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Analysis of the community compositions of rhizosphere fungi in soybeans continuous cropping fields

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ABSTRACT

We used rhizosphere soil sampled from one field during zero year and two years of continuous cropping of high-protein soybean to analyze the taxonomic community compositions of fungi during periods of high-incidence of root rot. Our objectives were to identify the dominant pathogens in order to provide a theoretical basis for the study of pathogenesis as well as control tactics for soybean root rot induced by continuous cropping. A total of 17,801 modified internal transcribed spacer (ITS) sequences were obtained from three different soybean rhizosphere soil samples after zero year and 1 or 2 years of continuous cropping using 454 high-throughput sequencing. The dominant eumycote fungal were identified to be *Ascomycota* and *Basidiomycota* in the three soil samples. Continuous cropping of soybean affected the diversity of fungi in rhizosphere soils and increased the abundance of *Thelebolus* and *Mortierellales* significantly. *Thanatephorus, Fusarium*, and *Alternaria* were identified to be the dominant pathogenic fungal genera in rhizosphere soil from continuously cropped soybean fields.

Introduction

Studies have shown that there is a high incidence of root rot in soybeans that are continuously cropped, and it has become the major disease threatening soybean production (Liu et al., 2012). Continuous cropping can decrease soil pH, changing the soil from neutral to acidic. This benefits the growth of fungi and inhibits the reproduction of bacteria and actinomycetes, resulting in a soil microbial community in which fungi predominate (Vargas-Gil et al., 2011). Moreover, the continuous cropping of soybean can enrich the soil in root exudates, such as phenolic acids, which can cause an increase in abundance of the major pathogenic fungi such as *Fusarium* spp. (Guo et al., 2011). Also, an increase in the contents of organic compounds, such as sugars, amino acids, and organic acids in the rhizosphere can promote the growth of pathogens that cause soybean root rot, and is a major contributor to the occurrence of root rot (Li et al., 2006). Soybean root rot disease is mainly caused by the compound infection of multiple soil microbes, and the pathogens consist of *Fusarium oxysporum*, *F. semitectum*, *Gliocladium roseum*, *Rhizoctonia solani*, *Phytophthora sojae*, and *Pythium* spp. (Hashem et al., 2010). Continuous cropping can change the fungal community structure and cause the emergence of pathogens. Many pathogens cause soybean root rot. *Fusarium* species are the main pathogens that cause this disease in many countries (Cichy *et al.*, 2007). In the United States, the main pathogens that cause root rot disease in soybean are *Fusarium* and *Pythium* (Ellis et al., 2013). The main pathogenic fungi in Japan are *Pythium* (Sugimoto et al., 2007), while the main pathogenic fungi in Canada are *F. oxysporum* and *F. graminearum* (Thomas et al., 2007).

Although there are many traditional molecular biological methods for studying the diversity of microbes in soil, such as denaturing gradient gel electrophoresis (DGGE) (Shtienberg, 1991; Smalla et al., 2007), fluorescence in situ hybridization (FISH) (Eickhorst and Tippkötter, 2008), terminal restriction fragment length polymorphism (T-RFLP) (Chau et al., 2011) and clone libraries (Liebner et al., 2008). Although the advantage of all the molecular methods is that no need for pure culture, they cannot overcome the species limitation of traditional genomic studies or provide enough data for analysis. For example, a limitation of PCR-DGGE is that one band may contain more than one DNA fragment. More sensitive technologies are needed to better understand the microbial communities in soil. Next-generation massively parallel, high-throughput DNA sequencing is a method that has been developed independently for several instrument platforms, such as the Roche 454 FLX+ and the Illumina Miseq, and it can be

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used to sequence the highly variable regions in ribosomal RNA (16S/18S/ITS) to reveal the diversity of microbes in environmental samples, as well as their relative abundance and evolutionary relationships. At present, this technology has been widely applied to studies of microbial diversity in waste water, soil, and the human gut (Qin et al., 2010). However, there are few studies dealing with either the diversity of fungi in continuously cropped soil in soybean fields, or the diversity of soil-borne pathogens related to soybean root rot using high-throughput sequencing technologies.

In this study, a high-protein soybean cultivar was grown in the same field after zero year and 1 or 2 years of continuous cropping. By analyzing the diversity of fungi in rhizosphere soil during the branching period of soybeans planted in these three samples, and comparing the diversity of fungi in rhizosphere soil during periods of high incidence of root rot disease, the dominant fungi responsible for soybean root rot disease could be identified. Our results will provide theoretical and experimental evidence for the continued study of the pathogenic mechanisms underlying root rot, and also enable strategies to control this disease in soybean.

Material and methods

Preparation of samples

This study was performed at the Experimental Station of the Research Institute of Sugar Industry, Harbin Institute of Technology, China. It is located in 125°42′–130°10′E, 44°04′–46°40′N. The soybean line Heilong48, which is widely cultivated in Heilongjiang province, is a high-protein cultivar with 45.27% and 19.50% average protein and fat contents respectively. The total protein was determined by Kjeldahl method. The total fat content is determined by using the Soxhlet extraction method. Heilong48 was planted in the fields which were zero year and continuously cropped for 1 and 2 years. Soybean samples were harvested 60 d after planting (at the branching stage). Acquisition of soybean root was in the soil from the ground 0–20 cm, and the rhizosphere soil sample was collected by shaking. Soybean cultivars of each year randomly selected 5 plants, 3 sampling points per plant, and 3 soil points are fully mixed as 1 sample. The soil samples of zero year, continuously cropped for 1 year and 2 years were denoted Ft1y, Ft2y and Ft3y, respectively. The major physical and chemical soil indicators in the plots included: organic matter 26.13 g kg^{-1}; total N 1.69 g kg^{-1}; total K 25.4 g kg^{-1}; total P 5.5 g kg^{-1}; alkali-hydrolyzable N 133.1 mg kg^{-1}; rapidly available P 13.14 mg kg^{-1}; rapidly available K 206 mg kg^{-1}; and pH 7.0. Determination of organic matter, potassium, phosphorus, nitrogen by Ignition method, NaOH melt-flame photometry method, alkali fusion-Mo-Sb Anti spectrophotometric method, Kjeldahl method, respectively.

Disease incidence statistics

Root rot lesions were observed on the basal stem. The root of each soybean plant was collected, washed under running tap water, and assessed for the presence and severity of root rot symptoms on a 0–4 scale according to Zhou et al. (2014), where: 0 = no symptoms, 1 = mild symptoms (discoloration but no visible lesions), 2 = obvious lesions, 3 = severe lesions on the stem and diminished plant vigor, and 4 = stem rotten, plant dead. The soybean plants of 1–4 scale are pathogenetic plant. Disease Incidence Statistics were dealed with 100 plants of each soil sample.

DNA extraction, PCR and 454 pyrosequencing

Total DNA was extracted from soil samples with the E.Z.N.A Soil DNA kit (Omega USA). For the internal transcribed spacer (ITS) region located between the 18 S rDNA and 28S rDNA, two fused primers were designed consisting of 5′-(454 A, B adaptor)-(specific primer)-3′: the sequences were 5′-TCCGTAGGTGAACCTGCGG-3′ and 5′-TCCTCCGCTTATTGATATGC-3′ for ITS1 and ITS4, respectively (White et al., 1990). The A adaptor-containing end was the sequencing end, and a 10 bp barcode was added between the adaptor and the forward primer sequence to allow for discrimination of the samples during sequencing (Liebner et al., 2008). The PCR amplification was done with TransStart Fastpfu DNA polymerase in 20 μl reaction volumes, which consisted of 4 μl 5× FastPfu Buffer, 2 μl 2.5 mM dNTPs, 0.8 μl Forward Primer (5 μM), 0.8 μl Reverse Primer (5 μM), 0.4 μl FastPfu Polymerase, and 10 ng Template DNA. The reaction program was: two min at 95 °C, followed by 30 cycles of 30 s at 95 °C, 30 s at 55 °C, and 30 s at 72 °C, with a final elongation step of five minutes at 72 °C, and a hold at 10 °C. After PCR amplification, the products were examined by 1% agarose gel electrophoresis (AGE). Each amplification was repeated three times and the products pooled. PCR products were electrophoresed on 2% agarose gels and purified with the AxyPrepDNA gel extraction kit (Axygen, USA) following the manufacturer's instructions. Based on the primary quantification results of AGE, the PCR products were quantified with the QuantiFluor™ -ST fluorescent quantitative system (Promega, USA) and pooled based on the sequencing proportion. Pyrosequencing was performed on the Roche Genome Sequencer FLX platform (Roche, Basel, Switzerland) (McKenna et al., 2008).

Pyrosequencing data analysis

The pyrosequencing data was initially processed as follows: (i) the terminal adaptor sequences and the reverse primer sequences were removed, as were reads containing multiple Ns, poly A/T tails, and low quality sequences; (ii) the barcode index sequences and forward primer sequences were removed; (iii) sequences with lengths <200 bp or that contained uncertain bases, or reads in which the average quality score was lower than 25 were removed. Seqcln software (http://sourceforge.net/projects/seqclean/) was used for adaptor detection and end trimming, and the mothur software, which was downloaded from the website http://www.mothur.org/wiki/Main_Page, was used to filter the sequence reads.

The modified sequences were OUT (Operational Taxonomic Units) clustered and divided at 97% sequence identity with mothur (Schloss et al., 2009). Based on the results of OTU cluster analysis, the Alpha-diversity of the three samples, the community richness (Chao http://www.mothur.org/wiki/Chao, Ace http://www.mothur.org/wiki/Ace), the community diversity (Shannon http://www.mothur.org/wiki/Shannon, Simpson http://www.mothur.org/wiki/Simpson), and sequencing coverage (http://www.mothur.org/wiki/Coverage) were calculated. Sequences in each OTU were ranked from large to small according to the OTU richness, and Rank-abundance curves were drawn with the relative percentages of each OTU ranked against the OTU ranks.

For each OTU cluster, consistency analysis was performed for those sequences with 97% sequence identity with others in order to determine to which species they belong. The community structures were then analyzed and compared among the three samples so that a histogram could be drawn of the community structures of multiple samples at the genus level. The common and unique OTUs with <97% sequence identity in these three samples were counted, and the distributions shown in a VENN diagram. A heatmap was calculated after the clustering to display the relative differences in OTU abundances between the three samples (Li et al., 2006; Chen et al., 2012; Kolde, 2012).