Transgenic Res
DOI 10.1007/s11248-016-9938-4

ORIGINAL PAPER

Impact of long-term cropping of glyphosate-resistant transgenic soybean [*Glycine max* (L.) Merr.] on soil microbiome

Letícia Carlos Babujia · Adriana Pereira Silva · André Shigueyoshi Nakatani · Mauricio Egidio Cantão · Ana Tereza Ribeiro Vasconcelos · Jesuí Vergilio Visentainer · Mariangela Hungria

Received: 4 December 2015 / Accepted: 4 February 2016

Abstract The transgenic soybean [*Glycine max* (L.) Merrill] occupies about 80 % of the global area cropped with this legume, the majority comprising the glyphosate-resistant trait (Roundup Ready®, GR or RR). However, concerns about possible impacts of transgenic crops on soil microbial communities are often raised. We investigated soil chemical, physical and microbiological properties, and grain yields in long-term field trials involving conventional and nearly isogenic RR transgenic genotypes. The trials were performed at two locations in Brazil, with different edaphoclimatic conditions. Large differences in physical, chemical and classic microbiological parameters (microbial biomass of C and N, basal respiration), as well as in grain production were observed between the sites. Some phyla (Proteobacteria, Actinobacteria, Acidobacteria), classes (Alphaproteobacteria, Actinomycetales, Solibacteres) and orders (Rhizobiales, Burkholderiales, Myxococcales, Pseudomonadales), as well as some functional subsystems (clustering-based subsystems, carbohydrates, amino acids and protein metabolism) were, in general, abundant in all treatments. However, bioindicators related to superior soil fertility and physical properties at Londrina were identified, among them a higher ratio of Proteobacteria:Acidobacteria. Regarding the trans-

Jesuí Vergilio Visentainer and Mariangela Hungria have equally contributed to this work.

Electronic supplementary material The online version of this article (doi:10.1007/s11248-016-9938-4) contains supplementary material, which is available to authorized users.

L. C. Babujia · J. V. Visentainer
Department of Chemistry, Universidade Estadual de Maringá, Av. Colombo 5790, Maringá, Paraná 87020-900, Brazil
e-mail: leticiacb_@hotmail.com

J. V. Visentainer
e-mail: jvvisentainer@uem.br

L. C. Babujia · A. P. Silva · A. S. Nakatani · M. Hungria (✉)
Embrapa Soja, C.P. 231, Londrina, Paraná 86001-970, Brazil
e-mail: mariangela.hungria@embrapa.br; biotecnologia.solo@hotmail.com; hungria@cnpq.br

A. P. Silva
e-mail: drikapera@yahoo.com.br

A. S. Nakatani
e-mail: andrenakatani@yahoo.com.br

M. E. Cantão
Embrapa Suínos e Aves, C.P. 21, Concórdia, Santa Catarina 89700-000, Brazil
e-mail: mauricio.cantao@embrapa.br

A. T. R. Vasconcelos
Laboratório Nacional de Computação Científica, Rua Getúlio Vargas 333, Petrópolis, Rio de Janeiro 25651-071, Brazil
e-mail: atrv@lncc.br

Published online: 12 February 2016

gene, the metagenomics showed differences in microbial taxonomic and functional abundances, but lower in magnitude than differences observed between the sites. Besides the site-specific differences, Proteobacteria, Firmicutes and Chlorophyta were higher in the transgenic treatment, as well as sequences related to protein metabolism, cell division and cycle. Although confirming effects of the transgenic trait on soil microbiome, no differences were recorded in grain yields, probably due to the buffering capacity associated with the high taxonomic and functional microbial diversity observed in all treatments.

Keywords *Glycine max* · Transgenic soybean · Soil microbial diversity · Soil metagenomics · Functional biodiversity

Introduction

Soybean [*Glycine max* (L.) Merrill] has become the most important legume crop on the international market, with Brazil occupying the second position in the ranking of world production (OECD 2015). Currently, both conventional and transgenic soybeans are grown in the main producing countries, but transgenic genotypes already occupy 82 % of the cropped area (James 2014). Glyphosate-resistant soybean (also known as Roundup Ready®, GR or RR) carries the enolpyruvoil-5-shikimate-3-phosphate synthase (EPSPS, EC 2.5.1.19, CP4) gene, which confers resistance to the herbicide glyphosate and represents the major transgenic trait incorporated into commercial soybean cultivars.

The technology of genetically modified plants has raised many questions regarding biosafety and biodiversity. One of the concerns relates to possible impacts on soil microorganisms, responsible for important microbial processes such as biological nitrogen fixation, nutrient cycling and xenobioses. Theoretically, the insertion of genes for tolerance of herbicides or insecticides should not affect soil microorganisms, but plausible effects could come from the release of new proteins into the rhizosphere, followed by gene transfer to soil microorganisms (O'Donnell and Gorres 1999), or by stimulating the growth of specific organisms able to use these molecules (Dunfield and Germida 2004).

Reports on the impact of transgenic plants on soil microorganisms and microbial processes indicate that, in general, the effects are minor or transitory, with the major effects being attributed to factors such as soil type, climate and cropping system (Dunfield and Germida 2004; Mulder et al. 2006; Souza et al. 2013a; Nakatani et al. 2014). In our research group, we have recently reported results from large sets of field trials performed in Brazil comparing conventional and transgenic pairs of soybean genotypes, in which no consistent differences on microbial quantitative (microbial biomass), functional (soil enzymes) or qualitative (16S rDNA-DGGE profiles) attributes were related to either the *ahas* gene [imidazolinone-tolerant acetohydroxyacid synthase (AHAS) gene from *Arabidopsis thaliana*] (Souza et al. 2013a), or to the glyphosate-resistance trait (Nakatani et al. 2014). However, differences were verified regarding biological nitrogen fixation parameters due to the glyphosate-resistance (Hungria et al. 2014), but not to the *ahas* transgene (Hungria et al. 2015). Nevertheless, one limitation from these studies is that they did not include detailed surveys of microbial communities.

Microbial diversity can represent an important indicator of soil quality, and the metagenomic analysis plays a key role in increasing our knowledge about the diversity of each soil or particular ecosystem (Kakirde et al. 2010; Souza et al. 2013b; 2014; Spang et al. 2015). The metagenomic shotgun approach is particularly interesting, as it captures both taxonomic and functional diversities.

Brazil encompasses one of the greatest biodiversities on the planet in a variety of ecosystems covering a continental area (Pylro et al. 2014; OECD 2015). The country also stands out in terms of its global role in agriculture (OECD 2015); furthermore, it is second in terms of cultivating transgenic crops, mostly soybeans (James 2014). Studies using soil metagenomics are increasing worldwide, but there are no reports comparing conventional and transgenic soybean crops in Brazil. Long-term experiments present opportunities for clarifying possible impacts on microbial communities. Therefore, in this study, we made comparisons using two long-term field trials (a decade in duration) performed under different edaphoclimatic conditions in Brazil with conventional and nearly isogenic RR transgenic pairs of soybean cultivars treated with the same set of conventional herbicides. We evaluated soil chemical and physical properties, grain yields, and microbial communities by using classical microbiological methods and the shotgun metagenomic

approach. The main purpose was to determine if long-term cropping of glyphosate-resistant soybean can affect soil microbiome.

Materials and methods

Experiments description

The experiments were established in the 2003/2004 crop season in a Latossolo Vermelho Eutroférrico (Brazilian classification; Rhodic Eutrudox, American classification) at Londrina, Paraná State (630 m altitude; 23°18′S, 51°09′W; climate Cfa according to Köppen–Geiger's classification) and in a Latossolo Vermelho Distrófico (Brazilian classification; Typic Hapludox, American Classification) at Ponta Grossa, Paraná State (886 m altitude; 25°05′S, 50°09′W; climate Cfa according to Köppen–Geiger's classification).

The experiments were established with an experimental design in randomized blocks, with six replicates per treatment, represented by combinations of parental conventional soybean cultivars and their respective nearly isogenic glyphosate-resistance (RR) transgenic counterpart, with different weed control treatments. A complete description of the experiment, the soil properties and crops managements in the first 3 years is available elsewhere (Hungria et al. 2014). From the fourth year, all plots with conventional or transgenic cultivars were cropped with soybean cultivar BRS 133 (parental conventional genotype) and the nearly isogenic transgenic BRS 245 RR counterpart, respectively, with the same set of conventional herbicides. In the winter, non-transgenic wheat (*Triticum aestivum* L.) was cropped in all plots. The areas were always managed under the no-till system.

Soil sampling

Soil sampling was carried out in the 2012, immediately after the soybean harvest. Samples were taken from the plots that have been grown since the establishment of the trials with conventional and its nearly isogenic counterpart treated with conventional herbicides. The purpose was to detect effects that could be attributed exclusively to the transgene. Conventional herbicides consisted of 0.5 L ha^{-1} of Select 240 (Clethodim, Milenia), mixed with Assist mineral oil at 0.5 % of the volume (narrow-leaf weeds), and Classic (Chlorimurum-ethyl, DuPont) at 80 g ha^{-1} (broad-leaf weeds)]. Consequently, for this study we have considered four treatments, each with six replicates, in a completely randomized block design, composed by 24 plots. For each plot, six subsamples were randomly collected from the top layer (0–10 cm) between cropped lines and mixed to obtain a composite sample. The composite samples were then sieved (4 mm, 5 mesh).

Arriving at the laboratory, each replicate was split for the analyses. Those destinated to the chemical analyses were air dried. For classic microbiological analyses [microbial biomass of C and N (MB-C, MB-N), basal respiration induced or not with glucose (BR and BRi)], samples were maintained at 4 °C. For DGGE and metagenomic analyses samples were kept on ice during collection and were then transferred to a freezer at −20 °C and DNA extractions were performed on the next day. Soil moisture content was determined by drying a 10 g subsample for 12 h at 105 °C.

Grain yield

The experiments continued for one more cropping season after soil sampling, with the same treatments; therefore, grain yield data referring to the 2012/2013 crop were also obtained. At the physiological maturity stage, pods were taken from the plants from two central 5.0-m long rows in each plot, and seeds removed, cleaned, weighed and soybean grain yield evaluated after adjusting moisture content to 13 %.

Grain yield data were considered since the establishment of the experiments in 2003/2004.

Soil chemical and physical properties

Before being analyzed for chemical parameters, soil samples were dried (60 °C for 48 h), sieved through a 2 mm sieve, and evaluated as described before (Hungria et al. 2013). Basically, pH was determined in 0.01 mol L^{-1} $CaCl_2$; exchangeable Ca, Mg and Al were determined in the extract obtained with 1 mol L^{-1} KCl; P and K contents were evaluated in the Mehlich-1 (0.05 mol L^{-1} HCl + 0.0125 mol L^{-1} H_2SO_4) extract; Al was determined by titration with 0.015 mol L^{-1} standardized NaOH, using bromothymol blue as indicator. Concentrations of Ca and Mg were determined in an

atomic absorption spectrophotometer; K in a flame photometer and P by colorimetry, using the molybdenum-blue method and ascorbic acid as reducing agent. Total nitrogen and organic matter were determined by the method of EMBRAPA (2009). Basically, organic C concentration was evaluated by the Walkley–Black potassium dichromate sulfuric acid oxidation procedure (Nelson and Sommers 1996) and total nitrogen was quantitated by the Kjeldahl's method. Soil density was determined according to Blake and Hartge (1986).

Microbial biomass parameters: microbial biomass of C and N (MB-C and MB-N) and basal respiration non-induced (BR) and induced with glucose (BRi)

Before the analyses, soil moisture was adjusted to 40 % water holding capacity (WHC), and the results are expressed on an oven-dry basis (105 °C overnight).

Microbial biomass-C and -N (MB-C and MB-N) were evaluated by the fumigation-extraction (FE) method after (Vance et al. 1987) and Brookes et al. (1985), slightly modified as previously described (Hungria et al. 2009; Silva et al. 2014). Basically, subsamples (20 g) of each composite soil sample were weighed and placed in glass receptacles (300 mL). Half of those subsamples were submitted to fumigation as described by Vance et al. (1987), and the other two were not fumigated. Fumigated and non-fumigated samples were kept in the dark at 25 ± 2 °C for 16 h. After that, the C was extracted from the samples by adding 50 mL of extractor solution (0.5 M K_2SO_4), shaking (175 rpm, 1 h), centrifuging (3000 rpm, 10 min) and filtering. Carbon content in the extracts was determined by the oxidation with Mn^{3+}, and evaluation on a spectrophotometer. The MB-C was estimated from the difference between fumigated and non-fumigated samples, with a K_{CE} of 0.38 (Feigl et al. 1995; Oliveira et al. 2001). MB-N was evaluated by the spectrophotometric determination of NH_4–N, by using the indophenol blue method (Feije and Anger 1972), with the correction factor K_{NE} 0.54 (Brookes et al. 1985).

The basal respiration (BR) was estimated in non-fumigated soil samples as described for the fumigation–incubation (FI) method (Jenkinson and Polwson 1976), slightly modified as described before (Brandão-Junior et al. 2008; Hungria et al. 2009). For the evaluation of induced basal respiration (BRi), 1 % of glucose was added and the soils were incubated for 7 days; a correction factor of K_{CE} 0.41 (Anderson and Domsch 1978) was employed. More details about the incubation procedures were given elsewhere (Hungria et al. 2009).

16S rDNA-DGGE profiles obtained by DGGE (denaturing gradient gel electrophoresis)

Microbial DNA was extracted from 0.25 g of each of each of the six replicates per treatment, using the UltracleanTM Soil DNA Kit (MoBio Laboratories, Inc., CA, USA), according to the manufacturer's instructions; DNA quantification and verification of purity in agarose were performed as described before (Silva et al. 2013; Babujia et al. 2014).

Two successive amplifications were carried out for each DNA sample regarding the 16S rRNA region. First, the soil DNA was amplified with primers fD1 (5′-AGAGTTTGATCCTGGCTCAG-3′) and rD1 (5′-AAG GAG GTG ATC CAG CC-3′), which amplify almost the entire region of the DNA encoding the 16S rDNA (approximately 1500 bp). The second amplification was performed using 1.0 µL (~20 ng) of the products of the first reaction as template. The primers F-968 (5′-CGCCCGGGGCGCGCCCCGGGCGGGGCGGGGG CACGGGGGGAACGCGAAGAACCTTAC-3′), with a GC-clamp (underlined) and R-1401 (5′- GC GTGTGTACAAGACCC-3′) were used to amplify the 16S rDNA region of approximately 430 bp, corresponding to the V3 hypervariable region. Details about the amplification conditions in both steps are given elsewhere (Silva et al. 2013; Babujia et al. 2014). Amplification was confirmed by running 2.0 µL of PCR product in a 1 % (w/v) agarose gel in 1× TBE, staining with ethidium bromide (0.3 µg mL^{-1}) and visualizing under UV light. The products of the amplification were subjected to the DGGE analysis.

DGGE was carried out in a D-Code System (Bio-Rad, Hercules, CA, USA), by loading 25 µL from the last PCR product of each replicate. In addition, a standard mix of soil bacteria was prepared in the laboratory, consisting of equal proportions of *Burkholderia*, *Bradyrhizobium*, *Methylobacterium*, *Azorhizobium* and *Rhizobium* and the mixture was also amplified for the 16S rRNA region. The mixture of bacteria was loaded in three lanes of each gel, on the boarders and in the central lane. The 6 % (w/v)

polyacrylamide gels were made up with a denaturing gradient ranging from 35 to 55 %, using a mixture of 100 % denaturing solution [7 mol L^{-1} urea and 40 % (v/v) formamide] and 0 % solution (no urea and formamide added). The electrophoresis was run in 0.5× TAE buffer [10 mmol L^{-1} Tris–acetate and 0.5 mmol L^{-1} disodium EDTA (pH 8.3)], first with a pre-running at 60 °C and 100 V for 1 h and then at a constant voltage of 100 V for 16 h. After electrophoresis, the gels were stained for 4 min with ethidium bromide and photographed under UV light.

Metagenomics: DNA amplification and pyrosequencing

DNA extraction for the metagenomic analyses was performed with 10 g of soil from each plot, following the specifications of the manufacturer of the kit PowerMax™ Soil DNA Isolation Kit (MoBio). The DNA samples were quantified on a Qubit® fluorometer, and their purity checked by NanoDrop spectrophotometer at 260/280 ratio. Some samples had their concentration adjusted to 50 ng μL^{-1} by SpeedVac® Eppendorf Concentrator Plus. Metagenomic DNA was submitted to sequencing analysis in the 454 platform (GS-FLX Titanium Roche Applied Science) at the Labinfo of LNCC (Petrópolis, Rio de Janeiro, Brazil, http://www.labinfo.br). For the pyrosequencing, DNA was randomly fragmented by nebulization with compressed nitrogen gas. Fragments of 300–800 bp were selected (Bioanalyzer DNAChip), prepared and submitted to sequence analysis with the Titanium kit, and the support PicoTiterPlate (Roche Applied Science).

Data analyses

Statistical analysis of chemical data, microbial biomass parameters and grain yields

Analysis of variance was performed using ANOVA (GenStat® for Windows 8.0, VSN Int. Ltd, UK, 2005), and Tukey's test was performed to compare averages using the Statistica 7.0 program (StatSoft, USA).

In addition, the data were analysed with non-metric multidimensional scaling (NMS) (Sokal 1979) using the program PC-ORD v 6.0 (McCune and Mefford 2011), considering four microbial parameters (MB-C, MB-N, BR, BRi). The number of dimensions to be interpreted was selected considering the criteria of stress and stability of the graphical solutions. Pearson correlation coefficients between the samples scores of NMS axis 1 and 2 and the values of microbial attributes were calculated. Significant differences in four microbial parameters analyzed together between treatments were determined by permutational multivariate analysis of variance (PERMANOVA).

Analysis of DGGE profiles

The 16S rDNA-DGGE profiles were analyzed using Bionumerics software (Applied Mathematics, Kortrijk, Belgium, v.4.6). Each gel image was normalized by identifying the bands of the mixture of bacteria used as reference in the three lanes (borders and central). Similarities between fingerprints were analyzed statistically using the unweighted pair-group method with arithmetic averages (UPGMA) and the Jaccard (J) coefficient, with a tolerance of 5 % to create a distance matrix, as defined before (Silva et al. 2013; Babujia et al. 2014).

Analysis of metagenomic data

The metagenomic fragments were submitted to FastX-trimmer (http://hannonlab.cshl.edu/fastx_toolkit/) in order to remove low quality sequences and reads ≤50 bp. The duplicated reads were filtered using the Replicates software (Gomez-Alvarez et al. 2009). The generated sequences from metagenome were submitted to the MG-RAST v.3.3 (MetaGenomics RAST—http://metagenomics.anl.gov) server (fast metagenomics Subsystem Annotation Technology), that consists of a pipeline broadly used for the metagenomic data generated by shotgun sequencing (Meyer et al. 2008). In the analysis with MG-RAST the abundance data was identified through the lowest common ancestor (LCA), with defined parameters (max. e-value cutoff 1e−5, min. % identity cutoff = 60 % e min. alignment length cutoff = 15) (Overbeek et al. 2005).

The statistical analysis for distinct taxonomic levels from MG-RAST was conducted using the Statistical Analyses of Metagenomic Profiles (STAMP) (Parks and Beiko 2010) software. In order to identify differences between treatments on taxonomic distribution the Fisher's test (1958) ($p < 0.05$) was applied, the statistical significance was estimated by the

Benjamin–Hochberg FDR (Benjamini and Hochberg 1995) correction method, the confidence interval was calculated using the Newcombe–Wilson method (Newcombe 1998), and unclassified reads were removed from analyses.

Functional analysis was performed by the MG-RAST server, comparing the sequences against the standard energy efficiency data (SEED) (Meyer et al. 2008) database (max. e-value cutoff 1e−5, min. % identity cutoff = 60 % e min. alignment length cut-off = 15) (Overbeek et al. 2005). According to the SEED website (http://theseed.org/wiki/Home_of_the_SEED), a subsystem represents a set of functional roles that make up a metabolic pathway, a complex, or a class of proteins, and in our study we considered the highest level (1) and the second highest level (2). Data were also statistically analyzed with the STAMP software, considering the level 1 of and the subsystem using the same parameters described for taxonomic distribution.

To test differences between the samples based on a distance measure in the principal coordinate analysis (PCoA) chart, the nonparametric test ANOSIM was conducted. This pattern results in a ratio R, where R = 1 means that there is separation of the community structures, or if there was no separation (R = 0). The values R < 0.2 are interpreted as 16S rDNA-DGGE profiles with no separation between the groups (Clarke 1993). We searched for differences that could be attributed to different edaphoclimatic conditions and to the transgenic trait.

Accession numbers

The sequences were uploaded in the MG-RAST server and received the IDs: PG BRS 133 (4499912.3); PG BRS 245 RR (4499915.3); LD BRS 133 (4507029.3) and LD BRS 245 RR (4507011.3).

Results

Soil chemical and physical properties

Chemical properties of the soils at Londrina (LD) and Ponta Grossa (PG) are given in Table S1. Values of Ca, Mg, K, organic matter (OM), N, P, cation exchange capacity (CEC), Mn, Fe, Cu, Zn and pH were significantly different between the two sites, with LD showing better fertility than PG. Differences resulting from conventional and transgenic cultivars were observed only for soil pH, with higher values in soils cropped with the conventional cultivar (Table S1).

In relation to the physical properties, the soil in PG showed higher density in comparison to LD (PG with BRS 133 = 2.22 g cm^{-3}; PG with 245 RR, 2.29 g cm^{-3}; LD with BRS 133 = 1.12 g cm^{-3}; LD with 245 RR = 1.14 g cm^{-3}), but with no differences due to the transgenic trait. Soil granulometry also varied with the site, but with no effect due to the transgene. The average values were (%): 71.0 (clay), 8.2 (silt), 20.8 (sand) in LD and 23.8 (clay), 30.0 (silt) and 73.2 (sand) in PG.

Grain yield

Significant differences on soybean grain yield were observed between sites. Differences between the cultivars were observed in only one crop season, but the transgenic genotype had a better performance in LD, whereas higher yields were achieved with the conventional genotype in PG (Table S2). The averages of ten cropping seasons (2003/2004–2012/2013) indicate that higher yields were achieved in LD (BRS 133 = 2798 kg ha^{-1}, BRS 245 RR = 2744 kg ha^{-1}) than in PG (BRS 133 = 2397 kg ha^{-1}, BRS 245 RR = 2473 kg ha^{-1}). Differences in grain yield between sites (Table S2) probably resulted from the superior fertility (Table S1) and lower compaction of the soil at LD.

Microbial biomass parameters: MB-C, MB-N, BR, BRi

The results for the MB-C, MB-N, BR and BRi analyses (Table S3) indicated significant differences attributable to the location (PG and LD) in all parameters, which were consistently higher in the soil at LD. Contrarily, no differences in microbial biomass parameters were attributable to the genotypes (Table S3).

The multivariate NMS and PERMANOVA analyses confirmed the results of the univariate statistical analyses, showing effects attributable only to location; axis 1 explained most of the variability (80 %) (Table S4), showing a clear separation of the sites (Fig. S1). Moreover, all microbiological parameters were significantly correlated with axis 1 (Table S4).

16S rDNA-DGGE profiles

The DGGE analysis (Figs. S2A and S2B) indicated high homogeneity among replicates, with similarity of 100 % among replicates of each treatment. Differences were observed between the cultivars in both sites, resulting in final similarities of 91.5 % at LD, and 93.5 % at PG. One purpose of the DGGE analysis was to verify the homogeneity among replicates. As replicates were identical in the four treatments, the metagenomic DNAs of the replicates of each treatment were pooled and homogenized to form a single composite sample per treatment. A similar approach has been used in our previous studies (Souza et al. 2013b, 2014).

Microbial diversity evaluated by metagenomics

Genetic diversity detected in the four metagenomes

Shotgun sequencing analysis generated about one million sequences for each treatment, with an average of 340 bp per read, as follows: PG BRS 245 RR (1,169,983 sequences); PG BRS 133 (942,625); LD BRS 245 RR (916,009); LD BRS 133 (903,335). The rarefaction curves from samples generated by MG-RAST showed that, even with this high number of sequences, saturation was not reached, indicating high genetic diversity (Fig. S3). The transgenic treatment showed the highest values of alpha-diversity (which estimates the distribution of species annotated in a dataset) at both sites (Fig. S3).

In order to compare the treatments, the data were normalized by the MG-RAST (0–1) (Wilke et al. 2013), and a comparative study was performed based on the M5NR database (Wilke et al. 2012), available at the MG-RAST server. The majority of the sequences was attributed to the Bacteria (28.94 %) domain, followed by Archaea (15.88 %) and Eukaryota (15.75 %), and with a considerably lower number of sequences attributed to Viruses (3.73 %) (Fig. S4A). The number of sequences in each domain was, in general, similar between the treatments, with a slightly lower number of Eukaryota at LD. Noticeable was the higher number of unclassified sequences (24.02 %), the second highest group, and also of unassigned sequences (Fig. S4A).

Within the Bacteria domain, 28 phyla were detected, and the highest in abundance were Proteobacteria (7.96 %), Actinobacteria (7.11 %), Acidobacteria (6.55 %), Firmicutes (6.02 %), Bacteroidetes (5.78 %), Chloroflexi (5.52 %), Verrucomicrobia (5.44 %), Planctomycetes (5.38 %) and Cyanobacteria (5.35 %) (average values of the four treatments) (Fig. S4B). In the domain Eukaryota, 32 phyla were detected, and the most abundant were Ascomycota (7.82 %), Chordata (7.65 %), Streptophyta (7.65 %), Unclassified (6.81 %) and Arthropoda (6.41 %) (Fig. S5A). In the domain Archaea, only five known phyla were detected, Euryarchaeota (28.89 %), Crenarchaeota (22.77 %), Thaumarchaeota (20.41 %), Korarchaeota (12.00 %) and Nanoarchaeota (2.48 %), in addition to 13.45 % of unclassified sequences (Fig. S5B). Finally, in the domain Viruses the order Caudovirales was the most abundant (53.51 %), in addition to unclassified sequences (36.01 %) (Fig. S5C).

In the three dominant phyla of the Bacteria domain, higher abundance was detected in four classes of Proteobacteria (alpha, beta, delta and gamma) (Fig. S6A), in the Actinomycetales class of Actinobacteria (Fig. S6B) and in the Solibacteres class of Acidobacteria (Fig. S6C). In general Proteo- and Deltaproteobacteria showed similar abundance in all treatments, whereas beta- and gamma-proteobacteria were lower in LD than in PG (Fig. S6A). In contrast, the most abundant classes of Actinobacteria, as well as of unclassified Acidobacteria were higher at LD than in PG (Fig. S6B).

Within the most abundant phylum of Proteobacteria, prevalence of some orders within the classes was observed. In the Alphaproteobacteria class, the most abundant order was of Rhizobiales (22.65 %), with relevant abudances also of Rhodobacterales, Sphingomonadales, Rhodospirillales, and Caulobacterales (Fig. S7A). In the Betaproteobacteria class there was a clear dominance of the order Burkholderiales (42.50 %) (Fig. S7B), while in the Deltaproteobacteria there was a predominance of the order Myxococcales (27.55 %), followed by the Desulfuromonadales, Desulfovibrionales, Syntrophobacterales and Desulfobacterales (Fig. S7C). In the Gammaproteobacteria class several orders were present in all treatments, and in general were lower at LD than in PG (Fig. S7D).

Genetic diversity associated with the transgenic trait

As expected and observed at the domain, phyla, class and order levels (Figs. S4 to S7), several differences

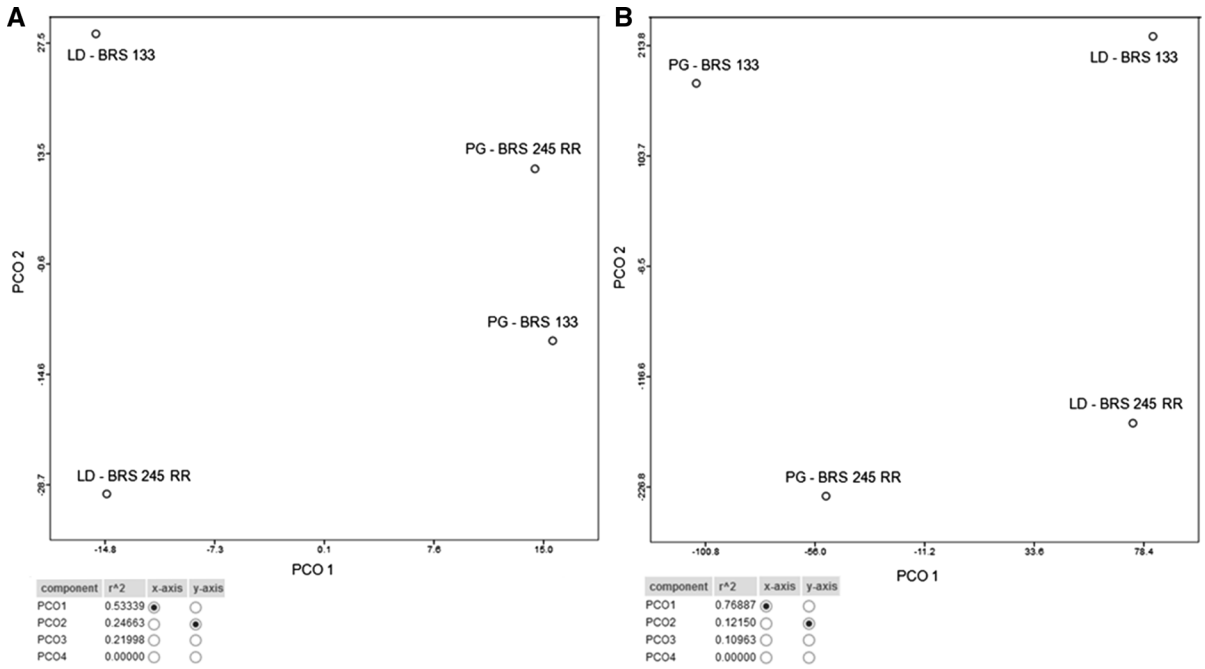

Fig. 1 Principal coordinate analysis (PCoA) of the four metagenomes (data normalized for the number of sequences of each metagenome) using an e-value cut-off of 0.001. **a** PCoA using the M5nr database of the MG-RAST, **b** PCoA using the subsystems (SEED, level 1) database of MG-RAST. Treatments consisted of two sites, Londrina (LD) and Ponta Grossa (PG), and two soybean genotypes, the parental conventional BRS 133 and the nearly isogenic RR transgenic BRS 245 RR

were observed between the two sites, LD and PG. However, the main purpose of our study was to investigate differences due to the RR transgene. Comparisons of all four metagenomes by PCoA based on the representative hit classification against the M5nr database in MG-RAST (Fig. 1a) and the representative hit classification against the subsystems (Level 1) database in MG-RAST (Fig. 1b) indicated significant differences (R^2) associated with both the location and the transgenic trait.

Statistical analyses were also performed with the STAMP software. At LD, differences between the transgenic and conventional genotypes were detected in 16 orders of microorganisms (Fig. 2), and in 20 orders at PG (Fig. 3). Proteobacteria were more abundant in the treatment with RR transgenic soybean both at LD and PG, and Firmicutes and Chlorophyta were higher too. In addition, five other orders at LD and ten at PG were higher under the transgenic trait. On the contrary, Actinobacteria, Acidobacteria and unassigned sequences were higher in the conventional treatment at both sites. The conventional genotype also resulted in higher abundance of five other orders at LD and four at PG (Figs. 2, 3).

Microbial functional diversity evaluated by metagenomics

Functional diversity detected in the four metagenomes

Metabolic profiles were generated by the MG-RAST server, and the metagenome annotated sequences were distributed in 28 functional categories (level 1 SEED subsystems) (Fig. S8). The most abundant genes were those related to the metabolism of clustering-based subsystems (6.19 %), carbohydrates (5.74 %), amino acids and derivatives (5.46 %), miscellaneous (5.38 %) and protein metabolism (5.32 %) (averages of the four treatments) (Fig. S8). In general the lowest functional abundance was at LD with the transgenic cultivar, whereas the highest was observed at PG with the transgenic cultivar (Fig. S8).

At level 2 of the subsystems, for the amino acids and derivatives, there was higher abundance related to the metabolism of lysine, threonine, methionine, and branched-chain amino acids, but, in general, sequences related to the metabolism of all amino acids were abundant (Fig. S9A). For another important subsystem, of protein metabolism, the highest

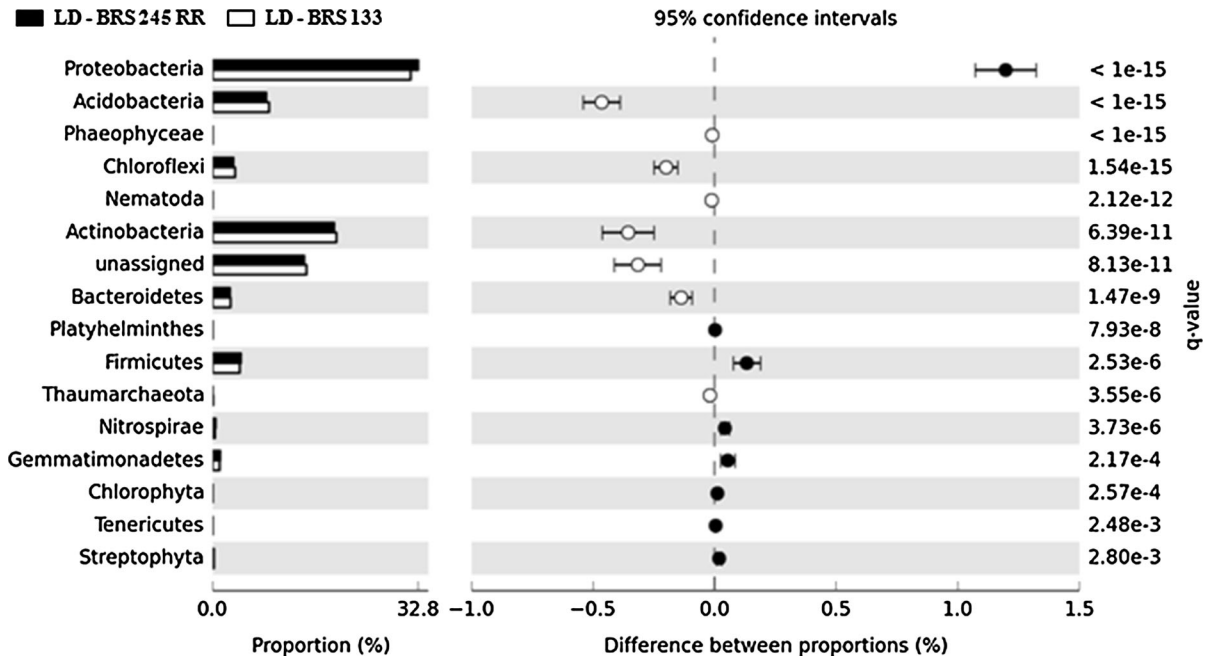

Fig. 2 Statistically significant differences between orders of microorganisms obtained with the STAMP software, at the soil of Londrina (LD), in the comparison of two soybean genotypes, the parental conventional BRS 133 and the nearly isogenic RR transgenic BRS 245 RR. The *graphic* shows only the proportions of sequences with statistical differences with a confidence interval of 95 %

abundance was associated with protein biosynthesis (Fig. S9B). For cell division and cell cycle, cell cycle in prokaryota represented the most abundant subsystem at level 2, and PG had more sequences than LD; the YgjD and YeaZ are proteins involved in DNA repair (Fig. S9C).

Functional diversity associated with the transgenic trait

The comparative analysis of metabolic profiles using level 1 of classification with the STAMP software revealed statistically significant differences in the subsystems of RNA metabolism and phages, prophages, transposable elements and plasmids associated with the conventional cultivar at LD (Fig. 4), while at PG cropped with the conventional cultivar higher abundances of six other subsystems were associated with the conventional trait (Fig. 5). Two subsystems were higher in the transgenic treatments at both LD and PG: protein metabolism and cell division and cell cycle. In addition, three other subsystems were higher at LD and four others at PG (Figs. 4, 5).

Discussion

Microbial diversity in agricultural soils of southern Brazil

The importance of Brazil and other South American countries as grain producers and reservoirs of biodiversity is being increasingly recognized; however, very few studies have evaluated soil metagenomic diversity in the region (Pylro et al. 2014). In our study, two oxisols under contrasting edaphoclimatic conditions in Brazil were studied, at Londrina (LD), with a warmer climate, high clay content, lower soil compaction and higher soil fertility, and at Ponta Grossa (PG), with contrasting properties. The better conditions at LD implicated in higher yields than at PG, and also favored quantitative traits of soil microbial biomass: MB-C, MB-N, BR and BRi. Therefore, our results confirm previous observations of good correlations between these microbial parameters and grain yield (e.g. Hungria et al. 2009; Silva et al. 2010; Lopes et al. 2013), exemplifying the relevance of their use as bioindicators of soil quality (Babujia et al. 2010; Kaschuk et al. 2010, 2011).

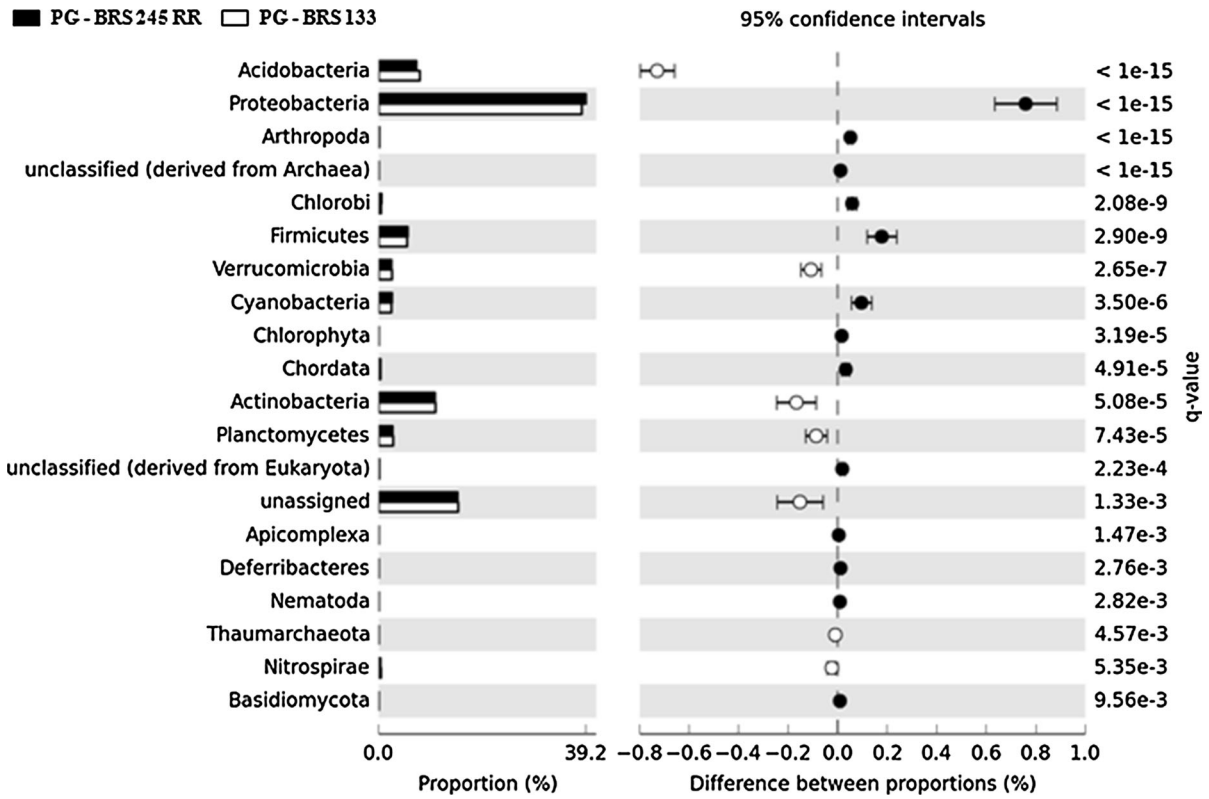

Fig. 3 Statistically significant differences between orders of microorganisms obtained with the STAMP software. Figure obtained with the STAMP program, at the soil of Ponta Grossa (PG), in the comparison of two soybean genotypes, the parental conventional BRS 133 and the nearly isogenic RR transgenic BRS 245 RR. The *graphic* shows only the proportions of sequences with statistical differences with a confidence interval of 95 %

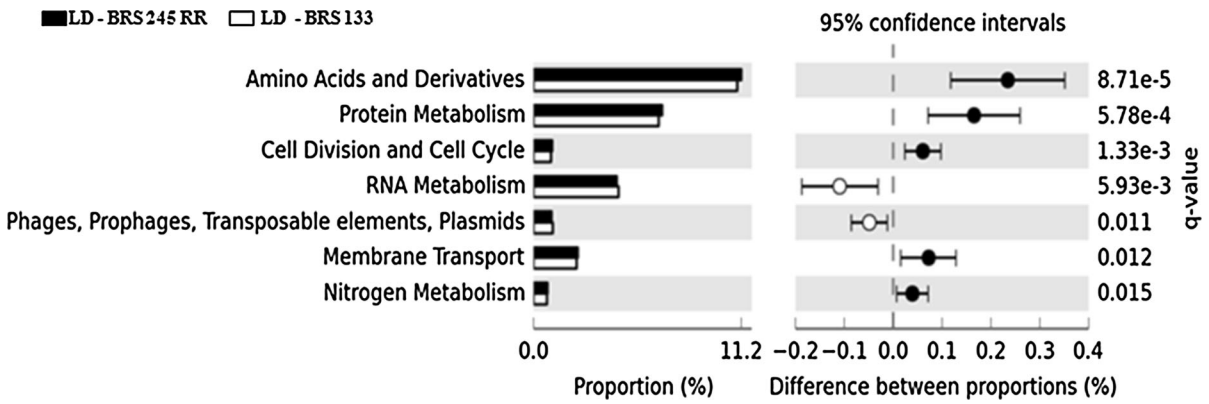

Fig. 4 Statistically significant differences in the functional subsystem in the level 1 of classification using the MG-RAST annotation platform and STAMP statistical software, in the comparison of conventional (BRS 133) and the nearly isogenic (BRS 245 RR) soybean cultivars at Londrina (LD). The figure shows only the subsystems with statistical differences between the proportions of sequences in each treatment, with a confidence interval of 95 %

The metagenomic analyses of both soils revealed the predominance of the domain Bacteria and of the phyla Proteobacteria, Actinobacteria and Acidobacteria, corroborating other studies performed worldwide under a variety of edaphoclimatic conditions (e.g. Liles et al. 2003; Delmont et al. 2012, Fierer et al. 2012; Steven et al.

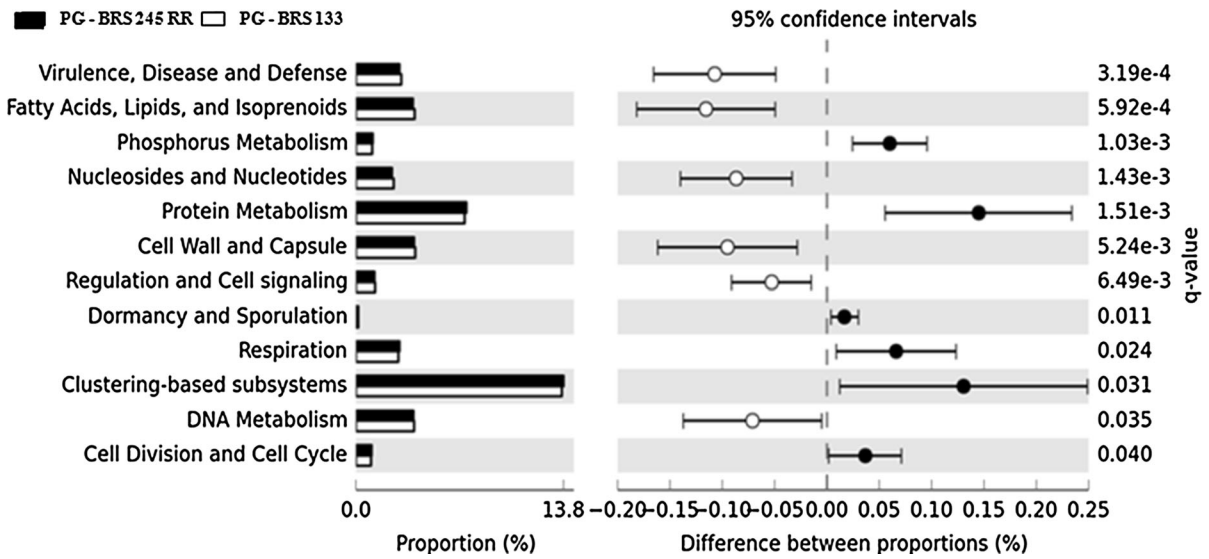

Fig. 5 Statistically significant differences in the functional subsystem in the level 1 of classification using the MG-RAST annotation platform and STAMP statistical software, in the comparison of conventional (BRS 133) and the nearly isogenic (BRS 245 RR) soybean cultivars at Ponta Grossa (PG). The figure shows only the subsystems with statistical differences between the proportions of sequences in each treatment, with a confidence interval of 95 %

2012; Souza et al. 2013b). Proteobacteria are well known as critical components of soil fertility due to their roles in C, N and S cycles (Kersters et al. 2006). Within the Alphaproteobacteria class, the most abundant order was of Rhizobiales in all treatments, probably favored by the annual massive inoculation of soybean seeds with *Bradyrhizobium* strains. Biological nitrogen fixation is a key process for soybean sustainability in Brazil, but requires annual reinoculation to achieve optimal symbiotic performances, resulting in increases in soil *Bradyrhizobium* population of 1000- to 10,000-fold (Hungria et al. 2006, 2007; Hungria and Mendes 2015).

The prevailing order of the Betaproteobacteria class was of Burkholderiales, which performs a variety of functions in the soil, including biological control of pathogens, xenobiotics and bioremediation (Coenye and Vandamme 2003; Zuleta et al. 2014), plant growth promotion (Estrada-de los Santos et al. 2001) and biological nitrogen fixation (Gyaneshwar et al. 2011; Zuleta et al. 2014). Among the Deltaproteobacterias class, the dominant order was Myxococcales, which has been related to several biotechnological applications, including the production of secondary metabolites, the solubilization of large molecules, cellular and other organic debris, and the production of lytic enzymes, all of which are of commercial interest (Reichenbach 2003).

The second most abundant phylum was of the Actinobacteria, with the Actinomycetes as the most abundant class, composed of microorganisms well adapted for survival in harsh environments and with multiple functions, such as the production of antibiotics and the decomposition of toxic material (Pawlowski et al. 2003); they are abundant in several Brazilian soils, with an emphasis on the Brazilian Cerrados (Vargas and Hungria 1997). The third most abundant phylum was of the Acidobacteria, represented by bacteria with high metabolic versatility (Quaiser et al. 2003) but, for which, few studies of their genetics and physiology in the tropics have been reported. Interesting, recently Lladó et al. (2016) have pointed out the importance of Acidobacteria in acid soils, emphasizing that they were responsible for the widest range and the highest enzymatic activities in an acidic forest soil.

It has been repeatedly shown that microbial diversity can be strongly influenced by abiotic factors such as soil temperature, humidity and pH (e.g. Manzoni et al. 2012; Vargas and Hungria 1997). According to Zhalnina et al. (2015), pH is be the most important factor determining soil microbial communities' structure (Osborne et al. 2011; Chaparro et al. 2012; Kuramae et al. 2012; Zhalnina et al. 2015). Indeed, high correlations between soil pH and the

compositions of bacterial communities have been reported, including studies based on DNA fingerprinting (Fierer and Jackson 2006), gene cloning (Lauber et al. 2008), and pyrosequencing (Lauber et al. 2009). In our study, several differences in microbial diversity were observed in the comparison of the two sites, possibly related to differences in soil chemical and physical properties, as well as differences in climatic conditions. For example, we found lower Eurkaryota, Proteobacteria and higher Actinobacteria numbers at LD, characterized by higher soil fertility—including higher pH—and lower compaction. According to Smit et al. (2001), soils with high levels of available nutrients have a positive selection for the Gammaproteobacteria, while in soils of low fertility or high content of recalcitrant substrates, the phylum Acidobacteria is more abundant. However, in our study, we observed the opposite, with LD showing lower abundance of Gammaproteobacteria and higher numbers of Acidobacteria, indicating that the dominance of some phyla might vary not only with soil fertility, but with a combination of edaphoclimatic conditions.

There have been many attempts to associate the microbial diversity with soil quality. Smit et al. (2001) used sequence data from the 16S rDNA of five phyla of the domain Bacteria (Acidobacteria, Proteobacteria, Nitrospira, Cyanobacteria and green sulfur bacteria) to compare the abundances of microbial groups and soil fertility. Now, our study suggests that the ratio of Proteobacteria:Acidobacteria might be a good indicator, as the better soil properties at LD were associated with a higher ratio of Proteobacteria:Acidobacteria for both cultivars (BRS 133 = 2.96; BRS 245 RR = 2.89) in comparison to Ponta Grossa (BRS 133 = 2.89; BRS 245 RR = 2.48).

As observed in a previous metagenomic study at LD (Souza et al. 2013b), the number of sequences of Eukaryota might be underestimated, most certainly due to the low number of species currently identified and the low number of sequences deposited in the databases. This limitation is critical for our knowledge about soil function. Eukaryota are considered as the primary decomposers of plant residues in terrestrial ecosystems, with an emphasis on the Ascomycota phylum (Ma et al. 2013), which were also more abundant in our study, together with Streptophyta and Chordata. Finally, although Viruses are important for the regulation of the composition of microbial communities in many environments (Breitbart and Rohwer 2005; Reyes et al. 2010), very few sequences have been deposited in databases, and, at both sites of our study, Caudovirales was the only order identified.

In the functional analyses, the higher abundances of some subsystems (carbohydrates, virulence, amino acids and derivatives, protein metabolism and DNA metabolism), were also reported in studies performed in a variety of edaphoclimatic conditions (e.g. Delmont et al. 2012; Lavery et al. 2012; Souza et al. 2014), suggesting their central role in soil functioning. However, the large number of unclassified sequences observed in our study, representing 24 % of the sequences, highlights that important functions of microbial communities remain unknown. In general, subsystems were slightly higher at PG than at LD, which may indicate an adaptation to the lower soil fertility and limitation on physical properties, requiring higher functional abundance for the maintenance of microbial processes.

Effects of long-term cropping of transgenic soybean on soil physical, chemical and biological properties

The comparison of cropping for 9 years at LD and 8 years at PG with non-transgenic and nearly isogenic RR soybean cultivars indicated no differences in soil density and granulometry, and, in relation to soil chemical properties, the only detected difference was on soil pH, slightly, but significantly lower, with the transgenic genotype. How the RR transgene affects soil pH is unclear. Contrary to our results, an increase in soil pH has been reported in transgenic *Bt* cotton (*Gossypium hirsutum* L.) (Dong et al. 2015), while in chitinase-transgenic tobacco (*Nicotiana tabacum* L.) differences in soil pH varied from increases to decreases in soil pH, depending on the plant growth stage (Wang et al. 2013). In our study, the decrease in soil pH did not affect grain yield, such that, in the long-term, no differences were detected between the conventional and the transgenic genotypes at both sites. Similar results have been previously reported by our group in comparisons of nearly isogenic genotypes carrying either the RR (Hungria et al. 2014), or the *ahas* (Hungria et al. 2015) genes. In a global analysis of 169 yield results with several genetically modified crops, including 17 RR soybean genotypes, Carpenter (2010) showed that, in most cases, there were increases in yield, followed by lack of differences and only a

small percentage of decreases; our results can be added to the second category, of lack of differences.

Concerns about possible impacts of transgenic crops on soil microbial communities are often raised, and our study offered a good opportunity of showing possible long-term effects of cropping RR soybean. We found no differences in quantitative classical microbial parameters—MB-C, MB-N, BR and BRi—confirming previous reports by our group on the analyses of both the RR (Nakatani et al. 2014) and the *ahas* (Souza et al. 2013a) genes. However, differences were detected in microbial diversity, in both 16S rDNA-DGGE and metagenomic analyses.

Examples of differences in soil microbial diversity included Proteobacteria, Firmicutes, Chlorophyta, that were more abundant with the transgenic soybean, while higher abundances of Actinobacteria and Acidobacteria were associated with the conventional genotype. Changes might be at least partially associated with the decrease in soil pH observed in the transgenic treatments, as soil microorganisms are very sensitive to this factor. For example, in a study by Dong et al. (2015), an increase in soil pH was considered as the main factor responsible for an increase in abundance and diversity of soil denitrifiers. Therefore, a slight modification in soil pH could be sufficient to alter soil microbial community structure, by favoring some microorganisms. However, as the differences in microbial diversity detected between LD and PG were far greater than those associated with the transgene, we may also conclude that other factors than soil pH—e.g. soil granulometry and compaction, both of which have profound effects on oxygen availability, as well as climate—strongly contributed to the differences observed between the two sites.

Few differences between the transgenic and non-transgenic treatments were detected in the functional metagenomic analyses. Some differences in subsystems were associated with the transgene either in LD or in PG, but only two subsystems were different at both sites: the protein metabolism and the cell division and cell cycle. As our study has highlighted a high level of genetic diversity in all four treatments analysed, it could well be that several of these microorganisms play multiple roles, indicating functional redundancy towards building a buffering capacity of the soil. That might explain similar grain yields even though some important classes of microorganism were decreased or increased due to the continuous cropping with the transgenic genotype.

In conclusion, although we detected differences in taxonomic and functional microbial diversity in soils cropped for almost a decade with conventional and nearly isogenic RR soybean genotypes using the same set of herbicides, differences were far less frequent than those associated with the locations.

Acknowledgments The project was partially financed by Embrapa (02.13.08.003.00.00) and CNPq (National Council for Scientific and Technological Development) (Universal, 470515/2012-0). L. C. Babujia acknowledges a PhD fellowship from CAPES (Coordenação de Aperfeiçoamento de Pessoal de Nível Superior), A.P. Silva a pos-doc fellow from CPNq, and A. Nakatani a pos-doc fellow from Fundação Araucária. A. T. R. Vasconcelos, M. A. Nogueira, J. V. Visentainer and M. Hungria are grateful for research fellowships from CNPq. We also thank Dr. Donovan Parks and M.Sc. Santiago Revale for help with the software STAMP and Dr. Allan R. J. Eaglesham for reviewing the manuscript.

References

Anderson TH, Domsch KH (1978) A physiological method for the quantitative measurement of microbial biomass in soils. Soil Biol Biochem 10:215–221

Babujia LC, Hungria M, Franchini JC, Brookes PC (2010) Microbial biomass and activity at various soil depths in a Brazilian oxisol after two decades of no-tillage and conventional tillage. Soil Biol Biochem 42:2174–2181

Babujia LC, Silva AP, Nogueira MA, Hungria M (2014) Microbial diversity in an oxisol under no-tillage and conventional tillage in southern Brazil. Rev Ciênc Agron 45:863–870

Benjamini Y, Hochberg Y (1995) Controlling the false discovery rate: a practical and powerful approach to multiple testing. J R Stat Soc 57:289–300

Blake GR, Hartge KH (1986) Particle density. In: Klute C (ed) Methods of soil analysis: part 1, physical and mineralogical methods, 2nd edn. ASA/SSSAJ, Madison, pp 377–382

Brandão-Junior O, Hungria M, Franchini JC, Espindola CR (2008) Comparação ente os métodos de fumigação-extração e fumigação-incubação para determinação do carbono da biomassa microbiana em um latossolo. Rev Bras Ci Solo 32:1911–1919

Breitbart M, Rohwer F (2005) Here a virus, there a virus, everywhere the same virus? Trends Microbiol 13:278–284

Brookes PC, Landman A, Pruden G, Jenkinson DS (1985) Chloroform fumigation and the release of soil nitrogen: a rapid direct extraction method to measure microbial biomass nitrogen in soil. Soil Biol Biochem 17:837–842

Carpenter JE (2010) Peer-reviewed surveys indicate positive impact of commercialized GM crops. Nat Biotechnol 28:319–321

Chaparro JM, Sheflin AM, Manter DK, Vivanco JM (2012) Manipulating the soil microbiome to increase soil health and plant fertility. Biol Fertil Soils 48:489–499

Clarke KR (1993) Non-parametric multivariate analyses of changes in community structure. Austral Ecol 18:117–143

Coenye T, Vandamme P (2003) Diversity and significance of *Burkholderia* species occupying diverse ecological niches. Environ Microbiol 5:719–729

Delmont TO, Prestat E, Keegan KP, Faubladier M, Robe P, Clark IM (2012) Structure, fluctuation and magnitude of a natural grassland soil metagenome. ISME J 6:1677–1687

Dong L, Meng Y, Wang J (2015) Effects of planting transgenic Bt + CpTI cotton on rhizosphere denitrifier abundance and diversity. Wei Sheng Wu Xue Bao 55:358–365

Dunfield KE, Germida JJ (2004) Impact of genetically modified crops on soil- and plant-associated microbial communities. J Environ Qual 33:806–815

EMBRAPA (Empresa Brasileira de Pesquisa Agropecuária) (2009) Manual de análises químicas de solos, plantas e fertilizantes. Embrapa Informação Tecnológica/Embrapa Solos, Brasília

Estrada-De Los Santos P, Bustillos-Cristales RO, Caballero-Mellado J (2001) *Burkholderia*, a genus rich in plant-associated nitrogen fixers with wide environmental and geographic distribution. Appl Environ Microbiol 67:2790–2798

Feigl BJ, Sparling GP, Ross DJ, Cerri CC (1995) Soil microbial biomass in Amazonian soils: evaluation of methods and estimates of pool size. Soil Biol Biochem 27:1467–1472

Feije F, Anger V (1972) Spot tests in inorganic analysis. Elsevier, Amsterdan

Fierer N, Jackson RB (2006) The diversity and biogeography of soil bacterial communities. Proc Natl Acad Sci USA 103:626–631

Fierer N, Leff JW, Adams BJ, Nielsen UN, Bates ST, Lauber CL (2012) Cross-biome metagenomic analyses of soil microbial communities and their functional attributes. Proc Natl Acad Sci USA 109:21390–21395

Fisher WD (1958) On grouping for maximum homogeneity. J Am Stat Assoc 53:789–798

Gomez-Alvarez V, Teal TK, Schmidt TM (2009) Systematic artifacts in metagenomes from complex microbial communities. ISME J 3:1314–1317

Gyaneshwar P, Hirsch AM, Moulin L, Chen WM, Elliott GN, Bontemps C (2011) Legume-nodulating betaproteobacteria: diversity, host range, and future prospects. Mol Plant Microbe Interact 24:1276–1288

Hungria M, Mendes IC (2015) Nitrogen fixation with soybean: the perfect symbiosis? In: de Brujin F (ed) Biological nitrogen fixation. Wiley, New Jersey, pp 1005–1019

Hungria M, Franchini JC, Campo RJ, Crispino CC, Moraes JZ, Sibaldelli RNR, Mendes IC, Arihara J (2006) Nitrogen nutrition of soybean in Brazil: contributions of biological N_2 fixation and of N fertilizer to grain yield. Can J Plant Sci 86:927–939

Hungria M, Campo RJ, Mendes IC (2007) A importância do processo de fixação biológica do nitrogênio para a cultura da soja: componente essencial para a competitividade do produto brasileiro. Embrapa Soja, Londrina

Hungria M, Franchini JC, Brandão-Junior O, Kaschuk G, Souza RA (2009) Soil microbial activity and crop sustainability in a long-term experiment with three soil-tillage and two crop-rotation systems. Appl Soil Ecol 42:288–296

Hungria M, Nogueira MA, Araujo RS (2013) Co-inoculation of soybeans and common beans with rhizobia and azospirilla: strategies to improve sustainability. Biol Fertil Soils 49:791–801

Hungria M, Mendes IC, Nakatani AS, Reis-Junior FB, Moraes JZ, Oliveira MC, Fernandes MF (2014) Effects of glyphosate-resistant gene and herbicides on soybean crop: I. Field trials monitoring biological nitrogen fixation and yield. Field Crop Res 158:43–54

Hungria M, Nakatani AS, Souza RA, Sei FB, Chueire LM, Arias CA (2015) Impact of the *ahas* transgene for herbicides resistance on biological nitrogen fixation and yield of soybean. Transgenic Res 24:155–165

James C (2014) Global status of commercialized biotech/GM crops. ISAAA, Ithaca

Jenkinson DS, Polwson DS (1976) The effect of biocidal treatment on metabolism in soil. V. A method of measuring soil biomass. Soil Biol Biochem 8:209–213

Kakirde KS, Parsley LC, Liles MR (2010) Size does matter: application-driven approaches for soil metagenomics. Soil Biol Biochem 42:1911–1923

Kaschuk G, Alberton O, Hungria M (2010) Three decades of soil microbial studies in Brazilian ecosystems: lessons learned about soil quality and indications for improving sustainability. Soil Biol Biochem 42:1–13

Kaschuk G, Alberton O, Hungria M (2011) Quantifying effects of different agricultural land uses on soil microbial biomass and activity in Brazilian biomes: inferences to improve soil quality. Plant Soil 338:467–481

Kersters K, Lisdiyanti P, Komagata K, Swings J (2006) The family Acetobacteraceae: the genera Acetobacter, Acidomonas, Asaia, Gluconacetobacter, Gluconobacter, and Kozakia. In: Falkow S, Rosenberg E, Schleifer H, Stackebrandt E (eds) Prokaryotes, vol 5. Springer, Berlin, pp 163–200

Kuramae EE, Yergeau E, Wong LC, Pijl AS, van Veen JA, Kowalchuk GA (2012) Soil characteristics more strongly influence soil bacterial communities than land-use type. FEMS Microbiol Ecol 79:12–24

Lauber CL, Strickland MS, Bradford MA, Fierer N (2008) The influence of soil properties on the structure of bacterial and fungal communities across land-use types. Soil Biol Biochem 40:2407–2415

Lauber CL, Hamady M, Knight R, Fierer N (2009) Pyrosequencing-based assessment of soil pH as a predictor of soil bacterial community structure at the continental scale. Appl Environ Microbiol 75:5111–5120

Lavery TJ, Roudnew B, Seymour J, Mitchell JG, Jeffries T (2012) High nutrient transport and cycling potential revealed in the microbial metagenome of Australian sea lion (*Neophoca cinerea*) faeces. PLoS One 7:e36478

Liles MR, Manske BF, Handelsman J, Goodman RM (2003) A census of rRNA genes and linked genomic sequences within a soil metagenomic library. Appl Environ Microbiol 69:2684–2691

Lladó S, Žifčákpvá L, Větrovský T, Eichlerová I, Baldrian P (2016) Functional screening of abundant bacteria from acidic forest soil indicates the metabolic potential of Acidobacteria subdivision I for polysaccharide decomposition. Biol Fertil Soils 42:251–260

Lopes AAC, Souza DMG, Chaer GM, Reis Junior FB, Goedert WJ, Mendes IC (2013) Interpretation of microbial soil

indicators as a function of crop yield andorganic carbon. Soil Sci Soc Am J 77:461–472
Ma A, Zhuang X, Wu J, Cui M, Lv D, Liu C, Zhuang G (2013) Ascomycota members dominate fungal communities during straw residue decomposition in arable soil. PLoS One 8:e66146
Manzoni S, Taylor P, Richter A, Porporato A, Ågren GI (2012) Environmental and stoichiometric controls on microbial carbon-use efficiency in soils. New Phytol 196:79–91
McCune B, Mefford MJ (2011) PC-ORD multivariate analysis of ecological data. MJM, Gleneden Beach
Meyer F, Parmann D, D'Souza M, Olson R, Glass EM, Kubal M, Paczian T, Rodriguez A, Stevens R, Wilke A, Wilkening H, Edwards RA (2008) The metagenomics RAST server—a public resource for the automatic phylogenetic and functional analysis of metagenomes. BMC Bioinform 19:386
Mulder C, Wouterse M, Raubuch M, Roelofs W, Rutgers M (2006) Can transgenic maize affect soil microbial communities? PLoS Comput Biol 2:1165–1172
Nakatani AS, Fernandes MF, Souza RA, Silva AP, Reis-Junior FB, Mendes IC, Hungria M (2014) Effects of the glyphosate-resistance gene and of herbicides applied to the soybean crop on soil microbial biomass and enzymes. Field Crop Res 162:20–29
Nelson DW, Sommers LE (1996) Total carbon, organic carbon, and organic matter. In: Black CA (ed) Methods of soil analysis. Part 3. Chemical methods. ASA/SSSAJ, Madison, pp 961–1010
Newcombe RG (1998) Interval estimation for the difference between independent proportions: comparison of eleven methods. Stat Med 17:873–890
O'Donnell AG, Gorres H (1999) 16S rDNA methods in soil microbiology. Curr Opin Biotechnol 10:225–229
OECD/Food and Agriculture Organization of the United Nations (2015) OECD-FAO agricultural outlook 2015. OECD Publishing, Paris. doi:10.1787/agr_outlook-2015-en. Accessed 03 December 2015
Oliveira JRA, Mendes IC, Vivaldi L (2001) Carbono da biomassa microbiana em solos de cerrado sob vegetação nativa e sob cultivo: avaliação dos métodos fumigação-incubação e fumigação-extração. Rev Bras Ci Solo 25:863–871
Osborne CA, Zwart AB, Broadhurst LM, Young AG, Richardson AE (2011) The influence of sampling strategies and spatial variation on the detected soil bacterial communities under three different land-use types. FEMS Microbiol Ecol 78:70–79
Overbeek R, Begley T, Butler RM, Choudhuri JV, Chuang HY, Cohoon M, de Crecy-Lagard V, Diaz N, Disz T, Edwards R et al (2005) The subsystems approach to genome annotation and its use in the project to annotate 1000 genomes. Nucleic Acids Res 33:5691–5702
Parks DH, Beiko RG (2010) Identifying biologically relevant differences between metagenomic communities. Bioinformatics 26:715–721
Pawlowski J, Holzmann M, Berney C, Fahrni J, Gooday AJ, Cedhagen T, Habura A, Bowser SS (2003) The evolution of early Foraminifera. Proc Natl Acad Sci USA 100:11494–11498
Pylro VS, Roesch LFW, Ortega JM, Amaral AM, Tótola MR, Hirsch PR, Rosado AS, Góes-Neto A, Silva ALC, Rosa CA, Morais DK, Andreote FD, Duarte GF, Melo IS, Seldin L, Lambais MR, Hungria M, Peixoto RS, Kruger RH, Tsai SM, Azevedo VAC (2014) Brazilian microbiome project: revealing the unexplored microbial diversity—challenges and prospects. Microbial Ecol 67:237–241
Quaiser A, Ochsenreiter T, Lanz C, Schuster SC, Treusch AH, Eck J, Schleper C (2003) Acidobacteria form a coherent but highly diverse group within the bacterial domain: evidence from environmental genomics. Mol Microbiol 50:563–575
Reichenbach H (2003) The Myxococcales. In: Garrity GM (ed) Bergey's manual of systematic bacteriology, part 3: the alpha-, beta-, delta-, and epsilon-proteobacteria, 2nd edn. Springer, New York, pp 1059–1143
Reyes A, Haynes M, Hanson N, Angly FE, Heath AC (2010) Viruses in the faecal microbiota of monozygotic twins and their mothers. Nature 466:334–338
Silva AP, Franchini JC, Babujia LC, Souza RA, Hungria M (2010) Microbial biomass under different soil and crop managements in short- to long-term experiments performed in Brazil. Field Crop Res 119:20–26
Silva AP, Babujia LC, Matsumoto LS, Guimarães MF, Hungria M (2013) Microbial diversity under different soil tillage and crop rotation systems in an oxisol of southern Brazil. TOASJ 7:40–47
Silva AP, Babujia LC, Franchini JC, Ralisch R, Hungria M, Guimarães MF (2014) Soil structure and its influence on microbial biomass in different soil and crop management systems. Soil Till Res 142:42–53
Smit E, Leeflang P, Gommans S, van den Broek J, van Mil S, Wernars K (2001) Diversity and seasonal fluctuations of the dominant members of the bacterial soil community in a wheat field as determined by cultivation and molecular methods. Appl Environ Microbiol 67:2284–2291
Sokal RR (1979) Testing statistical significance of geographic variation patterns. Syst Zool 28:227–231
Souza RA, Babujia LC, Silva AP, Guimarães MF, Arrabal CA, Hungria M (2013a) Impact of the *ahas* transgene and of herbicides associated with the soybean crop on soil microbial communities. Transgenic Res 22:877–892
Souza RC, Cantão ME, Vasconcelos ATR, Nogueira M, Hungria M (2013b) Soil metagenomics reveals differences under conventional and no-tillage with crop rotation or succession. Appl Soil Ecol 72:49–61
Souza RC, Hungria M, Cantão ME, Vasconcelos ATR, Nogueira MA, Vicente VA (2014) Metagenomic analysis reveals microbial functional redundancies and specificities in a soil under different tillage and crop-management regimes. Appl Soil Ecol 86:106–112
Spang A, Saw JH, Jorgensen SL, Zaremba-Niedzwiedzka K, Martijn J, Lind AE, Eijk JK, Schleper C, Guy L, Ettema TJG (2015) Complex archaea that bridge the gap between prokaryotes and eukaryotes. Nature 521:173–184
Steven B, Gallegos-Graves LV, Yeager CM, Belnap J, Evans RD, Kuske CR (2012) Dryland biological soil crust cyanobacteria show unexpected decreases in abundance under long-term elevated CO_2. Environ Microbiol 14: 3247–3258
Vance ED, Brookes PC, Jenkinson DS (1987) An extraction method for measuring soil microbial biomass C. Soil Biol Biochem 19:703–707

Vargas MAT, Hungria M (1997) Biologia dos solos dos cerrados. EMBRAPA Cerrados, Planaltina

Wang B, Shen H, Yang X, Guo T, Zhang B, Yan W (2013) Effects of chitinase-transgenic (McChit1) tobacco on the rhizospheric microflora and enzyme activities of the purple soil. Plant Soil Environ 59:241–246

Wilke A, Harrison T, Wilkening J, Field D, Glass EM, Kyrpides N, Mavrommatis K, Meyer F (2012) The M5nr: a novel non-redundant database containing protein sequences and annotations from multiple sources and associated tools. BMC Bioinform 13:141

Wilke A, Glass E, Bischof J, Braithwaite D, Souza M, Gerlach W (2013) MG-RAST technical report and manual for version 3.3.6–Rev 1. http://www.mcs.anl.gov/papers/ANL/MCS-TM-333_1.pdf. Accessed 03 December 2015

Zhalnina K, Dias R, Quadros PD, Davis-Richardson A, Camargo FOA, Clark IM, McGrath SP, Hirsch PR, Triplett EW (2015) Soil pH determines microbial diversity and composition in the park grass experiment. Microb Ecol 69:395–406

Zuleta LFG, Cunha CO, Carvalho FM, Almeida LGP, Clapina LP, Souza RC, Mercante FM, Faria SM, Baldani JI, Hungria M, Vasconcelos ATR (2014) The complete genome of the symbiotic nitrogen-fixing *Burkholderia phenoliruptrix* strain BR3459a: is it possible to delineate the borders between symbiosis and pathogenicity? BMC Genom 15:535