

Changes of bacterial communities in the rhizosphere of sugarcane under elevated concentration of atmospheric CO₂

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Abstract

It is believed that climate change will influence most of interactions that sustain life on Earth. Among these, the recruitment exerted by plants in their roots vicinity can change, leading to differential assemblages of microbiomes in the rhizosphere. We approached this issue analyzing the variations in the composition of bacterial communities in the rhizosphere of sugarcane cultivated under two concentrations of atmospheric CO₂ (350 or 700 ppm). In addition to the analysis of bacterial community, the use of DNA-SIP allowed the comparison of bacterial groups assimilating roots exudates (based on ¹³C-labeled DNA) in both conditions, in a period of 8 days after the CO₂ pulse. The separation of ¹³C-DNA indicated the low but increasing frequency of labeling in the rhizosphere, as averages of 0.6, 2.4 and 5.0% of total DNA were labeled after 2, 4, and 8 days after the ¹³CO₂ pulse, respectively. Based on large-scale sequencing of the V6 region in the gene 16S rRNA, we found an increase in the bacterial diversity in the ¹³C-DNA along the sampling period. We also describe the occurrence of distinct bacterial groups assimilating roots exudates from sugarcane cultivated under each CO₂ concentration. Bacilli, Gammaproteobacteria, and Clostridia showed high affinity for the C-sources released by sugarcane under 350 ppm of CO₂, while under elevated concentration of CO₂, the assimilation of roots exudates was prevalently made by members of Bacilli and Betaproteobacteria. The communities became more similar along time (4 and 8 days after CO₂ pulse), in both concentrations of CO₂, electing Actinobacteria, Sphingobacteriia, and Alphaproteobacteria as the major cross-feeders on sugarcane exudates. In summary, we described the bacterial groups with higher affinity to assimilate roots exudates in the rhizosphere of sugarcane, and also demonstrated that the rhizosphere community can be differentially assembled in a future scenario with increased contents of CO₂.

Keywords: carbon cycling, climate change, microbial diversity, plant microbiome, plant–bacteria interactions, stable isotope probing

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Introduction

The increase in the contents of CO₂ in the atmosphere is one of the major components of climate change, first acting as a triggering component, leading to increases in average temperature of Earth, what consequently affects the climate and the rain regimes worldwide (Chen *et al.*, 2013; Vasskog *et al.*, 2015; Castello & Macedo, 2016; Worn & Paine, 2016; Runting *et al.*, 2017).

The rapid increase in the concentration of atmospheric CO₂ can affects the plant metabolism, changing photosynthetic rates (Jia & Zhou, 2011; Philippot *et al.*,

2013). It is postulated that it can change plant physiology, for example, leading to higher accumulation of biomass (Guenay *et al.*, 2013) or determining differential patterns of diseases and pests occurrences (Mendes *et al.*, 2013). In addition, as plants invest part of the assimilated carbon to feed microbes in their rhizosphere (Jia & Zhou, 2011), climate change can interfere in the interactions between plants and beneficial microbes in the soil.

Rhizosphere is the ultimate soil portion in the roots vicinity, where microbial community assemblage and activity are modulated by the release of roots exudates (Drigo *et al.*, 2013; Jiang *et al.*, 2017). Plants pose a selection in this niche, aiming to increase the abundance of beneficial organisms, as those that prompt plant

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development, either by nutritional supply or protection against stressors (Medeiros *et al.*, 2006; Mendes *et al.*, 2013). Changes in the concentration of atmospheric CO₂ have been shown to influence the rhizosphere composition in native fields composed by grass plants (Drigo *et al.*, 2010). However, it is important to make similar inferences in plants used for agriculture, what can serve for a better knowledge about the microbiomes of cultivated plant species, directly connecting climate change with food supply efficiency.

Sugarcane (*Saccharum officinarum* L.) is an important crop in Brazil, serving the energetic matrix, and used for the production of sugar and ethanol (Carvalho *et al.*, 2017). Its high potential to produce biomass also includes this plant use for electrical and/or thermal energy (Lozano & Lozano, 2017). It presents a C4 metabolism, characterized by the linear increase in photosynthesis rate along the augmentation of the atmospheric CO₂ concentration (Eisenhauer *et al.*, 2012, 2013). Recent studies have suggested the potential role of several bacterial groups influencing sugarcane development (Bhardwaj *et al.*, 2017). These characteristics elect sugarcane as an important model plant to study the responsiveness of microbial communities in the rhizosphere for the increase in the CO₂ contents in the atmosphere.

Here, we used it to build an experimental design, complemented by the application of proper analytical methods to achieve the following objectives: (i) the description of bacterial groups that rapidly respond to the exudation of sugarcane roots; (ii) the detection of variations in the frequencies of these groups in a scenario with higher contents of atmospheric CO₂. These objectives derived from the central hypothesis that changes in atmospheric concentration of CO₂, as promoted by climate change, can influence the composition of bacterial community in the rhizosphere of sugarcane.

Materials and methods

Soil and plant sources

The soil used in this experiment was collected at the farm located in 'Luiz de Queiroz' College of Agriculture (ESALQ) farm, a campus of the University of São Paulo, located in Piracicaba, Brazil (22°41' 42.96"S; 47°38' 28.68"E). The soil was classified as Kandiuclafic Eutrudox with clayey texture (Nvef) (Santos *et al.*, 2006) and characterized by 44.7% of clay, 26.4% of silt and 28.9% of sand, density of 1.29 g cm⁻³; pH 5.0 in CaCl₂; 2.7% of organic matter, and 86% of bases saturation.

Sugarcane seedlings (variety RB86-7515) were generated from original material also collected in the same farm. The selection of this variety was also based on its wide cultivation along the last 10 years in the main region for sugarcane production in Brazil (the State of São Paulo) (Costa *et al.*, 2014).

Experimental setup, ¹³C labeling, and samples collection

The experiment was set using sugarcane seedlings with approximately 60 days after stalks germination in vases containing 3 kg of soil – approximately 30–40 cm height, with 3–4 leaves. These seedlings were then incubated into two sealed cabinets, made of acrylic (polymethyl methacrylate – PMMA), located in a greenhouse with temperature control at 24 °C, providing the internal temperature in the cabinets between 26 and 28 °C (monitored by a internal placed thermometer), limiting temperature stresses for the plant. When temperature raises above 28 °C cabinets were opened until temperature stabilization.

The seedlings were then acclimatized for 30 days under greenhouse conditions (26–28 °C, 16-h photoperiod), in treatments with two concentrations of CO₂: 350 (±17.5) or 700 (±35) ppm (daily checked and adjusted). During acclimatization, cabinets were opened for 1 h every day, and the amount of CO₂ inside chambers were monitored by gaseous chromatography, calculating the average rate of CO₂ fixation per plant. The amendments of CO₂ were made based on the amount needed by the plant to perform photosynthesis. These concentrations of CO₂ were adopted following a previous study where such increase in the CO₂ contents was able to induce significant changes in the structure and composition of the microbial communities in the rhizosphere of pristine plants (Drigo *et al.*, 2010).

Plants were then labeled with pulses of ¹³CO₂ (¹³C, 99% CO₂, Cambridge Isotope Laboratories) or ¹²CO₂ (for control plants), injected in the period of greatest solar intensity – connecting it with higher assimilation by photosynthesis. In total, 36 plants were used; each nine plants receiving a pulse of ¹³CO₂ or ¹²CO₂, at concentrations of 350 or 700 ppm.

Three rhizosphere samples (one per plant) were collected 48, 96, and 192 h after pulses of CO₂ (either ¹²CO₂ or ¹³CO₂). During this period plants did not shown any visual differences concerning its development. The rhizosphere was considered as the soil fraction highly adhered to roots. To proper access this soil portion, plants were removed from soils, roots were vigorously shaken and rinsed. Approximately 1.0 g gram of soil portions adhered to the roots were then removed using a spatula and stored in 2 mL plastic microtubes.

DNA extraction and separation of 'light' and 'heavy' fractions

The rhizosphere samples were subjected to total DNA extraction using the commercial kit Mobio Powersoil™ DNA Isolation (Mobio Laboratories Inc, Carlsbad, CA, USA). The quantity and integrity of the DNA were checked in agarose gel electrophoresis 1% (w/v) in 1× TAE buffer (Tris, acetic acid, EDTA), stained with in Sybr Green I (Invitrogen, Waltham, MA, USA), and further visualized under UV light.

The separation of labeled DNA (¹³C-DNA) was performed by the ultra-centrifugation of 500 ng (in volume of 10 µL) of soil DNA in a gradient of cesium trifluoroacetate (CsTFA; Amersham Biosciences) (Manfield *et al.*, 2002). Briefly, aliquots

of DNA from each sample were added to 2.2-mL tubes containing 2.0 mL of CsTFA solution with a density of 1.62 g mL⁻¹. Control tubes were also prepared containing 10 µL of ultrapure water. All tubes were centrifuged in a bench Optima TLX ultracentrifuge 120 000 (Beckman, Brea, CA, USA) with a fixed angle rotor TLA 120.2 at 64 000 rpm for 40 h at 20 °C.

After centrifugation, samples were fractionated by sample depletion from the bottom to the top of tubes using fractionation recovery system (Beckman), with a syringe attached to an infusion pump (flow rate of 0.2 mL min⁻¹). A total of 25 fractions of 80 µL of solution were collected for each sample.

The density of each fraction was determined at the AR200 Refractometer (Reichert, Depew, NY, USA) as a measure of the refractive indices (RI), further used to the construction of the density curves (g mL⁻¹). Total DNA present in each fraction was also quantified in each fraction using NanoDrop® ND-1000 UV-Vis Spectrophotometer (Uniscience, Hialeah, FL, USA). Fractions containing light and heavy portions of DNA were subjected to precipitation with isopropanol at 25 °C for 2 h followed by 15 min of centrifugation (17 000 g at 4 °C). Pelleted DNA was rinsed twice in chilled ethanol 70%, dried on air, and suspended in ultrapure sterilized water (10 µL). The total amount of DNA in each of these purified fractions was determined by quantification with Qubit® 2.0 Fluorometer using Quant-iT dsDNA HS kit (Thermo®, Waltham, MA, USA).

Due to the low amount of DNA obtained in heavy fractions, aliquots of 10 to 30 ng of DNA – either from light or heavy samples – were submitted to amplification of the complete genome, promoted by the Whole Genome Amplification kit (WGA4) (Sigma, St. Louis, MO, USA), following the manufacturer instructions.

Sequencing the V6 region of the of bacterial gene 16S rRNA

Fractionated DNA (light and heavy fractions) was subjected to sequencing of amplicons containing the V6 region of the gene 16S rRNA. The amplification reactions were performed using primers Bac967F (combined with different 5-nucleotide tag for each sample) and Bac1046R as previously described (Sogin *et al.*, 2006). Amplicons were purified with ChargeSwitch® PCR Clean-Up Kit (Life Technologies, Carlsbad, CA, USA) and quantified using the Qubit® 2.0 Fluorometer (Life Technologies). Sequencing was performed using the Ion Torrent personal genome machine (PGM) (Ion Torrent, Life Technologies) system with Ion 316 Chip (Ion Sequencing Kit User Guide v2.0, Life Technologies). See Kavamura *et al.* (2013) for a detailed description of this method.

The sequences analysis was carried out using the software QIIME (Quantitative Insights Into Microbial Ecology) (Caporaso *et al.*, 2010). The removal of poor quality sequences (below 50 pb) and primers, as well as the separation of samples by their tags (barcodes), was carried out from the *split_libraries.py* command. High quality sequences were binned into OTUs using a 97% nucleotide identity threshold with the *uclust* method (Edgar, 2010). The OTU table was rarified 17 000 sequences per samples (the lowest number found in one sample), decreasing the effects of sampling effort on the analysis. The representative

sequences for each OTU were subjected to taxonomic assignments using the *Basic Local Alignment Search Tool* (BLAST) against the Caporaso Reference OTUs (Greengenes, 2014).

Data analysis of OTUs and taxa matrices

QIIME was used to generate weighted/unweighted UniFrac distance matrices (Lozupone *et al.*, 2006) and α -diversity metrics, including Shannon-Weaver diversity indices. Values of bacterial diversity were compared by variance analysis (ANOVA), and average values were compared by Tukey test ($P < 0.05$).

To assess the variation in the phylogenetic beta-diversity among different samples we used the Principal Coordinate Analysis (PCoA) derived from the weighted UniFrac distances of bacterial communities. The separations between the groups were tested by the similarity analysis (ANOSIM) based on the distance matrix of OUT's transformed by Gower's algorithm. The intervals of R -values can indicate total separation ($R > 0.75$), separation with overlap ($0.5 < R < 0.75$), and approximation ($R < 0.5$) (Clarke & Gorley, 2001).

We used data from phyla and class *taxa summary* matrices to determine by *Similarity Percentages* (SIMPER) (Clarke, 1993) the taxa that primarily respond to variations of the communities in each concentration of CO₂, or between light and heavy fractions of the DNA.

Using data from groups named as responsive by SIMPER, the percentages of the marked phylum/classes (¹³C-DNA) were divided by the respective percentages of unlabeled (¹²C-DNA), and this ratio was subtracted from 1. The same was done between taxa assessed in standard atmosphere and with high CO₂ concentrations. The data were normalized in interval -1 and 1, representing the values of predominance (P) of each taxon as a function of the isotopic and atmospheric variations. $P > 0$ indicates a predominance of the contribution of the marked or cultivated taxon in 700 ppm of CO₂ over the unmarked and cultivated in 350 ppm of CO₂, respectively. $P < 0$ indicates the inverse of these predominance, and $P = 0$ refers to equivalent contributions of the taxon between the isotopic and atmospheric variants. The *VEGAN* package (Oksanen *et al.*, 2011) was used to run all these analyzes in R environment.

Results

Obtaining light and heavy fractions of rhizosphere DNA

The quantification of sugarcane carbon assimilation by the injection of CO₂ determined a peak of activity where plants fix approximately 210 mg CO₂ h⁻¹ (Fig. S1). This information was later used to provide the CO₂ pulse for plant assimilation on appropriate period of the day (around noon).

We found similar percentages of ¹³C labeled DNA in pulses with 350 or 700 ppm of CO₂, with average values of 0.6%, 2.4%, and 5.0% of total DNA at two, four, and eight days after pulses, respectively. These percentages correspond to ¹³C-DNA concentrations between 0.02–

1.00 mg mL⁻¹, collected from fractions 0.161 to 0.166 g mL⁻¹ within a density of CsTFA solution (Fig. S2). Similarly, lightweight DNA was concentrated in fractions of density ranging from 0.157 to 0.160 g mL⁻¹.

¹²C-DNA- and ¹³C-DNA-labeled bacterial structure and diversity

A total of 445 853 valid sequences were used to support the further inferences (170 566 from ¹²C-DNA and 275 287 from ¹³C-DNA). These sequences were binned in 41.130 OTUs, which revealed a lower bacterial diversity in ¹³C-DNA (3.92–6.68) than in ¹²C-DNA (7.61–7.78) (Fig. 1). Comparing the labeling patterns in 350 or 700 ppm, only at 4 days after CO₂ pulse, a difference is observed among treatments, with higher values of bacterial diversity for plants cultivated under 700 ppm of CO₂ (Fig. 1). Considering ¹²C-DNA samples, the diversity was similar along the period of the experiment, and also independently on CO₂ concentrations. Evaluating ¹³C-DNA samples, the tendency of diversity was similar between samples on different CO₂ concentrations, but it was possible to observe lower values on the beginning of incubation (2 days) and the progressive increase in diversity along the experimental period (4 and 8 days after the CO₂ pulse).

The PCoA shows a clear separation between the light and heavy fractions of DNA, represented in the first axis with 31% of variations explanation. Additionally, differences between samples from plants cultivated

under distinct CO₂ concentrations were detected in the second axis, where 9.3% of the variation was plotted (Fig. 2).

The analysis of ANOSIM confirmed these differences in the community structures, with differentiation between light and heavy bacterial communities in both concentrations of CO₂ ($R > 0.94$). In the same context, the differences between communities derived from atmospheres with 350 ppm or 700 ppm of CO₂ were confirmed for ¹²C-DNA ($R = 0.70$), and described as subtle for analysis based on the ¹³C-DNA ($R = 0.14$) (Table 1).

Bacterial succession driven by C consumption in the rhizosphere of sugarcane

We succeeded to detect differential bacterial communities acting as early assimilators in the rhizosphere of sugarcane cultivated under distinct CO₂ concentrations. After 2 days of the 350 ppm ¹³CO₂ pulse, the most frequent bacterial classes were Bacilli (32.6%), Gammaproteobacteria (27.5%) and Clostridia (29.3%), while prevalent groups cultivated under 700 ppm of CO₂ were affiliated to Bacilli (36.6%) and Betaproteobacteria (30.9%) (Fig. 3a).

The temporal variation in the taxonomic composition of the labeled bacteria in sugarcane rhizosphere was also observed. Either in samples from plants cultivated under 350 or 700 ppm of CO₂, communities revealed similar dynamics in samples collected at 4 and 8 days

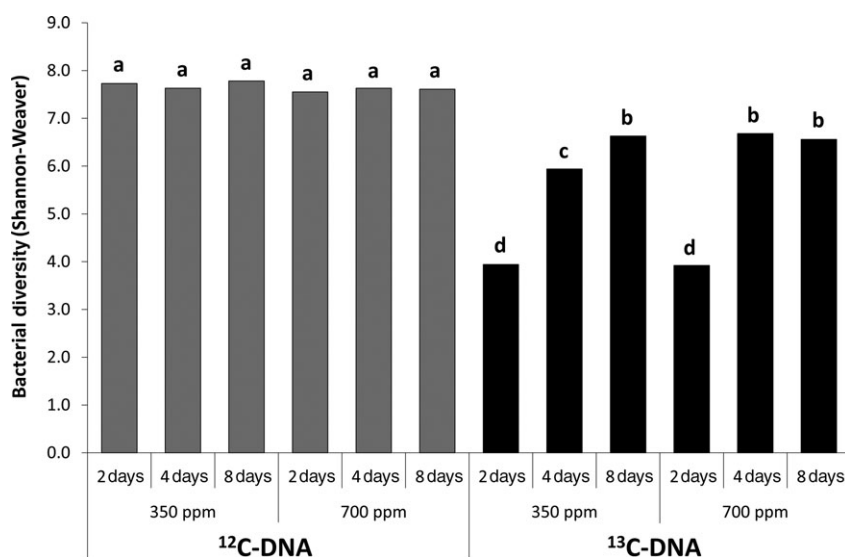


Fig. 1 Values of bacterial diversity, based on Shannon-Weaver estimator, for light (¹²C-DNA) and heavy (¹³C-DNA) fractions of DNA used to depict bacterial communities in the rhizosphere of sugarcane cultivated under standard (350 ppm) or elevated (700 ppm) concentrations of CO₂, collected 2, 4, and 8 days after the CO₂ pulse. Data represent means of three repetitions, and different letters indicate statistical differences as determined by the Tukey test ($P < 0.05$).

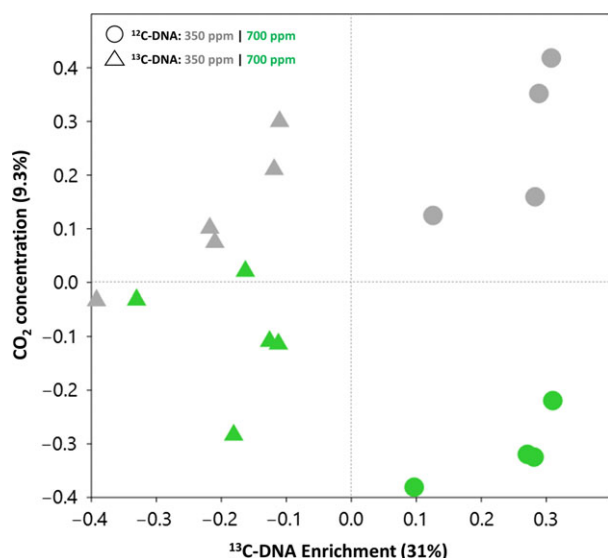


Fig. 2 Beta-diversity analysis based on PCoA derived from weighed Unifrac distances of bacterial communities found in ¹²C- or ¹³C-labeled DNA on the rhizosphere of sugarcane cultivated under standard (350 ppm) or elevated (700 ppm) concentrations of CO₂. Values in axes indicate the amount of variance plotted in each axis.

after the ¹³CO₂ pulse (Fig. 3a). In both cases, it is remarkable the increase in the frequencies of Actinobacteria, Sphingobacteriia, and Alphaproteobacteria, elected as the major cross-feeders, or secondary assimilators of the sugarcane exudates (Fig. 3a). The dynamics revealed by the labeled fraction of the DNA was not evident in the community derived from ¹²C. Data from ¹²C-DNA suggest a constant pattern of bacterial communities along the sampling period of 2, 4, and 8 days after the CO₂ pulse (Fig. 3b). These patterns were also similar in plants cultivated under 350 or 700 ppm of CO₂. Remarkable is the frequent occurrence of Anaerolinea in samples of ¹²C-DNA, a group not found in labeled DNA (Fig. 3b).

The SIMPER analysis revealed the complete set of bacterial groups supporting the differentiation of the communities described above (Table 2). Comparing the communities under distinct CO₂ concentrations, it is

possible to describe the response of 17 bacterial groups along time (Table 2) or the differentiation between ¹²C- and ¹³C-DNA (Table 3).

Discussion

Plants rely on their associate microbiota to accomplish several of the functions responsible for their proper and healthy development. The rhizosphere is the main sensor of this interaction, where plants and microbes complement themselves nutritionally and protect each other from stressors (Pietersen *et al.*, 2016). The knowledge on major players in this arena is essential either for better use this resource in technological innovations, and to predict changes in the plant association in future scenarios, as those promoted by climate change.

Several studies have shown the consequences of the increase in the CO₂ contents in plant metabolism and connections to soil. For example, an increase in the CO₂ to 850 ppm is correlated with increases in plant biomass (García-Palacios *et al.*, 2015). Also, Eisenhauer *et al.* (2012, 2013) showed that the increase in CO₂ concentrations increases the microbial biomass in soils. Together, such differentiations could induce shifts in plant metabolism, modulating the composition of roots exudates either quantitative or qualitatively (Van Veen *et al.*, 1991; Medeiros *et al.*, 2006; Drigo *et al.*, 2010, 2013), leading to differentiation on plant associated microbiota (Jia & Zhou, 2011; Philippot *et al.*, 2013). Drigo *et al.* (2010) have used SIP-DNA to determine this effect in the composition of the rhizosphere microbial community in pristine gramineous species. Here, we used a similar analysis to infer on the bacterial communities found in the rhizosphere of sugarcane, a widely cultivated crop, responding for more than 20 million hectares of land use worldwide (<http://www.fao.org/faostat/en/>). In this case, consequences of climate change may possibly have a strong impact on plant development and viability, besides possible impact on the carbon cycle and storage in agricultural soils.

Results indicate the efficacy of the SIP approach to track the bacterial groups assimilating C direct from sugarcane roots. The increases along the 8 days of

Table 1 The significance of the separations between the groups of samples by the similarity analysis (ANOSIM) based on the distance matrix of OUTs transformed by Gower's algorithm

	¹² C (350 ppm)	¹² C (700 ppm)	¹³ C (350 ppm)	¹³ C (700 ppm)
¹² C (350 ppm)		0.70*	0.94*	0.90*
¹² C (700 ppm)	0.70*		0.99*	0.96*
¹³ C (350 ppm)	0.94*	0.99*		0.14
¹³ C (700 ppm)	0.90*	0.96*		

*Significant *P*-values by analysis of similarities with Gower's distance (*R* = 0.737).

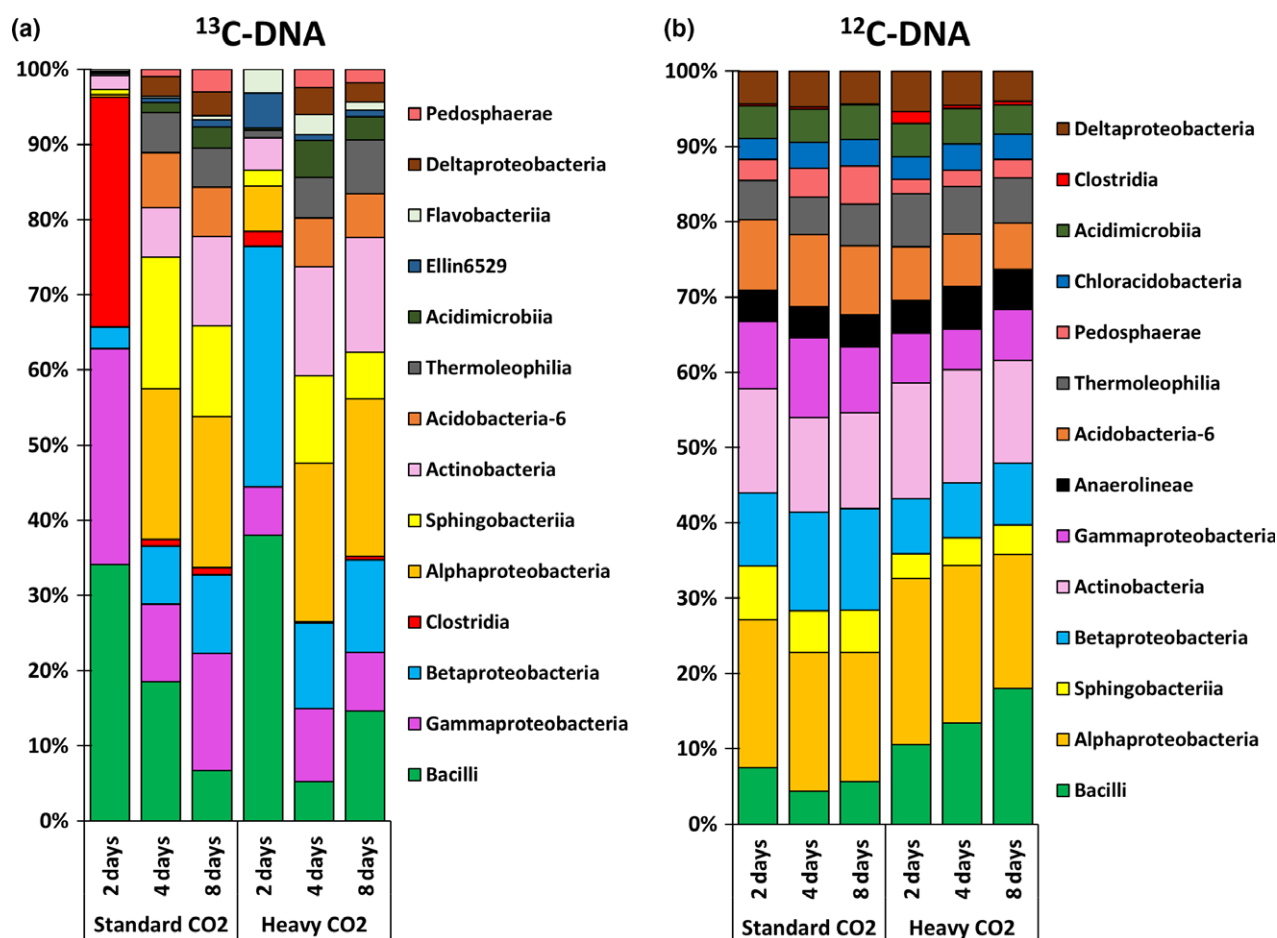


Fig. 3 Taxonomic composition of bacterial communities found in the ¹²C-DNA and ¹³C-DNA from the rhizosphere of sugarcane cultivated under standard (350 ppm) (a) or elevated (700 ppm) (b) concentrations of CO₂, collected 2, 4, and 8 days after the CO₂ pulse. Data represent means of three repetitions, and different letters indicate statistical differences as determined by the Tukey test ($P < 0.05$).

sampling in the amount of DNA labeled, and also in the diversity of bacterial community (Fig. 1) validate our approach. The sensitiveness of the SIP approach is indicated by the results, where bacterial community revealed by ¹²C-DNA is similar, but ¹³C-DNA showed very distinct patterns of bacterial community along samplings. The faster increase in bacterial diversity with labeled DNA also suggests that C release in the rhizosphere of plants cultivated under 700 ppm of CO₂ is more intense than those cultivated under 350 ppm of CO₂. Similar analyzes of microbial communities in the rhizosphere have also used small periods of high CO₂ treatment to make inferences on this issue. For example, Gschwendtner *et al.* (2015) showed that incubation of beech trees (*Fagus sylvatica* L.) for only two days in high CO₂ concentrations (1100 ppm) significantly changes the diversity of bacteria in the rhizosphere.

Modification of quantity and quality of plant exudation pattern may lead on a recruitment of differential

groups of bacteria in their roots vicinity (Van Veen *et al.*, 1991; Drigo *et al.*, 2010, 2013). We identified differential microbial assemblages in plants cultivated under distinct CO₂ concentration. The prevalent groups assimilating ¹³C in the rhizosphere of plants grown under 350 ppm of CO₂ were Bacilli, Gammaproteobacteria, and Clostridia, while in plants under higher contents of CO₂, most of bacteria labeled by ¹³C were Bacilli and Betaproteobacteria (Fig. 3). The major bacterial groups found in the rhizosphere of sugarcane in this study were similar to those previously described as associated with this plant species (Dini-Andreote *et al.*, 2010; Rachid *et al.*, 2013; Durrer *et al.*, 2017). Besides the major responders, the approach used here also named the major cross-feeders in the rhizosphere of sugarcane (Fig. 3). Members of Actinobacteria, Sphingobacteriia, and Alphaproteobacteria seem to properly get carbon from metabolites generated by the primary assimilators in the sugarcane rhizosphere, either under 350 or 700 ppm of CO₂.

Table 2 The SIMPER determination of major bacterial groups contributing to distinctions of bacterial communities, and its percentage along the eight days of plants incubation after the pulse of CO₂

	Class	Contribution (%)	2 days	4 days	8 days
350 ppm	Bacilli	21.1	32.6	16.3	5.6
	Clostridia	18.1	29.3	0.8	0.8
	Gammaproteobacteria	15.5	27.5	9.1	13.1
	Others	9.5	2.6	7.6	12.0
	Alphaproteobacteria	8.3	0.3	17.6	16.9
	Sphingobacteriia	7.0	0.7	15.4	10.1
	Acidobacteria	4.5	1.8	5.8	10.0
	Acidobacteria-6	3.3	0.0	6.5	5.5
	Betaproteobacteria	2.7	2.7	6.8	8.8
	Thermoleophilia	2.3	0.2	4.7	4.4
	Pedospaerae	1.6	0.0	0.9	2.5
	Deinactinobacteria	1.3	0.0	2.2	2.6
	Acidimicrobiia	1.3	0.1	1.2	2.3
	Chloracidobacteria	1.0	0.0	1.1	1.9
	Fusobacteria	1.0	1.6	0.0	0.1
	Anaerolineae	0.8	0.0	1.5	1.5
	Spartobacteria	0.8	0.8	2.5	1.7
700 ppm	Bacilli	27.5	36.6	4.5	12.7
	Betaproteobacteria	19.6	30.9	9.8	10.7
	Others	10.5	1.2	12.3	11.7
	Alphaproteobacteria	6.9	5.8	18.1	18.2
	Actinobacteria	5.4	4.2	12.5	13.3
	Sphingobacteriia	4.2	2.0	10.0	5.4
	Acidobacteria-6	3.8	0.0	5.6	5.0
	Thermoleophilia	3.7	1.0	4.7	6.3
	Ellin6529	3.3	4.5	0.7	0.8
	Flavobacteriia	2.3	3.1	2.3	1.0
	Acidimicrobiia	2.2	0.2	4.2	2.7
	Epsilonproteobacteria	2.0	2.6	0.0	0.0
	Gammaproteobacteria	1.9	6.2	8.4	6.8
	Spartobacteria	1.9	0.0	0.9	2.3
	Deltaproteobacteria	1.7	0.0	3.1	2.2
	Gemmatimonadetes	1.7	0.0	2.8	0.6
	Clostridia	1.4	1.9	0.2	0.4

Besides the composition of roots exudates, the differential dynamics of microbial communities observed in rhizosphere emerging from distinct concentrations of CO₂ could be also explained by lifestyle of some bacterial groups identified (Ho *et al.*, 2017). Bacilli presented a behavior classified as stress-tolerant life style (Ho *et al.*, 2017), as these organisms tolerate variable substrate variability, as indicated by the known features found in this groups of bacteria, such as desiccation and heat-resistant cells (Grime, 1977). Betaproteobacteria are classified as r-type life strategy or competitors – ruderal (Ho *et al.*, 2017). In this case, bacterial groups are prone to multiply in the presence of abundant sources of nutrients (Fierer *et al.*, 2007; Ho *et al.*, 2017). This group was responsive for the increase in the CO₂ contents in the atmosphere, what possibly increased plant exudation in the roots vicinity. The major cross-feeders

– members of Actinobacteria, Sphingobacteria, and Alphaproteobacteria – are possibly versatile in substrate utilization, able to use nutrient from recalcitrant soil organic matter, or derived from primary assimilators, a feature associated with stress-tolerant – ruderal life strategy (k-type) (Ho *et al.*, 2017). Considering that some of these groups (e.g., Betaproteobacteria) occur differentially in the rhizosphere of plants under distinct concentrations of CO₂, we need further to investigate the consequences of this shift for plant development.

Unfortunately, the sequencing approach used in this survey does not allow a better inference on the taxonomical identification of bacterial cells living in distinct rhizosphere, what could support more detailed inferences on the roles of bacteria when recruited by plant roots. Whether the changes in taxonomical composition identified here lead to a differential functioning of the

Table 3 The SIMPER determination of major bacterial groups contributing to distinctions of bacterial communities, and its percentage in communities depicted based on light (^{12}C -DNA) and heavy (^{13}C -DNA) fractions of DNA

	Class	Contribution (%)	^{12}C -DNA	^{13}C -DNA	Normalized proportion
350 ppm	Bacilli	16.1	2.6	14.7	0.83
	Clostridia	14.3	8.5	16.0	0.47
	Gammaaproteobacteria	13.1	11.8	8.5	-0.20
	Others	10.7	1.9	8.7	0.79
	Alphaproteobacteria	10.0	0.1	8.2	1.00
	Sphingobacteriia	6.9	13.7	7.1	-0.47
	Acidobacteria	6.6	18.9	12.7	-0.24
	Acidobacteria-6	4.8	8.9	4.3	-0.53
	Betaproteobacteria	4.3	10.8	6.9	-0.29
	Thermoleophilia	3.5	4.6	1.5	-1.00
	Pedospaerae	2.3	5.6	3.4	-0.33
	Destaproteobacteria	2.0	3.8	1.9	-0.51
	Acidimicrobiia	1.4	1.8	0.7	-0.75
	Chloracidobacteria	1.3	2.0	1.6	-0.26
	Fusobacteria	1.1	1.3	1.6	0.22
700 ppm	Anaerolineae	0.9	2.0	1.1	-0.76
	Spartobacteria	0.8	1.7	1.2	-0.41
	Bacilli	21.1	6.6	17.3	0.72
	Betaproteobacteria	15.5	6.3	15.6	0.69
	Others	15.0	10.7	4.7	-0.34
	Alphaproteobacteria	6.3	20.9	15.0	-0.16
	Actinobacteria	6.2	1.9	5.3	0.75
	Sphingobacteriia	5.5	14.0	10.8	-0.12
	Acidobacteria-6	4.2	6.7	3.9	-0.31
	Thermoleophilia	3.9	6.7	4.7	-0.18
	Ellin6529	3.6	3.6	1.1	-1.00
	Flavobacteriia	3.6	4.4	1.8	-0.61
	Acidimicrobiia	2.8	6.3	6.9	0.10
	Epsilonproteobacteria	2.5	2.7	1.0	-0.76
	Gammaaproteobacteria	2.4	0.2	1.7	1.00
	Spartobacteria	2.4	4.2	2.3	-0.34
	Deltaproteobacteria	1.9	0.8	1.7	0.63
	Gemmatimonadetes	1.6	2.0	0.8	-0.58
	Clostridia	1.5	1.9	5.3	0.75

rhizosphere, promoting or inhabiting plant growth remain to be targeted in future studies. Metagenomics also could support deeper studies of taxonomical identification and function of bacterial cells living in distinct rhizosphere.

In summary, this study is the first attempt to show changes in the rhizosphere of cultivated plants due to an increase in the CO_2 contents in the atmosphere. By approaching it using sugarcane as a model, we can suggest that in future scenario cropped plants may differentially assemble their rhizosphere. Intriguingly, we do not have information about the maintenance of the environmental services, or about the plant development supported by microbial communities in the rhizosphere in this new condition. Based on the model proposed here, we can move forward in this area of study in

order to properly predict changes in the interactive system between plants and microbes in the rhizosphere due to the climate change.

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Supporting Information

Additional Supporting Information may be found online in the supporting information tab for this article:

Figure S1. The rate of CO₂ fixation by sugarcane along the daylight hours. Under the conditions of this experiment, the plants reached the peak of fixation from 12 to 14 h.

Figure S2. Enrichment of DNA with ¹³C in the rhizosphere after 48 h (a), 96 h (b) and 192 h (c) after the pulse of ¹³CO₂ in concentrations of 350 ppm or 700 ppm.