Occurrence of diverse *Bradyrhizobium* spp. in roots and rhizospheres of two commercial Brazilian sugarcane cultivars

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Abstract

The genus *Bradyrhizobium* harbors many endosymbionts of legumes, but recent research has shown their widespread presence in soils and in non-legumes, notably in roots of sugarcane. This study aimed to investigate the *Bradyrhizobium* sp. community density in the endosphere and the rhizosphere of two commercial sugarcane cultivars. Samples of the rhizosphere and root endosphere of two Brazilian sugarcane cultivars (RB867515 and IACSP95-5000) were collected, serially diluted, and inoculated on axenic cowpea (*Vigna unguiculata*) and the induction of nodules was evaluated. Based on the results, a density was estimated of at least 1.6×10^4 rhizobia g root⁻¹ in rhizosphere samples and up to 105 rhizobia g root⁻¹ in endosphere. BOX-PCR profiling of 93 *Bradyrhizobium* isolates revealed genetic variability, with some dominant (up to 18 representants) and less dominant genotypes. 16S rRNA and ITS sequence analyses confirmed nine phylotypes, six of which pertained to the *B. elkanii* clade and three to the *B. japonicum* clade. Five isolates were genetically similar to the recently described species *B. sacchari*. There was no effect of the factors “plant cultivar” and “root compartment” on *Bradyrhizobium* sp. community composition and the most abundant genotypes occurred both in rhizosphere and endosphere of both cultivars. Therefore, this study confirms the natural presence of diverse *Bradyrhizobium* spp. in sugarcane root systems (mainly the rhizosphere) and indicates that certain *Bradyrhizobium* phylotypes have a special affinity for sugarcane root colonization.

Keywords Endophytic bacteria · Rhizobia · Non-legumes · Plant growth–promoting bacteria · Symbiotic bacteria · Most probable number

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Introduction

Rhizobia from genera like *Rhizobium* and *Bradyrhizobium*, for example, have been long known for their role as microsymbionts of many plants from the *Fabaceae* family, on which they induce root nodules involved in biological nitrogen fixation (BNF) [1]. However, it has become clear that these bacteria are present in a variety of different environments, including in association with non-leguminous plants. For example, Yanni and collaborators [2] found *Rhizobium leguminosarum* bv. *trifolii* in rice roots and found that inoculation with these bacteria can promote rice growth. Photosynthetic *Bradyrhizobium* sp. isolates were also found as endophytes in African wild rice [3]. Culture-independent studies with sugarcane have shown the presence of *nifH* cDNA phylotypes related

to *Rhizobium* and *Bradyrhizobium* in sugarcane tissues, suggesting that these bacteria are active in plant-associated BNF [4–6]. More recently, the presence of rhizobia (genera *Rhizobium* and *Bradyrhizobium*) in sugarcane tissues has been confirmed by 16SrRNA amplicon sequencing for sugarcane growing in Brazil [7], China [8], and Australia [9], confirming the ubiquitous occurrence of rhizobia in sugarcane microbiomes.

Culture-independent studies indicating the presence of rhizobia sugarcane tissues induced us to focus on the cultivation of these bacteria, because cultivation is a pre-requisite for more detailed studies and biotechnological application of such bacteria, for example as inoculants. An advantage of using rhizobia as inoculants for non-legumes, as compared to other bacterial genera, is that Brazilian inoculant industry is well adapted to producing these bacteria, facilitating their adoption in industrial processes. A diverse collection of symbiotic and non-symbiotic *Bradyrhizobium* sp. isolates has been obtained from surface disinfected roots, but not from shoots, of sugarcane cultivar RB867515 [10]. Some isolates, including the recently described *B. sacchari* [11] presented in vitro nitrogenase activity, thus indicating a role of these bacteria in non-symbiotic BNF. However, the population density of these sugarcane-inhabiting rhizobia has not been determined and the distribution of rhizobia over rhizosphere and root endosphere compartments is unknown. In other plant species, it is known that the composition of the associated microbial community can be influenced by the host genotype and compartment [12, 13]. Recently, evidence was also found that root-associated bacterial communities may differ at the genus level among *Saccharum* spp. (ancestors of modern sugarcane hybrids [8]).

Therefore, the present study aimed to improve our understanding of the ecology of *Bradyrhizobium* spp. associated with the sugarcane root system by investigating the abundance and diversity of *Bradyrhizobium* spp. in rhizosphere and endosphere of two sugarcane cultivars through culture-dependent methodology and genetic characterization. Two cultivars were studied: RB867515, the most planted and harvested sugarcane cultivar in Brazil and IACSP95-5000, a cultivar mainly used in the state of São Paulo [14].

Materials and methods

Sampling of sugarcane

Samples of sugarcane root systems were taken from sugarcane plants planted in September 2014 at the experimental field of Embrapa Agrobiologia in Seropédica, Brazil (coordinates S 22° 33' 53.8"; O 43° 40' 15.4"). The layout consisted of four experimental blocks, each containing eight lines of different sugarcane cultivars, including RB867515 and IACSP95-5000, used in this study. Distance between lines was 1 m and each block measured 8 × 8 m. Blocks were separated by

3-m unplanted strips with some spontaneous weeds. Root and rhizosphere were sampled from randomly chosen plants of each of the two cultivars from three of the four experimental blocks in April 2016, 5 months after the last cutting, when plants were approximately 1.5–2 m high. For practical reasons, the three experimental blocks were sampled and processed on different days. Plants were uprooted and root systems with adhering soil were transported to the lab in plastic bags.

Rhizosphere and root endosphere sampling

Samples of the rhizosphere and root endosphere compartments were obtained from both sugarcane cultivars from three experimental blocks (replicates), giving a total of 3 (replicates) × 2 (cultivars) × 2 (compartments) = 12 samples in total. Rhizosphere samples were obtained mainly as described by Barillot and collaborators [15] with some modifications. Using latex gloves, excessive soil was removed from the root systems and 1 g of non-lignified young roots, with only firmly adhering soil particles, was selected per sample and placed in 50-ml capacity conical tubes containing sterile saline solution (0.7% NaCl) amended with tween 20 (0.01%) to obtain a total volume of 5 ml. The tubes were shaken using a vortex device for 15 min. The resultant suspensions were considered as rhizosphere samples.

To obtain endosphere samples, the root samples used to obtain the rhizosphere samples were rinsed with sterile saline solution and incubated in sodium hypochloride solution (2.5%) for 5 min in order to kill surface-associated bacteria and then washed 10 times with sterile water. Samples of the last washing step were plated on yeast extract mannitol agar (YMA) plates, which were incubated to verify the absence of surviving bacteria [16]. The roots were macerated in autoclaved mortars and suspended in saline solution in a total volume of 5 ml in 15-ml capacity conical tubes. These suspensions were considered the endosphere samples.

Serial dilution of samples and inoculation of gnotobiotic *Vigna unguiculata* plants

The rhizosphere and endosphere samples were diluted serially with base 5. The suspensions obtained as described above were considered as dilution 5^{-1} and these were diluted serially until level 5^{-6} using sterile plastic ware and saline solution.

V. unguiculata (cowpea) seeds were surface-disinfected by immersion in ethanol (70%) for 1 min, followed by 3 min in sodium hypochloride solution (2.5%). After 10 washing steps in sterile water, the seeds were placed on water agar plates (1%) and incubated at 28 °C for 3 days. After germination, seedlings with apparent absence of microbial contaminants were transferred to 150-ml capacity glass tubes containing a slope of 60-ml agarized sterile nitrogen-free nutrient solution [17]. Transferred plantlets were each inoculated with 1 ml of the

samples from the serial dilutions described above. For each of the 12 original sugarcane samples, serial dilutions were prepared and inoculated in triplicate. Plants were incubated in a growth chamber with artificial light during 12 h day⁻¹ at approximately 28 °C. The tubes were initially capped with polypropylene bags and when they grew out of the tubes, the bags were removed and the opening of the tubes was sealed around the stem with plastic film. Non-inoculated plants and plants inoculated with cowpea-strain BR 3262 [18] were included as negative and positive nodulation controls, respectively.

Evaluation of nodulation and estimation of rhizobium numbers

Twenty-six days after inoculation (DAI), the presence or absence of nodules on each individual cowpea plant was evaluated (Table 1). When possible, the most probable number (MPN) method, using the McCrady tables, was applied to estimate numbers of rhizobia in the original samples [19, 20]. Because this was not possible for several samples, a minimum number of rhizobia were also estimated for samples that were entirely positive until the last dilution step. The estimation was based on the assumption that the presence of nodules on a plant inoculated with a certain dilution level requires that at least one rhizobial cell be present in the inoculated 1-ml dilution aliquot. This number was thus determined as the inverse of the dilution factor (dilution factor⁻¹).

Isolation of rhizobia from nodules

Nodulated cowpea plants were gently removed from the tubes and around three nodules per plant were selected for isolation. The nodules were disinfected superficially with ethanol and hypochlorite solution as described above for cowpea seeds. After that, individual nodules were squashed on YMA using forceps and the content was spread using a platinum loop. The plates were incubated at 28 °C until bacterial colonies appeared. Cultures were re-streaked and pure cultures obtained. Isolates were named as follows: sugarcane cultivar (RB = RB867515 or IA = IACSP95-5000), followed by compartment (R = rhizosphere or E = endosphere), and followed by the number of the cowpea plant of origin (numbers 1–227). In the case where isolates were obtained for more than one nodule per plant, a lower-case letter was added to distinguish among them. Table S1 shows the relation between plant numbers and inoculant treatment.

Genetic characterization of isolates

Cell material was scraped from pure cultures on agar plates and suspended in ultrapure water in microtubes using a vortex manifold. The suspensions were centrifuged at maximum speed for 10 min, the supernatant was discarded, and the cell pellets were used for DNA extraction using the Wizard Genomic DNA Purification Kit (Promega), according to the manufacturer's recommendations. Genomic DNA integrity and quantity was estimated by gel electrophoresis. BOX-PCR was performed as described [21] using approximately 100-ng bacterial DNA as

Table 1 Numbers of nodulated cowpea plants after inoculation with different dilution levels of endosphere and rhizosphere samples from sugarcane cultivars RB 867515 and IAC SP95-5000

Block	1				2				3			
Sample	RBR	RBE	IAR	IAE	RBR	RBE	IAR	IAE	RBR	RBE	IAR	IAE
Dilution level /isolation source												
5 ⁻¹	2/3	1/3	2/3	2/3	2/2*	3/3	3/3	3/3	2/2*	2/2*	3/3	1/3
5 ⁻²	3/3	1/3	3/3	1/3	3/3	3/3	3/3	1/3	3/3	2/3	3/3	2/3
5 ⁻³	2/2*	2/3	3/3	0/3	3/3	2/3	3/3	1/3	3/3	2/3	3/3	1/3
5 ⁻⁴	3/3	1/3	3/3	0/2*	3/3	0/2*	2/3	0/3	3/3	0/3	3/3	0/3
5 ⁻⁵	2/3	0/3	3/3	0/3	3/3	0/3	2/3	0/3	3/3	0/3	3/3	0/3
5 ⁻⁶	3/3	0/3	3/3	0/3	2/2*	0/3	3/3	0/3	3/3	0/2*	3/3	0/3

For each sample/dilution level, three individually grown cowpea plants were inoculated. *One out of three plants did not develop well or died during the experiment. RBR, cultivar RB867515/rhizosphere; RBE, cultivar RB867515/endosphere; IAR, cultivar IACSP95-5000/rhizosphere; IAE, cultivar IACSP95-5000/endosphere

template and the Box A1R primer. BOX-PCR products (10 µl) were separated by electrophoresis on agarose (2%) gels at 70 V during 6 h. The 1-kb plus ladder (Invitrogen) was used as a reference. After electrophoresis, gels were stained with ethidium bromide, visualized, and photographed on a photo documentation system. The software BioNumerics 7.6 (Applied Maths, Belgium) was used to analyze the results with the “number of different band” similarity coefficient and UPGMA clustering method.

For sequence analyses, DNA samples (approximately 50 ng) were submitted to PCR reaction with gene-specific primers. For the 16S rRNA gene, primers 27F and 1492R were used as previously described [10]. ITS sequences were amplified using primers ITSF and ITSReub [22]. PCR products were evaluated using gel electrophoresis and post-reaction cleanup was performed by treatment with exonuclease I (Exo I) and thermosensitive alkaline phosphatase (FastAP) (ThermoFisher Scientific). Bi-directional Sanger sequencing reactions were performed using the BigDye Terminator v3.1 Cycle Sequencing kit (ThermoFisher Scientific) and the same primers used for PCR amplification. Samples were analyzed using an ABI 3500 genetic analyzer (ThermoFisher Scientific). BioNumerics 7.6 (Applied Maths, Belgium) was used to analyze and assemble DNA sequences. 16S rRNA and ITS sequences were deposited in the GenBank under accession numbers shown in Fig. S1. The accession numbers of 16S rRNA sequences for the four fast-growing isolates are as follows: IAE45b (MK776949), IAE71a (MK776950), IAR64 (MK776951), and RBR179a (MK776952).

Phylogenetic analyses

Sequence alignments and phylogenetic analyses were performed using MEGA 6 software package [24]. Multiple sequence alignments were generated using Muscle [25]. Phylogenetic trees were calculated using the maximum likelihood (ML) reconstruction method [26], using the complete deletion option. The most adequate model for calculating phylogenies was determined for each alignment using the integrated model selection tool of MEGA 6. The strength of the phylogenetic tree topologies was evaluated using the bootstrap method by applying 500 pseudo replicates.

Results

Nodule-inducing rhizobia associated with sugarcane roots

MPN could be determined for endosphere samples IAE block 1 (6.1 rhizobia g⁻¹ root), IAE block 2 (18 rhizobia g⁻¹ root), and RBE block 2 (105 rhizobia g⁻¹ root). MPN determination could not be performed for the other samples because there was no

consistent decrease in nodule numbers in relation to the extract dilution levels, or because nodulation occurred at all dilution levels (down to dilution 5⁻⁶), which was the case for rhizosphere samples (Table 1). Nevertheless, the fact that nodulation occurred in almost all the cowpea plants inoculated with the most dilute samples (5, 6) of rhizosphere extract suggests that there were at least $1 \times 5^6 = 1.6 \times 10^4$ rhizobia g root⁻¹ (Table 1). Similar numbers were observed for the roots of both sugarcane cultivars; therefore, similar numbers of rhizobia were present in both.

Isolation of sugarcane rhizobia from cowpea root nodules

A total of 97 bacterial isolates were obtained from root nodules formed on cowpea plants. These isolates were representative of the two sugarcane cultivars, the three experimental blocks from the field experiment, compartment of origin (rhizosphere and endosphere), and dilution levels (Table S1). Thirty-four isolates were obtained for rhizosphere samples of each sugarcane cultivar. Fifteen and 14 isolates were obtained from superficially disinfected root samples from cultivar IACSP95-5000 and RB867515, respectively (Table S1). The large majority (93 isolates) of the bacterial isolates presented morphocultural characteristics compatible with the genus *Bradyrhizobium*, such as slow growth and increase of medium pH after growth on bromothymol blue containing YMA plates. Four isolates (RBR179a, IAE71a, IAE45b, and IAR64a) displayed fast growth (colony formation in up to 3 days) and neutral to acidic pH reaction.

Characterization of putative *Bradyrhizobium* isolates using BOX-PCR genetic profiling

BOX-PCR genetic profiles could be obtained for 89 of the 93 *Bradyrhizobium*-like isolates (RBR, 32 isolates; RBE, 13 isolates; IAR, 32 isolates; and IAE, 12 isolates) (Fig. 1). BOX-PCR fingerprinting showed genetic variability within the collection, although some genetic profiles were dominant representing up to 18 isolates (20% of the total). No apparent relationship could be observed between sugarcane cultivar or compartment (endosphere or rhizosphere) and BOX-PCR genetic profile. For example, the most dominant profile represented 9 RBR isolates, 2 RBE isolates, 5 IAR isolates, and 2 IAE isolates obtained from different experimental blocks (Fig. 1). Therefore, the same *Bradyrhizobium* genotype was present in rhizosphere and endosphere of both sugarcane cultivars collected at inter-plant distances of at least 10 m.

Fig. 1 BOX-PCR fingerprint analysis of *Bradyrhizobium* sp. isolates from rhizosphere and endosphere of two sugarcane cultivars. ITS phylogeny groups are indicated with upper case letters A–I (see also Fig. 2). Curly brackets indicate the most abundant profile

16S rRNA and ITS phylogeny

In order to study the genetics of the collection in more detail, 36 slow-growing representative isolates from the collection and also the four fast-growing isolates were submitted to 16S rRNA sequence analysis. This analysis confirmed that all slow growers pertain to the genus *Bradyrhizobium* (Fig. 2 and Fig. S1). Among the fast growers, three (IAE45b, IAR64, and RBR179a) pertained to the genus *Rhizobium* and one (IAE71a) was highly similar to *Burkholderia sylvatlantica*. The genus *Bradyrhizobium* can be divided into two major deeply branching clades, the *B. japonicum* clade and the *B. elkanii* clade [27]. A minority (7 isolates) was classified in the *B. japonicum* clade, whereas all other 29 isolates were classified in the *B. elkanii* clade (Fig. S1).

16S rRNA sequence variability within the *Bradyrhizobium* genus is limited, so classification at the species level requires the study of other genetic features, such as ITS. ITS sequence analysis permitted the distinction of seven phylotypes, which received uppercase letters A–I (Fig. 2). The ITS phylogeny was congruent with the 16S rRNA sequence analysis,

grouping seven isolates into three phylotypes (G, H, and I) within the *B. japonicum* clade. The five isolates of the H and I phylotypes were related, but not identical, to the recently described *B. sacchari* strain BR 10280^T, which was isolated from sugarcane roots in the same experimental field from sugarcane cultivar RB867515 [11].

ITS phylotypes A to F were classified in the *B. elkanii* clade. When comparing the BOX-PCR analysis with the ITS phylogeny, it becomes apparent that the genotype of ITS phylotype F was the most abundant genotype, being isolated from both sugarcane cultivars from rhizosphere and endosphere (Fig. 1).

Discussion

In this study, we took advantage of the ability of many rhizobia (especially from the genus *Bradyrhizobium*) to nodulate the tropical legume *V. unguiculata* in order to estimate the population density of these bacteria in the rhizosphere and root endosphere of sugarcane. After inoculating

Fig. 2 Phylogenetic relationships among selected isolates from rhizosphere and endosphere samples of sugarcane cultivars RB867515 and IACSP95-5000 (in bold) and *Bradyrhizobium* type strains. Detailed view of the *B. elkanii* (a) and detailed view of the *B. japonicum* (b) clade. Curly brackets and uppercase letters indicate identified ITS phylotypes referred to in Fig. 1. The phylogeny is based on internally transcribed spacer (ITS) partial sequence (598 nucleotide positions). Evolutionary history was inferred using the maximum likelihood method, with 500 replications based on the Kimura 2-parameter model [23]. Numbers next to the branches represent the percentage of trees in which the shown topology occurred. Genbank accession numbers are given between parentheses

Fig. 2 (continued)

serially diluted samples on gnotobiotic cowpea plants and evaluating nodulation, it became apparent that many more rhizobia are present in the rhizosphere than in the endosphere of tested sugarcane cultivars. However, the MPN technique could be applied only to part of the data, because for several dilution series, results obtained did not follow the most common McCrady tables. This kind of methodological difficulty is inherent to the MPN technique and is possibly related to heterogeneity in the samples [28].

The data obtained in this study confirmed our previous results on the presence of diverse rhizobia (*Bradyrhizobium* spp. and *Rhizobium* spp.) in sugarcane roots [10]. These bacteria could be isolated both from the endosphere and from the rhizosphere. The density of rhizobia was around 100 times higher in the rhizosphere than in the endosphere of sugarcane roots, probably because bacteria generally occupy the endosphere after first colonizing the rhizosphere and rhizoplane [29]. The role of *Bradyrhizobium* spp. associated with non-legume plants has not been well understood, although some may have the ability to fix nitrogen in free-living state [10] and produce phytohormones and ACC deaminase [30, 31]. Several recent studies have demonstrated the presence of *Bradyrhizobium*-related bacteria in association with non-legume plants [5–9]. These studies applied culture-independent techniques and (16S rRNA) amplicon sequencing on (surface-disinfected) plant tissues. 16S rRNA sequence

analysis is a very useful tool to obtain a general understanding of bacterial diversity, but limited sequence variability within the 16S rRNA gene makes it little useful to study diversity below the genus level [32]. Another limitation of sequencing PCR amplicons obtained from plant tissues is that, even after chemically eliminating the surface-associated microbial community, DNA from such organisms may persist. Therefore, it is challenging to confirm the endophytic nature of bacteria detected by these approaches. Cultivation is one approach to assess specific microbial communities and characterize them at the species level. Apart from that, cultivation is a straightforward approach to obtain microorganisms for biotechnological studies and application.

Different from the *Bradyrhizobium* isolates described in this study, and which are restricted to the root system, other endophytic bacteria attain high numbers inside host-plant tissues and are less abundant in the soil around plants. This is the case, for example, for the sugarcane endophyte *Gluconacetobacter diazotrophicus*, which survives poorly in soil, but which can be found inside the root and shoot tissues at high densities (up to 10^6 – 10^7 bacteria g^{-1}) [33, 34]. This indicates that *Bradyrhizobium* spp. are predominantly rhizosphere colonizers in sugarcane.

Genetic analyses, including BOX-PCR and 16S rRNA and ITS sequence analysis, showed that the same phylotypes that occur in the rhizosphere are also present in the endosphere.

Therefore, no evidence was found for the existence of specific *Bradyrhizobium* phylotypes specialized for endophytic sugarcane colonization. Similarly, there was no evidence for differences between plant cultivars with respect to associated *Bradyrhizobium* phylotypes.

Some highly similar to identical bacterial phylotypes, possibly representing clones were isolated from samples from different experimental blocks with approximately 10-m distance between sampling sites (Figs. 1 and 2). This indicates that these phylotypes are present at the ecosystem level and not restricted to microenvironments and also that certain *Bradyrhizobium* strains may have a certain level of specialization in sugarcane root colonization. On the other hand, the most abundant phylotype isolated in this study was different from the previously described *B. sacchari*, isolated in 2012 from roots of sugarcane cultivar RB867515 cultivated at a site at approximately 50-m distance from the plots used in the present study [10, 11]. Still, ITS phylogenetic reconstruction showed genetic relatedness of five isolates from this study with *B. sacchari*, indicating the presence of this species or related strains in the experimental test area. In general, the majority (30 isolates) of the representative isolates submitted to 16S rRNA and ITS sequence analyses pertained to the *B. elkanii*-related clade and only seven to the *B. japonicum* clade.

Conclusions

Abundant and diverse *Bradyrhizobium* spp. were found inside roots at low densities and at around 100 times higher densities in the rhizosphere of two Brazilian sugarcane cultivars. There was no evidence for an effect of plant cultivar or compartment (rhizosphere or endosphere) on the composition of the sugarcane root-associated *Bradyrhizobium* community. The data confirm that the sugarcane root system is a natural habitat of diverse *Bradyrhizobium* spp. and suggest that certain *Bradyrhizobium* phylotypes have increased affinity for sugarcane root colonization. These bacteria may affect the growth and health of the plant and are potential targets for the development of inoculants in the future.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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