Soil Biology & Biochemistry 80 (2015) 70–78

Contents lists available at ScienceDirect

Soil Biology & Biochemistry

journal homepage: www.elsevier.com/locate/soilbio

Reduced dependence of rhizosphere microbiome on plant-derived carbon in 32-year long-term inorganic and organic fertilized soils

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ARTICLE INFO

Article history:
Received 5 June 2014
Received in revised form
23 September 2014
Accepted 29 September 2014
Available online 13 October 2014

Keywords:
Bacteria
Fertilization
Pyrosequencing
Root-derived carbon
Rhizosphere microbiome
Stable isotope probing
Wheat

ABSTRACT

Root-derived carbon (C) is considered as critical fuel supporting the interaction between plant and rhizosphere microbiome, but knowledge of how plant–microbe association responds to soil fertility changes in the agroecosystem is lacking. We report an integrative methodology in which stable isotope probing (SIP) and high-throughput pyrosequencing are combined to completely characterize the root-feeding bacterial communities in the rhizosphere of wheat grown in historical soils under three long-term (32-year) fertilization regimes. Wheat root-derived ^{13}C was dominantly assimilated by Actinobacteria and Proteobacteria (notably Burkholderiales), accounting for nearly 70% of root-feeding microbiome. In contrast, rhizosphere bacteria utilizing original soil organic matter (SOM) possessed a higher diversity at phylum level. Some microbes (e.g. Bacteroidetes and Chloroflexi) enhancing in the rhizosphere were not actively recruited through selection by rhizodeposits, indicating a limited range of action of root exudates. Inorganic fertilization decreased the dependence of Actinobacteria on root-derived C, but significantly increased its proportion in SOM-feeding microbiome. Furthermore, significantly lower diversity of the root-feeding microbiome, but not the SOM-feeding microbiome, was observed under both organic and inorganic fertilizations. These results revealed that long-term fertilizations with increasing nutrients availability would decrease the preference of rhizosphere microbiome for root-derived substrates, leading to a simpler crop–microbe association.

1. Introduction

Terrestrial plants impact soil by producing an array of substrates that originate from sloughed-off root materials including cells, tissues and mucilages, as well as active root exudates such as organic acids, sugars, amino acids and phytohormones (Dennis et al., 2010). In general such plant-derived carbon (C) ranges from <10% photosynthetically fixed C to 44% total plant C under nutrient stress (Bais et al., 2006). This has the potential to lead to relationships between plants and various soil microbiota in the rhizosphere, because bacteria are often limited by available C sources in bulk soils. Microbes inhabiting such niches subsequently impart the plant with beneficial or detrimental traits; accordingly, altering this balance is of great interest in agronomy (Lundberg et al., 2012). However, the role of crop roots in the selection mechanism of the rhizosphere microbiome is less elucidated in agroecosystems (Hirsch and Mauchline, 2012), in part because of a lack of analytical methods (Cardon and Gage, 2006). The core rhizosphere-inhabiting microbiotas in *Arabidopsis thaliana*, soybean and wheat have been recently revealed in great detail (Bulgarelli et al., 2012; Lundberg et al., 2012; Donn et al., 2014; Mendes et al., 2014). These studies indicated that the rhizosphere or endophytic microbiome was a subset of the bulk soil community and dominated by Proteobacteria, Actinobacteria and Bacteroidetes. As a result, considering the less complexity of bacterial groups in the rhizosphere, a higher specialization of rhizosphere bacterial functions would be expected (Mendes et al., 2014). However, evidence of whether these dominant microorganisms thriving in the rhizosphere that is directly

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http://dx.doi.org/10.1016/j.soilbio.2014.09.028
0038-0717/

recruited by plant-derived C or via preferences for specific conditions such as soil pH, mineral nutrients and physical structure is still limited.

Soil organic matter (SOM) and nutrient availability have been increased in numerous agricultural lands over the last decades as a results of organic and inorganic fertilizations (Ai et al., 2012; Maillard and Angers, 2014) and enhanced nitrogen deposition (Liu et al., 2013). However, the ecosystem-level influences of these cumulative nutrients on soil–plant–microbe processes are unclear. Nutrient availability can impact the rhizosphere microbiotas either directly by limiting their nutrition or, more indirectly, by altering root exudation and root morphology (Rengel and Marschner, 2005). For example, inorganic fertilization suppressed microbial respiration by 36–46% in the rhizosphere of three hardwood trees, possibly via a decreased rhizosphere C flux (Phillips and Fahey, 2007). Nitrogen also has the potential to shift metabolic capabilities of microbial communities in decomposing soil C pools (Mack et al., 2004; Ramirez et al., 2012). In contrast to inorganic fertilizers, application of organic manure supports the accumulation of SOM and development of soil microbial communities with greater biodiversity (Mäder et al., 2002). Developing a predictive understanding of the role of fertilization practices in governing plant–microbe interaction would thus be a key step towards enhancing the environmental sustainability of crop production.

At present, limited information is available to address the plant-associated rhizosphere microbiome and their roles in root-derived C utilization, although the impacts of inorganic fertilizer and organic manure on soil microbial community have been widely reported in recent years (Sun et al., 2004; Ramirez et al., 2012; Coolon et al., 2013). As a result, the aims of this study were (*i*) to identify the bacterial community actively utilizing root-derived C in wheat rhizosphere, (*ii*) to determine whether the enhancement of rhizosphere microbes was directly driven by root-derived C, and (*iii*) to assess how soil microbial community and plant–microbe interaction responded to long-term fertilization practices. In doing so, we collected soils from a long-term fertilization field experiment with three treatments (i.e. no-fertilization control; inorganic nitrogen, phosphorus and potassium, NPK; and organic manure plus NPK, MNPK). With an *in situ* external $^{13}CO_2$ pulse labeling to wheat plants growing in the middle rhizosphere soil chamber in a 3-chambered-microcosm, we tracked ^{13}C movement from aboveground to belowground through DNA-SIP (stable isotope probing), and then characterized the bacterial communities through 454 pyrosequencing in wheat rhizosphere and root-free (bulk) soils. We hypothesized that: (*i*) microbial assimilation of wheat root-derived C would be dominated by a small subset of rhizosphere microbiome; (*ii*) a more diverse group of soil microbes would be activated under organic manure than under chemical fertilizers in the bulk soil; and (*iii*) long-term fertilization practices with increasing nutrients availability would reduce the dependence of rhizosphere microbiome on plant-derived C source.

2. Experimental procedures

2.1. Soil collection

Soil for the greenhouse experiment was collected in three fertilization fields from a long-term fertilization site initiated in 1979 at the Malan Farm (37°55′N, 115°13′E, 37 m above the sea level), Hebei, China. This region has a temperate and monsoonal type climate with an annual average temperature and precipitation of 12.6 °C and 490 mm, respectively. The experimental soil was classified as aquic inceptisol with a sandy loam texture according to U.S. soil taxonomy, which is typical in the North China Plain. Three fertilizations (three replicates each) as no fertilization (Control), chemical fertilizer N, P and K (NPK), and organic manure plus inorganic fertilizer N, P and K (MNPK) were respectively implemented in plots (12 m × 6.7 m) (Ai et al., 2013). The cropping system has been a rotation of winter wheat (*Triticum aestivum* cv. Shimai 18) and summer maize (*Zea mays* cv. Zhengdan 958). After 32-year fertilization, soil biochemical and biological properties are substantially different under these three fertilizations (Table S1) (Ai et al., 2012, 2013). In October 2011, ten random soil cores (6 cm diameter) from each plot as a composite sample were taken at 0–20 cm depth. A total of nine composite samples were transported to laboratory on ice, sieved (2-mm), and then stored at 4 °C until greenhouse experiments.

2.2. Soil microcosm construction, wheat growth and ^{13}C labeling

Seeds of wheat (*T. aestivum* cv. Shimai 18) were sown in the middle chamber of three-chambered growth pots (see Fig. S1) at a density of seven plants per pot, which were filled with distinctive soils from the Control, NPK or MNPK fertilization treatments with three replicates for each soil. Pots were spatially randomized and plants were grown in a greenhouse at the Institute under 16/8 h and 23/18 °C (day/night). Soil moisture was maintained at 40–60% of water-holding capacity. The ^{13}C labeling started 40 days later when plants were in an active vegetative growth state. Plants in the rhizobox system were labeled with $^{13}CO_2$ (98 atom % ^{13}C, Shanghai Research Institute of Chemical Industry, Shanghai, China) between 9 am and 5 pm (8 h) for 7 consecutive days (Lu and Conrad, 2005). During the labeling period, the total CO_2 concentration inside the chamber were maintained at 300–400 mg kg^{-1} with additional $^{13}CO_2$. It is very important that wheat plants were exposed to a normal CO_2 concentration level, because an elevated CO_2 level might affect rhizosphere C flow and associated microbiota (Drigo et al., 2010). Parallel microcosms as controls were also constructed without $^{13}CO_2$ labeling, i.e. under the ambient $^{12}CO_2$ condition (Thereafter we defined this as a $^{12}CO_2$ labeling condition). At the end of $^{13}CO_2$ labeling, the rhizosphere and bulk soils were sampled from the rhizobox (see Supplementary Information Methods S1 for details).

2.3. DNA extraction, gradient fractionation, denaturing gradient gel electrophoresis (DGGE) and ^{13}C analyses

Soil genomic DNA was extracted using a Fast DNA SPIN Kit for soil (MP Biomedicals, Illkirch, France) according to the manufacturer's instruction. In 5.1 ml quick-seal polyallomer tubes in a VTi 90 vertical rotor (Beckman Coulter, CA, USA), the rhizosphere DNA was fractionated by the cesium chloride (CsCl) equilibrium density gradient centrifugation (Lueders et al., 2004). Ultracentrifugation conditions were 56,200 r.p.m. (228 166 g_{av}) for 24 h at 20 °C (Zhang et al., 2012). DNA (4.0 μg) was mixed in CsCl gradients with an initial density of 1.725 g ml^{-1}. At a flow rate of 440 μl min^{-1}, centrifuged gradient was fractionated from bottom to top into 24 equal volumes (~220 μl) by displacing the CsCl solution with sterilized water at the top of the tube, using a programmable syringe pump (New Era Pump Systems Inc., NY, USA). Buoyant density of each collected fraction was determined by using an AR200 digital refractometer (Reichert, NY, USA). Recovery of nucleic acids from the CsCl salts was conducted by precipitation in two volumes of polyethylene glycol (PEG) 6000 solution (30% PEG 6000, 1.6 M NaCl) (Neufeld et al., 2007), and precipitates were washed twice with 70% ethanol and re-dissolved in 30 μl nuclease-free water. Quantification of nucleic acids was performed using the Picogreen assays (Molecular Probes, Invitrogen, Eugene, OR, USA). DGGE analyses (see Supplementary Information Methods S1) of the 16S rRNA PCR products derived from gradient fractions were performed using