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Fungal pathogen accumulation at the expense of plant-beneficial fungi as a consequence of consecutive peanut monoculturing

Xiao-gang Li [a], Chang-feng Ding [a], Tao-lin Zhang [a], Xing-xiang Wang [a,b,*]

[a] Key Laboratory of Soil Environment and Pollution Remediation, Institute of Soil Science, Chinese Academy of Sciences, Nanjing 210008, China
[b] Jiangxi Key Laboratory of Ecological Research of Red Soil, Ecological Experimental Station of Red Soil, Chinese Academy of Sciences, Yingtan 335211, China

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ABSTRACT

Peanut yield and quality are seriously compromised by consecutive monoculturing in the red soil region of southern China. Soil fungi are, however, also critical to the ecological functioning of soils and plant health and were thus the study subject here. Using 454 pyrosequencing, the entire soil fungal communities of a field where peanut had been consecutively cultured for five years and another field, where peanut had been consecutively cultured for at least 20 years, and a control field with only a single peanut crop after a lay period were compared. Fungal richness, community composition, and relative taxon abundances in soil were compared among the fields and sampling times, the latter corresponding to the pod-bearing and the pod-maturing stages. Eight hundred fungal operational taxonomic units at 97% ITS sequence identity were found among 194,783 sequence reads derived from 18 separate soil samples. Members of the phylum Ascomycota strongly dominated the soil fungal communities and putative pathogens, such as *Fusarium oxysporum*, *Leptosphaerulina australis*, *Phoma* sp., and *Bionectria ochroleuca* showed higher relative abundances in the fields where peanut was consecutively monocultured, compared to the control field, at the expense of putatively plant-beneficial fungal groups, such as *Trichoderma* sp., a glomeromycotan fungus, and *Mortierella elongata*. The results suggest that the accumulations of fungal pathogen loads at the expense of plant-beneficial fungi in the soil appear likely explanations for yield declines as a consequence of consecutive peanut cultivation.

1. Introduction

Because of increasing land scarcity and as a consequence of agro-industrialization, peanut (*Arachis hypogaea* L.) is increasingly grown in repetition on the same land without any crop rotation, which is particularly widespread in the hilly red soil region of southern China (Wang and Chen, 2005). However, consecutive monoculturing may be the reason for continuous declines in yield and quality as a consequence of increasing disease pressure (Larkin, 2003; Li et al., 2012). This phenomenon is known as soil sickness or replanting disease (Huang et al., 2013).

Soil microorganisms are important for soil functions, such as mineral nutrient cycling, organic matter turnover, and soil structure formation (Brussaard et al., 2007). Fungi are an important and diverse group of these microorganisms (Fierer et al., 2007; Burke et al., 2009), comprise multiple functional groups such as decomposers and mycorrhizal fungi (Stajich et al., 2009; Hibbett et al., 2011; Yu et al., 2012). Many factors could contribute to the yield decline and increased disease pressure in consecutive peanut monoculturing, but shifts in soil fungal communities are a very likely reason (Li et al., 2010). Many studies have been conducted on the effect of consecutive monoculturing (Huang et al., 2013). Total bacterial abundance was found to decline and total fungal abundance was increased with consecutive monoculturing of soybean, compared to corn–soybean rotation (Chen et al., 1997). Rhizodeposition of peanut and root and leaf litter, as well as identical field management for many years could create a specific soil microecological environment favourable to fungal pathogen accumulation. In line with the findings for soybean, consecutive monoculturing of peanut has been found to lead to declines in the abundances of bacteria and actinomycetes (Li et al., 2012).

Consecutive peanut monoculturing tends to lead to severe disease incidence by peanut leaf spot and root rot diseases caused by *Leptosphaerulina* sp., *Fusarium* sp. Peanut productivity gradually declines over repeated planting. In fields, in which peanuts had been monocultured for 21 years, root rot and leaf spot disease became more prevalent at flowering (Wang and Chen, 2005).

* Corresponding author. Institute of Soil Science, Chinese Academy of Sciences, Nanjing 210008, China. Tel.: +86 25 86881200; fax: +86 25 86881000.
E-mail address: xxwang@issas.ac.cn (X.-x. Wang).

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The need for containment of diseases and pests as a consequence of consecutive monoculturing is a worldwide issue and various strategies consisting of chemical, genetic and agricultural management approaches have been devised to improve crop yields and fight against peanut disease (Pavlou and Vakalounakis, 2005; Gil et al., 2008; Mbarga et al., 2012). For example, biological control using beneficial microorganisms, including plant growth-promoting rhizobacteria (PGPR) and mycorrhizal fungi has been studied extensively in recent years (Bora et al., 2004; Hu et al., 2010). However, lack of a mechanistic understanding about the causes of the problem seems the reason that biological disease control largely failed.

Previous investigations into the problem were based on conventional isolation culturing and 16S/18S rRNA gene community fingerprinting, such as denaturing gradient gel electrophoresis (DGGE), which only detect certain dominant microbial groups (Li et al., 2010). The advent of next-generation sequencing (NGS), particularly 454 deep amplicon sequencing, opened the perspective for systematic and comprehensive studies of fungal communities (Buée et al., 2009). Among the regions of the ribosomal rRNA gene cistron, the internal transcribed spacers (ITS1&2) show the highest resolution (Jumpponen and Jones, 2010). Most studies on fungi have only targeted the ITS1 region as a DNA metabarcode (Nilsson et al., 2009; Lumini et al., 2010; Xu et al., 2012a; Yu et al., 2013). The release of the GS FLX Titanium series (454 Life Sciences), which extends the length of the sequence reads to 400 bp, has recently enabled analyses of also the potentially more informative ITS2 region (Amend et al., 2010; Jumpponen and Jones, 2009, 2010). Therefore, ITS1 together with ITS2 became recently targets of DNA metabarcoding in the study of soil fungal communities (Schoch et al., 2012; Toju et al., 2012).

In this study, the main hypotheses were that the consecutive peanut monoculturing altered the composition of soil fungal communities and was indicated by certain soil fungi, and that 454 pyrosequencing could track these effects. Therefore, we investigated the population sizes and community structures of the soil fungi in two peanut consecutive monoculturing systems, one of five years and the other one of 20 years using deep amplicon sequencing. A field of a single cropping event was used as control. The study aimed at providing baseline data about the causes for the yield declines as a consequence of consecutive peanut cultivation in the red soil region of southern China.

2. Materials and methods

2.1. Site description

The study was conducted in fields of the Ecological Experimental Station of Red Soil, Chinese Academy of Sciences, Yujiang, Jiangxi province (28°13′N, 116°55′E) during the cropping season 2012. The soil at the site is classified as Udic Ferrosol (*FAO*1998 classification) and is commonly known as red soil in China. The mean annual precipitation amounts to 1750 mm (average of records over 50 years), and the period of main rainfall is the time between April and June. The monthly average temperature varies from a minimum of 5.9 °C in January to a maximum of 30 °C in July.

Three peanut fields were selected for this study. The control field, CK, had previously been planted to peanut, but was kept under fallow for the last five years before the peanut crop for this study. The field P1 has been continuously monocultured with peanut for about 20 years, except for a watermelon crop every 5–6 years. Before sampling, this field had been consecutively monocultured with peanut for 5 years. The field P2 had been consecutively planted to peanut without any exception for about 20 years. In 2012, the peanut cultivar, Ganhua 1, the fertilization scheme, and all other field management practices were the same on all three studied fields. Sowing took place at the beginning of April and the harvest at the beginning of August. Before peanut sowing, about 300 kg ha^{-1} of urea, 750 kg ha^{-1} of calcium magnesium phosphate, 225 kg ha^{-1} of potassium chlorate and 15 kg ha^{-1} borax were applied along the opposite slope surface of furrows of 10 cm width and 10 cm depth into which peanut seeds were sown. Weeds were controlled with herbicides. Glyphosate (active ingredient of 41% for 3 L ha^{-1}) was employed before planting. After planting and just before crop emergence, glyphosate (active ingredient of 41% for 3 L ha^{-1}) or atrazine (active ingredient of 50% for 1.5 kg ha^{-1}) was applied. From September to the following March, all three fields lay fallow. The three fields were positioned next to each other at a hill slope and were separated by a 1 m grassland stripe. Each field was subdivided into three subplots, which accounted for differences in the micro-relief.

2.2. Soil sampling

Since the peanut leaf spot and root rot diseases break out at the later peanut growth stage, soil samples were collected twice at the pod-bearing and the pod-maturing stages. Five random soil cores of 5 cm diameter were collected from each of three subplots per field to a depth of 0–20 cm. Upon arrival to the laboratory the soil samples were immediately frozen at −80 °C.

2.3. DNA extraction and PCR amplification

Total soil DNA was extracted from 0.25 g subsamples of soil using the FastDNA® SPIN Kit for soil (MP Biomedicals, Santa Ana, CA, USA) according to the manufacturer's instructions. Amplicon libraries were prepared using tagged fungal universal primers (ITS5 and ITS4), which target the internal transcribed spacers 1 and 2 (ITS1&2) (Schoch et al., 2012). Each of the 18 DNA samples was amplified separately using the fusion primer pair ITS5 (5′- A (6-bp MID) ACC CGCTGAACTTA AGC-3′) and ITS4 (5′- B TCCTGAGG GAAAC TTCG-3′) to generate PCR fragments of approximately 700 bp, where A and B denote the two pyrosequencing primers and MID denote the multiplexing barcode tags for post-sequencing read. PCR reactions contained 1 × PCR reaction buffer, 1.5 mM $MgCl_2$, 0.4 mM dNTPs, 1 mM of each primer, 1 U Taq DNA recombinant polymerase (Takara Biotechnology Co., Ltd., Japan), and 2 μL DNA template in a final volume of 25 μL. All amplifications were conducted in a GeneAmp PCR System 9700 thermal cycler (Applied Biosystems Inc., Foster City, CA, USA) using an initial DNA denaturation step at 94 °C for 4 min, followed by 27 cycles of denaturation at 94 °C for 30 s, annealing at 58.3 °C for 45 s, extension at 72 °C for 1 min, and a final elongation at 72 °C for 7 min. The 18 tagged PCR products were purified using the MinElute PCR Purification Kit (Qiagen, Gmbh, Germany), separated by electrophoresis through a 1.5% agarose gel in 1 × TAE and purified from the gel using the Qiagen QIAquick Gel Extraction kit (Qiagen Gmbh, Germany). These cleaned PCR products were quantified on a NanoDrop 1000 spectrophotometer (Thermo Scientific), and pooled in equimolar concentrations to a final concentration of 10 ng μL^{-1}. The product pool was sequenced in one quarter of a sequencing plate on a GS-FLX sequencer (454 Life Sciences) at Personal Biotechnology Co., Ltd (Shanghai, China). The sequence reads were separated based on their tag sequences for the 18 different soil samples.

2.4. Operational taxonomic unit-based sequence analysis

Data were processed following the procedure described previously (Fierer et al., 2008), using the Quantitative Insights into Microbial Ecology (QIIME) pipeline (http://qiime.sourceforge.net)