to 12 bacterial cells. This is the first report on the use of real-time PCR to quantify endophytic bacteria in sugarcane plants grown under field conditions and inoculated or not

with bacterium. The design and validation of primers for real-time PCR that are specific for *G. diazotrophicus* is the first important step that allows the use of this methodology for quantification of bacterial colonization of different plant tissues. Although this study used sugarcane stalks from plants at different ages, the same strategy can be used to quantify bacteria in other plant tissues. Furthermore, in addition to 16S RNA, other genes such as *nifH* (Church et al., 2005; Coelho et al., 2009; Juraeva et al., 2006) and housekeeping genes (Galisa et al., 2012) can also be targets for PCR analysis.

The pair of primers designed here for *G. diazotrophicus* was very species-specific. In contrast to the culture-dependent method using semisolid LGI-P medium where no *G. diazotrophicus* population was detected in plant tissues (data not shown), real time PCR could detect between 6.5×10^4 and 1.2×10^5 bacteria per gram of fresh stem tissues. Therefore, the technique showed high sensitivity that is sufficient to allow quantification of endophytic diazotrophic species colonization in sugarcane plant tissues, even when the bacterial population is very small and undetectable by culture-dependent methods.

Conflict of Interests

The authors have not declared any conflict of interests.

ACKNOWLEDGEMENTS

Financial support from Embrapa, CNPq/INCT-FBN and CNPq/CT-Agro is greatly acknowledged. Thanks also to CAPES and CNPq for the fellowship of both the first and last two authors.

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