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Original article

Rhizosphere bacterial communities associated with healthy and *Heterodera glycines*-infected soybean roots

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ABSTRACT

Bacterial communities in rhizosphere soil of soybean plants that were healthy, infected with soybean cyst nematode (SCN, *Heterodera glycines*), and infected with SCN but treated with *Purpureocillium lilacinus* YES-2 were investigated with community-level physiological profile (Biolog) and 16S rDNA clone library analyses. Biolog data indicated significant differences in substrate utilization patterns of the rhizosphere bacterial communities associated with healthy, SCN-infected, and SCN-infected plus *P. lilacinus*-treated plants; among the three treatments, substrate richness and catabolic diversity were lowest in the rhizosphere of healthy soybeans. Analysis of 16S rDNA profiles placed the soybean rhizosphere bacteria into seven groups: *Proteobacteria*, *Actinobacteria*, *Acidobacteria*, *Firmicutes*, *Bacteroidetes*, *Chloroflexi*, and an unclassified bacterial clade. The percentages of Rhizobiales and *Actinobacteria* clones were greater in the rhizosphere of healthy plants than SCN-infected plants, while the opposite was true for the proportions of *Bacteroidetes* and *Firmicutes* clones. Addition of *P. lilacinus* did significantly affect the rhizosphere bacterial community of SCN-infected plants. These results suggest that rhizosphere bacterial community may play an important role in the changes of soybean rhizosphere biological conditions during the infection process. Further studies will identify more specific changes in the rhizosphere bacterial community during the establishment and progression of SCN disease, and relate these changes to potential effects on disease management, soybean health, and soybean productivity.

1. Introduction

The rhizosphere, which is defined as that part of the soil that is influenced by living roots, is characterized by high microbial activity [1]. Rhizosphere microorganisms can greatly affect plant growth by transforming nutrients, by contributing to soil organic matter formation, and by acting as root pathogens or as antagonists of root pathogens [2,3]. It even has been postulated that plants actively recruit beneficial rhizosphere microorganisms to counteract pathogen assault [4].

A recent study of the rhizosphere microbiome of sugar beet seedlings in soils suppressive to the plant-pathogenic fungus *Rhizoctonia solani* identified key bacterial taxa and genes associated with the suppression [5]. Although more than 33,000 bacterial and archeal species were detected, *Proteobacteria*, *Firmicutes*, and *Actinobacteria* were consistently associated with disease suppression. The authors suggested that, upon attack by a fungal root pathogen, plants can exploit microbial consortia for protection [5]. To date, studies about the interplay between plants and rhizosphere microorganisms have focused on pathogens, symbiotic rhizobia, and mycorrhizal fungi, but other groups of soil microorganisms can also affect plant growth and health [6], therefore understanding of the changes of plant rhizosphere bacterial community structure will yield perhaps new control approaches of soil-borne plant pathogens and diseases.

Although rhizosphere bacterial communities have been extensively studied with classical culture-based techniques [7–11], which can only detect a small proportion of the bacteria [12,13]. Alternatives or supplements to culture-based techniques including

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PLFA (phospholipid fatty acid) analysis, Biolog analysis, and PCR-based methods such as PCR-RFLP, PCR-DGGE, T-RLFP, and 16S rDNA clone library analysis [13–16] have been developed. These methods overcome or supplement the primary limitation of classical culture-based techniques and provide essential tools for the comprehensive understanding of the roles and functions of rhizosphere bacteria.

The soybean cyst nematode (SCN; *Heterodera glycines* Ichinohe) is one of the most devastating pests of soybean in most soybean growing regions in China. A number of widely and effective used chemical nematicides for SCN management have been banned in recent years due to environmental concerns [17]. This has fueled increased interest in finding alternative methods for nematode control such as biocontrol by natural enemies. Parasites and predators of SCN have been the primary focus for biocontrol of SCN. Some fungi and bacteria, such as *Purpureocillium lilacinus* (formerly *Paecilomyces lilacinus*) [18], *Hirsutella rhossiliensis*, *Hirsutella. minnesotensis* [19,20] and *Pasteuria* sp. [21], have shown to be potential biocontrol agents. However, field application of these antagonists often have failed to suppress nematode populations because their inability to compete with the indigenous microorganisms [22,23]. Therefore, it is essential for comprehensive understanding on the relationship between rhizosphere microbial community and SCN occurrence as well as the introduction of biocontrol agent. In the current study, rhizosphere bacterial community structures of healthy and SCN-infected soybeans, and SCN-infested soybeans treated with the biocontrol agent, *P. lilacinus* YES-2 were examined by Biolog analysis and 16S rDNA clone library method.

2. Materials and methods

2.1. Soil samples

In July 2010, rhizosphere soil samples were collected from a field (45° 40′ N, 126° 35′ E) that had been cropped with soybean (*Glycine max* L. Merr.) cultivar Hefeng 50 for three consecutive years at Heilongjiang Academy of Science in Heilongjiang Province, which is the major soybean-producing province in China. The field was naturally infested with soybean cyst nematode (SCN, *H. glycines* Ichinohe) in an aggregated (spotty or patchy) pattern. The soil was black type (Mollisol) with moderate fertility. Soil samples were collected from three locations in the field: the rhizospheres of healthy and SCN-infected soybean plants, the rhizosphere of soybean plants where plants were heavily infested with SCN and treated with an alginate pellet formulation of *P. lilacinus* YES-2 (2×10^8 CFUs per g pellets) at a ratio of 75 kg pellets ha^{-1} 40 days before planting soybean. Soil pH values from the three sampling locations were 6.84, 6.91 and 6.97, respectively. Rhizosphere soil samples were obtained from each location of healthy, SCN-infested, and SCN-infested with treatment of *P. lilacinus* YES-2. According to symptoms caused by soybean cyst nematode, the healthy soybean plants were selected as showing healthy leaves and without SCN cysts on roots, while the SCN-infected soybean plants were selected as stunted growth and with cysts on roots (average 36 cysts per plant roots). And the plants were also selected from SCN-infested plot with treatment of *P. lilacinus* YES-2 which showed no visible symptoms but with cysts on the roots (average 19 cysts per plant roots). Each sample consisted of five randomly selected plants at bloom stage. After gently shaking to remove the large soil particles and loose attached soils from the roots, the soil remaining on the roots was used as the rhizosphere soil sample. Rhizosphere soil from individual plants were placed into separate sterile bags and treated as individual samples for Biolog and 16S rDNA clone library analysis.

2.2. Biolog analysis

BIOLOG™ ECO plates (Biolog Inc., Hayward, CA, USA), which contained three replicate of 31 carbon sources and a water blank containing no carbon source [14], were used to generate community-level physiological profiles (CLPPs) for the three kinds of rhizosphere soil samples. A soil suspension was prepared by adding 10 g of soil to 100 mL of sterile saline solution (8.5 g kg^{-1} NaCl). The suspension was vigorously shaken for 30 min at 180 rpm on a wrist-action shaker (Innova 42, New Brunswick Scientific). Ten-fold serial dilutions were made and 10^{-3} dilution was used to inoculate the plates. 150 μL of soil suspension was added to each well of an ECO plate, and all plates were incubated at 28 °C for 7 days, and the optical density at 590 nm in each well was read every 24 h using an automated BIOLOG Microplate Reader (BIO-RAD Model 680); data were collected using the MicroLog 4.01 software.

Rhizosphere bacterial activity in each microplate, expressed as "average well-color development" (AWCD) was calculated as follow equation at different measurement times: $\mathrm{AWCD} = \sum_{i=1}^{n}(OD_i - C_i)/n$ where OD_i is the optical density at OD_{590} of the ith substrate, C is the optical density of the control well, and n is the number of substrates (equal to 31 in this study).

Substrate richness (R) was determined as the total of number of substrates in which OD > 0.10 [24]. The Shannon diversity index (H') was calculated as follows: Shannon index, $H' = -\sum p_i \cdot \ln p_i$ where P_i is the relative optical density.

Optical density values (OD) at 72 h after inoculation were used for further analysis. Values were normalized, and the created matrix data were used for principal component analysis (PCA) using CANOCO (Microcomputer Power, Ithaca, NY, USA). ANOVAs were performed for AWCD, substrate richness, and diversity with the SPSS 13.0 for Window; means were compared with Turkey test at the 0.05 significance level.

2.3. DNA extraction and 16S rDNA clone library construction

Soil samples were placed in a PowerSoil DNA isolation kit and subjected to 5100 g for 30 s in a Mini-Bead Beater (BioSpec Products) to release DNA. DNA was extracted from the soil with the Ultra Clean soil DNA kit (MOBIO Laboratories, Inc.) as described by the manufacturer. The extracted DNA was dissolved in 50 μl of TE buffer (10 mmol/L Tris–HCl, 1 mmol/L EDTA, pH 8.0) and used as template for PCR.

PCR amplification for bacterial 16S rDNA gene was performed with the primer set of 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-TACGGTTACCTTGTTACGACTT-3′) [25]. The reaction mixture (50 μl) contained 0.5 μL of forward and reverse primers (50 pmol each), 5.0 μL of 10 × ExTaq Buffer, 5.0 μL of dNTP (2.5 mM each, TaKaRa), 2.0 μL of DNA template, and 0.5 μL Taq DNA polymerase. The PCR program consisted of 5 min at 94 °C; followed by 30 cycles of 94 °C for 1 min, 52 °C for 90 s, and 72 °C for 90 s; and then a final extension at 72 °C for 10 min. PCR products were tested for the expected size on 1.0% agarose gels that were treated with GoldView™ nucleic acid stain and viewed with an ultraviolet light. PCR products were purified using the EasyPure Quick Gel Extraction Kit (Transgene, CHINA). The DNA fragments were ligated to the pMD18-T cloning vector and transformed into *Escherichia coli* DH5a. Two-hundred white colonies were randomly selected and grown in LB medium (Luria Broth) containing 50 mg/ml ampicillin at 37 °C for 6 h. The transformants were identified using clone PCR with primers M13F and M13R. Positive clones containing full-length inserts (1500 bp) were used to construct the 16S library. One bacterial 16S rDNA library was constructed for each of the three treatments including rhizosphere soil from healthy plants, from SCN-infected plants not treated with *P. lilacinus* YES-2, and from