Journal of Agriculture and Environmental Sciences
June 2015, Vol. 4, No. 1, pp. 148-154
ISSN: 2334-2404 (Print), 2334-2412 (Online)

Published by American Research Institute for Policy Development
DOI: 10.15640/jaes.v4n1a19
URL: http://dx.doi.org/10.15640/jaes.v4n1a19

Detection and Enumeration of Bradyrhizobia Cells by Real-Time PCR Quantification

Karla Cristina Stropa Goulart[1], Eliana Gertrudes de Macedo Lemos[2] & Jackson Antônio Marcondes de Souza[3]

Abstract

Bradyrhizobiumelkanii and Bradyrhizobiumjaponicum are nitrogen-fixing bacteria that establish symbiosis with soybean plants. The optimization of this symbiosis and the success of biological nitrogen fixation are reached by inoculating the seeds with strains of bradyrhizobia. The aim of this work consisted in the enumeration of bacterial cells of inoculants from different ages by real-time PCR quantification (qPCR) based on 16S rDNA and the evaluation of surviving cells rates under desiccation experiments. The results of counting by colony forming units (CFU) were correlated with those obtained by qPCR. All qPCR values were higher than those for CFU values in cell enumeration for all samples and conditions observed. Cell resistance to desiccation was shown until 24 hours onto soybean seeds, with considerable fall in all the inoculants after 48 hours under desiccation. This was probably due to biochemical and physiological changes in its metabolism, making use of defensive mechanism to the adverse conditions for its survival.

Keywords: Cellular enumeration, soybean inoculants, biological nitrogen fixation, bradyrhizobia

1.0 Introduction

The main sources of nitrogen (N) for soybean crops provide from application of nitrogen fertilizers and biological nitrogen fixation (BNF) that uses atmospheric N_2 through symbiotic association between legumes and rhizobia BNF is the process that involves the conversion of inert N_2 gas into ammonia (NH_3) through expending large amounts of energy to break triple chemical bonds N atoms (Oldroyd*et al.,* 2011). Bradyrhizobia are nitrogen-fixing bacteria that possess a complex enzyme named nitrogenase that is able to realize N_2 conversion to produced NH_3, which in turn is assimilated into organic compounds by soybean plants able to establish symbiotic interactions with those microrganisms (Kuykendal*et al.,* 1992). The maximization of the symbiosis events can be achieved by inoculation with selected strains of rhizobia performing the best and desirable characteristics, reducing the cost of soybean production. On the other hand, rhizobia inoculation is also a factor that contributes to reducing pollution in the environment by decreasing N fertilizers applications in the field (Abdel-Fattah I El-Shaarawi*et al.,* 2011). Official recommendation of *Bradyrhizobiumelkanii* and/or *Bradyrhizobiumjaponicum* strains for soybean's inoculants production in Brazil is made by Ministry of Agriculture, Livestock and Food, Supply (MAPA). These bradyrhizobia are genetic and physiological different (Soares and Passaglia 2010) and can be used separately or in combination. MAPA requires a periodic control of inoculants quality and found that in some cases there is a discrepancy between the numbers of cells detected by the CFU (colony forming units) traditional method of counting performed by different laboratories. In this paper, the development and applying of a new method for inoculants cell enumeration was tested by real-time PCR quantification (qPCR) based on 16S rDNA. This technique was applied both to pure cultures as to commercial inoculants for soybean crop and we compare results from traditional CFU method. Our intention is discuss and propose a novel technology to monitor and control the quality of inoculants production.

[1]Principal Scientist; Departmentof Technology, Faculdade de Ciências Agrárias e Veterinárias, Jaboticabal, Brazil, 14884-900. Fone: 55(16).
E-mail: karlabio@yahoo.com.br
[2]Research Associate, Department of Technology. Email: egerle@fcav.unesp.br)
[3]Technical Officer, Department of Biology Applied to Agricultural and Livestock. Email: jackson@fcav.unesp.br), Univ. EstadualPaulista, Unesp.

2.0 Materials and Methods

2.1 Biological Inoculants, Desiccation assay and Greenhouse

The composition of the inoculant consist of cells of *B. elkanii* SEMIA 587 and *B. japonicum*SEMIA 5079 maintained in a nutrient base formulation of amino acids, magnesium sulphate, monopotassium phosphate, glucose, sucrose, polymer, yeast extract, sodium chloride and Fe-EDTA. Inoculants presenting 1, 2, and 4 years after production date were named Inoculant 1, 2, and 4. Independent *B. japonicum* SEMIA 5079cells grown in YMB (Yeast Mannitol Broth) (Vicent, 1970), 48 h at 28°C by 140 rpm, were used as reference sample. The efficacy of nodulation was evaluated in the drying conditions after 4, 24 and 48 h of aerial exposition onto soybean seeds (M-SOY 8000RR) surface, previously treated 1 mg/ml cycloheximide. Inoculation was performed according to manufacturer recommendation. Cells adhered to 50 seeds were recovered by resuspension in sterile water after each incubation period. Genomic DNA of all samples was extracted according Marmur (1961) for subsequent qPCR analysis. In addition, seeds were planted in plastic pots containing vermiculite and maintained in greenhouse for 28 days for nodulation efficiency and most probable number (MPN) analysis (Oblinger and Koburger 1975). All pure and inoculants samples conditions were accompanied by CFU, pH, and turbidity analysis. CFU analysis were performed as official protocol recommended by MAPA. After serial dilutions and plating on solid YMB (Yeast Mannitol Broth) (Vicent, 1970) of 100 µl of bacterial suspensions at 10^{-4}, 10^{-5}, 10^{-6}, and 10^{-7}. Petri dishes were maintained for six days in B.O.D. at 28°C until appearance of colonies that were counted to determine cell number/ml. Turbidity analysis were executed using red filter in a Klett-Summerson equipment (Klett MFG).

2.2 qPCRStandard Curve Confection and Experiments

16S rDNA fragment amplified by fD1 and rD1 primers (Weisburg*et al.,* 1991) from genomic DNA of *B. elkanii* was purified by GFX kit (GE Healthcare) and cloned into pGEM-T easy vector (Promega, Madison, WI). The obtained plasmid was used in qPCR standard curve confection in order to determine the number of 16S rDNA molecules. DNA plasmid concentration was performed by NanoDropspectrophotometer (Thermo Scientific) and absolute qPCR standard curve was obtained by a [1:3] dilution factor. All amplifications were based on standard curve method performed by absolute quantification using a 7500 Real Time PCR System on Applied Biosystems (Foster City, CA) platform. Reactions were conducted in triplicate containing 12.5 µl SYBR® Green PCR Master Mix (Applied Biosystem) and run at (i) 40°C / 2 min; (ii) 95°C / 10 min; and (iii) 40 cycles of 95°C / 15 s and 60°C / 1 min. Primers (F: 5'-TTAGCCGTCGGGCAGTTTAC-3'; R: 5'-CCCAGGCGGAATGTTTAATG-3') were selected was based on homologous region combined by aligned sequences for rhizobiathat were deposited in Rhizobase. Consensus and primers specificity were confirmed by nucleotide BLAST comparisons.

2.3 Data Analysis and Cellular Enumeration

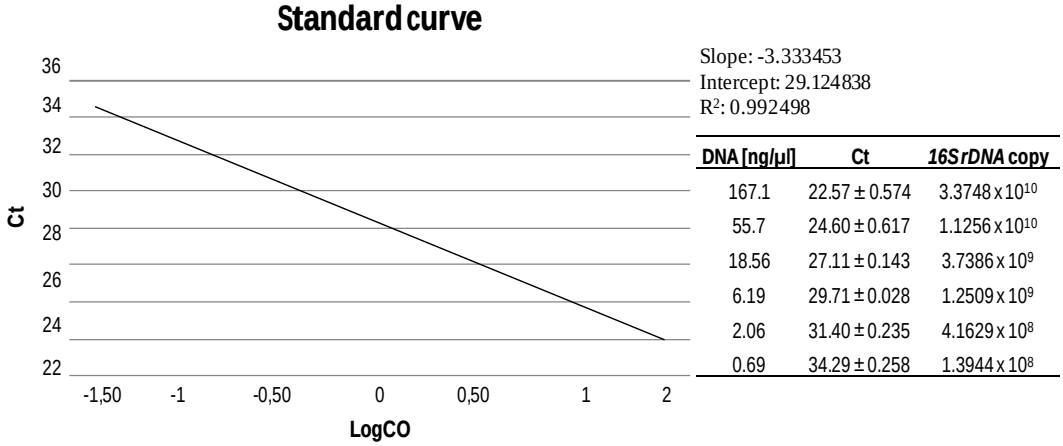
The number of standard plasmid molecules containing ribosomal fragment were calculated by the following formula based on Yun *et al.* (2006), $16S\ rDNA\ NC = [16S\ rDNA\ (g/\mu l)\ /\ (4515\ bp \times 660)] \times 6.022 \times 10^{23}$ where *NC* is number of copies; *16S rDNA (g/µl)* concentration was that obtained by qPCR; *4515 bp* correspond to molecular size of standard plasmid (3015 + 1500 base pairs); *660* is the average molecular weight of nucleotides in double-stranded DNA; and *6.022 x 10^{23}* is the Avogadro constant. For number of cells from CFU determination was used the formula *Cells/ml = CFU number x dilution factor x 10*. Relationship of qPCR and CFU data provide us with three significant parametric propositions: (i) 30 ml of bacterial suspension contained 13.1106 ng of 16S rDNA; (ii) 1.14 x 10^{10} cells present 0.43702 ng/ml of 16S rDNA by qPCR; and (iii) 2.96 x 10^{-4} % of 16S rDNA correspond to 0,3% of total quantified by NanoDrop. Finally, based on those parametric propositions unknown samples of interest might have defined their concentration values, number and percentage of 16S rDNA molecules, and number of bacterial cells. Data from pure culture of *B. elkanii* (Souza *et al*., 2012) were used as reference considering the lack of adverse factors that could interfere on the bacterial growth.

3.0 Results and Discussion

3.1 qPCR Data and 16S rDNA Quantification

In order to assure the specificity of 16S rDNA fragment applied in our studies, its sequence was resolved and *in silico* analysis was performed to compare results with those from databank revealing 97% of similarity with *Bradyrhizobiumsp.*

Ct values in function of DNA concentration representative of an unknown sample could be converted in number of copies of 16S rDNA sequences which in turn was related to number of cells as those based in reference samples (Fig. 01). The absolute quantification determines the copy number of a target of interest relating the signal emitted by qPCR with the data provided by the standard curve. In our work, since each plasmid contains only one insert, thus a copy of 16S rDNA allowed determination of the number of molecules based on high reliability for subsequent analyzes (Fig. 01). For this kind of purpose plasmidic DNA functions better than genomic DNA for qPCR determinations. The number of gene and transcript copies for 16S rDNA are highly variable per operon and they are also changeable according to species (Klappenbach*et al.*, 2000, Smith and Osborn, 2009). In prokaryotic cells genes for small (16S rRNA) and large (23S and 5S rRNA) ribosomal subunits are organized in multiple copies of a chromosomal operon fused in 16S/23S/5S linear arrangement. These rRNAs genes are cotranscribed in a single primary transcript ensuring they are produced in the same amounts (Tourova, 2003).



DNA [ng/µl]	Ct	*16S rDNA* copy
167.1	22.57 ± 0.574	3.3748×10^{10}
55.7	24.60 ± 0.617	1.1256×10^{10}
18.56	27.11 ± 0.143	3.7386×10^{9}
6.19	29.71 ± 0.028	1.2509×10^{9}
2.06	31.40 ± 0.235	4.1629×10^{8}
0.69	34.29 ± 0.258	1.3944×10^{8}

Figure 01: Standard curve generated after serial dilution of 16S rDNAamplicon previously quantified (ng/µl). Slope, intercept and R^2 are parameters for amplification efficiency and sensitivity. Cycle threshold (Ct) values are shown with standard deviation. The equation of the line is *y = -3.333453x + 29.124838*. This algorithm guides the following calculus for determination of unknown samples by plotting the Ct (Cycle threshold) values against the logarithm of the initial DNA template copy numbers.

Case *et al*. (2007) in order to explain the existence of multiple 16S rDNA operons raised the hypothesis that these arrays of operons allow a multiplier effect on transcription, allowing the bacterium to multiply rapidly in response to environmental changes. In addition, functional differentiation between rRNA operons has allowed the differential expression of them in response to environmental changes and resource availability (Klappenbach*et al*., 2000). Toledo *et al.* (2009) used 16S rDNA for the characterization of rhizobia suitable for the production of inoculants and stated that the molecular characterization of strains collections on the basis of conserved genes is essential to compare and identify soil rhizobia. Comparative studies based on 16S rDNA analysis are suitable not only among bacteria but also with 16S rDNA sequences from archeobacteria and the 18S rDNA from eukaryotes (Claridge III, 2004).

3.2 Cellular Enumeration and Physiological Aspects of Biological Inoculants

All qPCR values were higher than those for CFU values in cell enumeration for all samples and conditions observed (Table 01 and 02).

Table 01: Comparison of Methods for cell Enumeration in Soybean Inoculants, as Determined by CFU, qPCR, and MPN

Inoculant	CFU	qPCR	MPN
1	1.43×10^{9}	3.60×10^{10}	___
2	3.32×10^{9}	___	2.72×10^{7}
4	1.48×10^{8}	6.43×10^{12}	3.0×10^{7}

Table 02: Cell Recovery and Counting by CFU and qPCR after Desiccation Assay onto Soybean Seeds

Sample	*t* =0h		*t* =4h		*t* =24h		*t* =48h	
Analysis	CFU	qPCR	CFU	qPCR	CFU	qPCR	CFU	qPCR
Inoculant 1	2.09×10^4	9.50×10^{10}	___	6.24×10^{10}	___	3.64×10^{10}	___	2.07×10^{11}
Inoculant 2	2.18×10^4	1.80×10^{10}	___	7.83×10^{10}	___	___	___	6.82×10^{10}
Inoculant 4	1.06×10^4	2.16×10^{11}	___	5.86×10^{10}	___	___	___	4.85×10^{11}

Data from Inoculant 1 (Table 01) were according to commercial exigency by supervising bureau that establishes the minimum concentration of 1.0×10^9 viable cells per gram or milliliter within the expiration date. Inoculant 1 and Inoculant 2 had yet presented viable cells by CFU although expiration date was due in six and eighteen months ago, respectively (Table 01). However, Inoculant 4 had presented reduced number of cells by CFU counting method otherwise qPCR method (Table 01). Contradictory data were confronted by MPN counting method, which have proven the low performance of expired inoculants even if they were able to nodulate soybean roots (Table 01). In addition, it is important to notice that MPN method should be used to associate functional feat of inoculants. A number of parallel studies conducted by our group have shown a reduced expression of genes related to biological nitrogen fixation performed by *B. elkanii* in vitro and in vivo (Marcondes*et al.,* 2008) and also associated to soybean´s inoculants aging and greenhouse effects (Marcondes and Lemos 2011). Samples of commercial inoculants for soybean showed some alkalinization when stored for long periods above one year (Fig. 02).

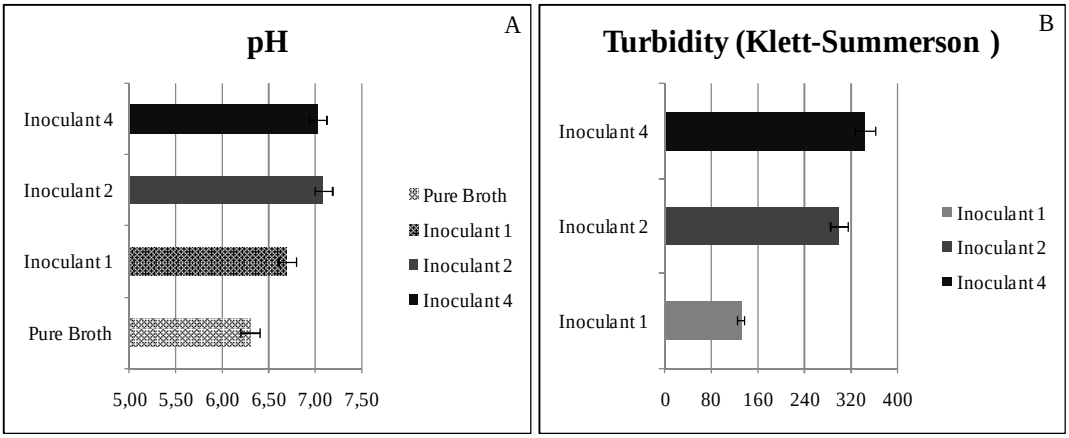


Figure 02: Physiological Indicators for Soybean Inoculants in Different Ages using pH (A) and Turbidity (B) Parameters

This confirms that bacteria tend to alkaline medium by using the available compounds and excreting cations, which gives an increase in pH in concern with storage time and availability of nutrients. A slight reduction in pH for Inoculant 4 when compared with Inoculant 2 indicates a possible diminution in the number of viable cells. The Inoculant 4 had also showed higher turbidity compared to the others (Fig. 02), raising the possibility that inoculants stored over the years may have exopolysaccharides (EPS) in high amounts in its composition. The older the inoculants, a higher concentration of this EPS it has (Castellane and Lemos 2007). The extracellular EPS can increase desiccation tolerance, as suggested by Deaker*et al.* (2004). This resistance to desiccation induces some cells to develop survival strategies that alter their physiology when subjected to stress conditions, as those induced by inoculants aging. The production of polysaccharides can act as a protective barrier against biotic and abiotic factors, allowing the survival and persistence of bacteria in the soil. The inconsistency observed among counting methods in our study not only reflects the best performance of qPCR technique but also led us to think if bradyhizobia could enter viable but nonculturable (VBNC) state (Oliver, 2005) and that dead cells are also being counted. Several factors could influence the measurements as those related to differences in DNA extraction efficiency and bacterial growth rate.

Kim and Wang (2009) reported the difficulty to detect *Candidatus*Liberibacterasiaticus using FISH (Fluorescent in situ hybridization) targeting 16S rDNA and associate that to the high ratio of dead/live cells which contributes to the unexpected low 16S rDNA copy number for this microrganism. Buy the other hand, cell counts determined by qPCR, FISH, and DAPI (4',6'-diamidino-2-phenylindole) staining methods were almost similar among them when applied to human intestinal *Bificobacterium*. In this last case, the authors suggest that the sensitivity of measurement by qPCR and DAPI staining may not vary greatly depending on the species or strain, while qPCR method should be 10 to 100 times more sensitive than the culture and FISH methods (Matsuki*et al.,* 2004).

3.3 Desiccation Resistance

CFU determination in this analyses was able just for samples at time of inoculation, *t*=0h, for all inoculants probably because long periods of air exposition were harmful for bacterial growth in Petri dishes. Thus, the qPCR method was more embracing and it reports better measurements and results (Table 02). Measurements of desiccation resistance cells by qPCR were superior to those obtained in pure inoculants (Table 01) and greenhouse tests showed sufficient nodulation on soybean roots as presented by MPN assay. Data from pure inoculants (Table 01) performed in liquid products according to the recommended dose by manufacturer was different from data from the same products in contact with seeds (Table 2). It is clear that the contact of bradyrhizobia cells with the surface of the seeds have affected their recovery even at *t*=0h. The age of inoculants and the time or air exposition seem be responsible for decreasing the number of detected cells (Table 02). Inoculant 4 presented contradictory results when compared to the others and these reflect it physiological constraints due the oldest age. Cells in inoculants could represent a physiological state similar to that observed in stationary phase. Bacterial growth in inoculants conditions should be characterized by starvation periods resulting in metabolically less active and more resistant cells. There are situations in which the number of cells/ml decreased considerably by the fact that oxygen restriction is so prolonged that prevents cell developing when exposed again to an atmospheric environment. In such case, cells must reprogram their respiratory metabolism by changing gene expression and it will reflect on nodulation efficiency (Marcondes and Lemos 2011).

We do not confirm that BNF process obtained through older inoculants is as efficient as those obtained by newer inoculants application, however, the composition of inoculants used in this study appears enhance the resistance of the cell wall of bradyrhizobia under adverse conditions for a certain period. Limitations and rapid death of strains of *Rhizobium* were observed when they are applied onto seeds or soil. In addition, it seems that rhizobia enter into VBNC state under stress for a certain period (Streeter *et al.,* 2007). The development of this technique makes clear that the qPCR method should be used in cases that require a confirmation of the number of CFU when there is some discrepancy between results from different laboratories. The proposed methodology should be used as a deciding factor when necessary once qPCR provides a reliable estimate of the number of cells so quickly. Considering this, the cell enumeration by 16S rDNA quantification is an innovative and efficient proposal since there was substantial difference in results between the techniques analyzed. The fact that physiological state of rhizobia inoculants is be sufficiently changeable throughout the storage period, the cellular enumeration for both methods do not reveal real state of the biological system in question and additional functional analysis as those performed by MPN counting method should be maintained. Thus, it is mandatory that the expiration time should be observed or reduced for the use of best quality products. Bradyrhizobia cells in liquid inoculants for soybeans used in this study certainly altered their physiology conditions for adapting to the storage period but without reduction of the number of cells.

Cells were also able to adapt and resist desiccation onto seeds presenting considerable number of nodules observed in root plants exposed to greenhouse effects. Expiration date was not deleterious in that inoculants formulation considering the number of cells as indicated by qPCR determination and nodulation efficiency, but no statement can be made to the efficiency of nitrogen fixation. The comparative analysis by both methods for cell enumeration has indicated that qPCR is more accurate once it expands quantification for cells in VBNC state even as we do not forget that many cells might be dead. Many discussions could be argumentative for VBNC state and there is no evidence of this case. However, a simple test for viability based on fluorescence microscopy technique will help to assure the percentage of truly dead cells.

Acknowledgements

The authorsthankto Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP) for financial supportand Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) for thescholarshipgrantedto Goulart, K.C.S. Thisworkregardsto Instituto Nacional de Ciência e Tecnologia de Fixação Biológica de Nitrogênio (INCT onBiologicalNitrogenFixation).

4.0 References

Case R, Boucher Y, Dahllöf I, Holmström C, Doolittle WF and Kjelleberg S (2007). Use of 16S rRNA and rpoB genes as molecular markers for microbial ecology studies. Applied and Environmental Microbiology**,** 73(1):278-288.

Castellane T and Lemos EGM (2007).Exopolysaccharides composition produced by rhizobia under different carbon sources.PesquisaAgropecuáriaBrasileira, 42:1503–1506.

Claridge III JE (2004). Impact of 16S rRNA gene sequence analysis for identification of bacteria on clinical microbiology and infectious diseases. Clinical Microbiology Reviews, 17(4):840-862.

Deaker R, Roughley RJ and Kennedy IR (2004). Legume seed inoculation technology-a review. Soil Biology & Biochemistry, 36:1275-1288.

El-shaarawi AI, Sabh AZ, Abou-Taleb SM and Ghoniem AE (2011).Effect of inorganic nitrogen and Bradyrhizobiumjaponicum inoculation on growth and yield of soybean. Australian Journal of Basic and Applied Sciences, 5(10): 436-447.

Kim JS and Wang N (2009). Characterization of copy numbers of 16S rDNA and 16S rRNA of Candidatusliberibacterasiaticus and the implication in detection in planta using quantitative PCR. BMC Research Notes**,** 2:37.

Klappenbach JA, Dunbar JM and Schmidt TM (2000).rRNA operon copy number reflects ecological strategies of bacteria. Applied and Environmental Microbiology**,** 66(4):1328-1333.

Kuykendall LD, Saxena B, Devine TE and Udell SE (1992). Genetic Diversity in Bradyrhizobiumjaponicum Jordan 1982 and a proposal for Bradyrhizobiumelaknii sp. Nov. Canadian Journal of Microbiology, 38:501-505.

Marcondes J, Cantão, M, Alves, MC, Lemos EGM (2008). Transcriptional profile of Bradyrhizobiumelkanii SEMIA 587 in symbiosis with soybean (Glycine max L. Merrill) analyzed by DNA microarray. Biological Nitrogen Fixation: Towards Poverty Alleviation through Sustainable Agriculture, pp 299-300. Dakora FD, Chimphango SBM, Valentine AJ, Elmerich C, Newton WE.Springer, Pretoria, South Africa.

Marcondes J, Lemos EGM (2011). DNA microarray applied to data mining of Bradyrhizobiumelkanii genome and prospection of active genes. Knowledge-oriented applications in data mining.pp 229-244. Funatsu K. In Tech, Vienna, Austria.

Marmur J (1961). A procedure for the isolation of deoxyribonucleic acid from microorganisms. Journal of Molecular Biology, 3:208-218.

Matsuki T, Watanabe K, Fujimoto J, Kado Y, Takada T, Matsumoto K and Tanaka R (2004). Quantitative PCR with 16S rRNA-gene-targeted species-specific primers for analysis of human intestinal bifidobacteria. Applied and Environmental Microbiology, 70(1):167-173.

Oblinger JL and Koburger JA (1975).Understanding and Teaching the Most Probable Number Technique. Journal Milk Food Technology, 38:540-545.

Oldroyd GED, Murray JD, Poole PS and Downie A (2011).The Rules of Engagement in the Legume-Rhizobial Symbiosis. Annual Review Genetic, 45:119-144.

Oliver JD (2005). The Viable but Nonculturable State in Bacteria. The Journal of Microbiology, 43:93-100.

Smith CJ and Osborn AM (2009).Advantages and limitations of quantitative PCR (q-PCR)-based approaches in microbial ecology. FEMS Microbiology Ecology, 67:6-20.

Soares RA and Passaglia LMP (2010).Application of Representaional Difference Analysis to Identify Genomic Differences between Bradyrhizobiumelkanii and B. japonicum species. Brazilian Journal of Microbiology, 41:1142-1151.

Souza JAM, Tieppo E, Magnani GS, Alves LMC, Cardoso LR, Cruz ML, Oliveira LF, Raittz RT, Souza EM, Pedrosa FO, Lemos EGM (2012). Draft Genome Sequence of Nitrogen-Fixing Symbiotic Bacterium Bradyrhizobiumelkanii. Journal of Bacteriology, 194:3547-3548.

Streeter JG (2007). Factors affecting the survival of Bradyrhizobium applied in liquid cultures to soya bean [Glycine max (L.) Merr.]seeds. Journal Applied Microbiology, 103:1282-1290.

Toledo BFB, Marcondes J and Lemos EGM (2009).Caracterização de rizóbios indicados para produção de inoculantes por meio do seqüenciamento parcial do 16S rRNA. PesquisaAgropecuáriaBrasileira, 4:384-391.

Tourova TP (2003). Copy number of ribosomal operons in prokaryotes and its effects on phylogenetic analyses. Microbiology, 72(4)389-402.

Vincent JM (1970). A manual for the practical study of root-nodule bacteria. pp164. Blackwell, Oxford.

Weisburg WG, Barns SM, Pelletier DA and Lane DJ (1991).16S ribosomal DNA amplification for phylogenetic study. Journal of Bacteriology, 173:697-703.

Yun JJ, Heisler LE, Hwang IIL, Wilkins O, Lau SK, Hyrcza M, Jayabalasingham B, Jin J, Mclaurin J, Tsao M and Der SD (2006). Genomic DNA functions as a universal external standart in quantitative real-time PCR. Nucleic Acids Research, 34(12).