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Lipopeptides produced by *B. amyloliquefaciens* NJN-6 altered the soil fungal community and non-ribosomal peptides genes harboring microbial community

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ABSTRACT

Lipopeptides have extensively been shown to be an efficient antifungal agent against many fungal pathogens. However, so far no studies have been reported focusing on their effect on both the soil fungal community and the abundance of genes encoding functions linked to lipopeptide. In this study, a lipopeptide mixture from *Bacillus amyloliquefaciens* NJN-6 was applied to soil to investigate these effects. The results showed lipopeptides could reduce the copy number of 18S rRNA gene and the α-diversity of the fungal community in soil using qPCR and ITS rRNA Illumina MiSeq sequencing platform technology. Further evaluation found that lipopeptides significantly increased the relative abundance of Basidiomycota and specifically sub-groups *Cryptococcus* and *Trichosporon*. At the same time, *Fusarium* spp., especially the relative abundance of the plant pathogen *Fusarium oxysporum* was significantly inhibited. In addition, the native genes encoding functions related to lipopeptide production, specifically genes encoding the non-ribosomal peptides synthase (NRPS), were also reduced in diversity and in abundance after evaluation by sequencing using a 454 GS-FLX Titanium platform. Several genes encoding NRPS annotated to the phyla Proteobacteria were increased in proportion, while some genes encoding NRPS annotated to the phylum Actinobacteria and Cyanobacteria were decreased in proportion at the same time. The results obtained in this study suggest that bacterial-source antifungal agent can change the soil fungal community. Moreover, the application of these antifungal agents may also disturb the balance of the native microbial community responsible for lipopeptide production.

1. Introduction

The soil microbial community is an important factor for the productivity and health of plants because the acquisition of nutritients and disease resistance of plants mainly rely on the microbial community of the rhizosphere (Chaparro et al., 2012). However, the soil microbial community can be disturbed by human activity, industrial waste water irrigation (Negreanu et al., 2012; Heinze et al., 2014), poisonous matter discharge (Rodrigues et al., 2013; Shrestha et al., 2013), and even some agricultural practices (Lin et al., 2012; Zhou and Wu 2012; Hartmann et al., 2015). Actually, with rapid population growth, high input of chemical fertilizers and pesticides were increasingly used to maintain a high level of agricultural productivity (Matson et al., 1997). The residual effect caused by the high input of chemicals has been proven to not only be toxic for humans, but also harmful for the soil habitat. Previous studies have reported that pesticides including both inorganic metal ions and industrially synthesized organic chemicals can cause changes in bacterial and fungal densities of soil (Wu et al., 2014), in soil enzyme activities (Lebrun et al., 2012). All the changes listed above may contribute to the loss of important soil functions and result in an imbalance within the soil microbial community. Furthermore, the broad application of pesticides may also result in the quick increase and spread of resistance genes (Kopmann et al., 2013), leading to a decrease or even the total lose in efficiency of some pesticides.

Recently, biocontrol strategies have become increasingly popular to avoid a high input of synthesized chemicals in soil. In particular, plant growth promoting rhizobactera (PGPR) as well as derived products, have been used for plant growth promotion and disease suppression worldwide (Mascher et al., 2003; Haas and Défago 2005). *Bacillus*

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amyloliquefaciens is one of the most extensively studied PGPRs that has also been widely implemented in agriculture (Chen et al., 2009; Yuan et al., 2013). *Bacillus amyloliquefaciens* produces antimicrobial compounds that were shown to benefit the soil micro-environment and to antagonize soil pathogens (Ongena and Jacques, 2008). Lipopeptides are a series of antifungal and antiprotist activity compounds produced by *Bacillus amyloliquefaciens* (Thimon et al., 1995), in liquid media or in the plant rhizosphere after colonization (Kinsella et al., 2009). Lipopeptides were shown to have a strong antagonistic effect on the growth of plant pathogenic fungi and displayed broad-spectrum inhibition of spore germination (Arguelles-Arias et al., 2009; Yuan et al., 2012a). Additionally, some compounds with a similar molecular structure to lipopeptides were synthesized and tested (Prass et al., 1987; Grünewald et al., 2004). Their presence in soil may become a threat to other fungi and even the whole fungal community in addition to only the target pathogens due to their broad antifungal spectrum. Thus, the effect of lipopeptides on non-target soil fungi and the whole microbial community was examined in this study.

Non-ribosomal peptides (NRPs) are one of the largest groups of natural microbial secondary metabolites, having antibiotic, anticancer, and immunosuppressant bioactivity. NRPs are important for the producer in terms of nutrient acquisition, chemical communication, and defense responses (Stein 2005; Raaijmakers et al., 2010). In modern agriculture, many antifungal members of NRPs can be directly used as fungicide or as a model in bionic synthesis research (Cao et al., 2010). In agricultural soil, the properties of the NRPs related to defense can be utilized for crop pathogen suppression. The NRPs as well as the microorganism containing genes encoding enzymes responsible for production of NRPs were shown to be one of the determinant factors for a soil to show pathogen suppressive properties (Mendes et al., 2011). The soil property is an important factor affecting the enrichment of NRPs genes, and the NRPs gene group can be stable both in diversity and in richness with similar soil types (Charlop-Powers et al., 2014). However, it is not known whether other factors such as the application of antifungal compounds could affect the abundance and diversity of these functional genes in the microbial community.

In the present study, antifungal lipopeptides from a PGPR strain were isolated and subsequently supplied to a distinct soil *in vitro* to test whether these micro-biogenic antifungal compounds can alter the soil fungal community, and also determine whether the genes encoding enzymes responsible for the production of antifungal NRPs are regulated by application of NRPs. The soil with addition of antifungal compounds and the control soil with no addition were sampled and the fungal ITS region of ribosomal DNA was analyzed by PCR amplification and sequenced using MiSeq sequencing technology. Furthermore, the section of the encoding the adenylation domain (AD) responsible for synthesizing NRPs was selected to represent the NRPS genes. 454 pyrosequencing was also used to analyze the NRPs gene diversity and richness of each sample. Additionally quantitative real-time PCR was used to determine the copy number of NRPS genes.

2. Materials and methods

2.1. Antifungal compounds isolation and detection

Strain NJN-6, isolated from banana rhizosphere soil and identified as *B. amyloliquefaciens* by 16S rRNA gene sequencing (Yuan et al., 2012b), was used to produce the antimicrobial compounds. For isolation of lipopeptides, strain NJN-6 was incubated in 1 L Erlenmeyer flasks with a 200 mL working volume of LB medium (10 g of tryptone, 5 g of yeast extract, and 10 g of NaCl per liter) at 37 °C and 170 rpm for 60 h. Thereafter, the cell-free supernatant was collected by centrifugation at 12,000*g* (4 °C) for 10 min, an Amberlite XAD-16 (Alfa Aesar, a Johnson Matthey Company, Ward Hill, MA) column (10 g) was used to adsorb the active compounds. The column was first washed with 150 mL deionized water, followed by 50 mL 30% methanol to remove the impurities. Finally, the lipopeptides were eluted with 100% methanol. The collected eluted fraction was concentrated by a rotary evaporator to remove the methanol. For further purification, the surplus liquid was adjusted to pH 2.0 with 6 M HCl and stored at 4 °C overnight. The precipitate was collected by centrifugation at 12,000*g* (4 °C) for 10 min. The obtained precipitate was then re-solved into 50 mL deionized water and re-adjusted to pH 7.0 with 6 M NaOH.

HPLC was performed using a HPLC 1200 device (1200 series, Agilent, Santa Clara, CA) to analyse the lipopeptides. A 5 μL sample was injected into the HPLC column (Eclipse XDB-C18, 4.6 × 250 mm, 5 μm, Agilent, Santa Clara, CA). The conditions were set up as described in our previous study (Yuan et al., 2012a), briefly, the column temperature was maintained at 20 °C throughout the analysis; the mobile phase was the solvent containing 60% A [0.1% (v/v) CH_3COOH] and 40% B (CH_3CN) at a flow rate of 0.6 mL/min; and an ultraviolet (UV) detector was used to detect peaks at 230 nm.

2.2. Soil supplementation assay

The soil was collected from the experimental site at the “Wan Zhong” orchard (18° 230′ N, 108° 440′ E) located in Le Dong County, Hainan Province, China. Bulk soil samples at the depths of 0–20 cm at three different positions were collected, and sieved (2 mm) to remove the above ground plant material and stones. All the soil samples were stored in 4 °C before this experiment was carried out. The soil treatment *in vitro* was performed with 15 g soil placed in Petri dish: (1) Control samples: the soil was amended with 1 mL sterile deionized water weekly; (2) treatment samples: the soil was amended with 1 mL sterile lipopeptides solution (67.72 mg/L) weekly. All the dishes were sealed with parafilm and placed at 30 °C. Three replicates were performed for each treatment. All the experiment lasted for four weeks, and soil was sampled after four weeks for DNA isolation.

2.3. Amplification and sequencing of the fungal ITS genes

DNA was extracted from each of the 6 soil samples (two treatments × three replications). Extractions were conducted on 500 mg of soil (wet weight) using the Power Soil DNA Isolation kit (Mo Bio Laboratories Inc., Carlsbad, CA, USA) according to the manufacturer's instructions. DNA extraction was performed with two technical replicates for each sample (two technical replicates) and stored at −20 °C until further use. Two successive DNA extractions of each sample were pooled before performing polymerase chain reaction (to minimize the DNA extraction bias). The DNA quality was assessed according to the 260/280 nm and 260/230 nm absorbance ratios using a NanoDrop ND-2000 spectrophotometer (NanoDrop, ND2000, Thermo Scientific, 111 Wilmington, DE). The concentration of extracted DNA was between 30 ng/μL and 59 ng/μL. The ITS1F (forward primer) and ITS2 (reverse primer) primer sets were used for fungal ITS1 region amplification. The primers used for final sequencing consisted of the appropriate Illumina adapter, pad linker, the gene-specific primer and a unique 6-nt barcode, being attached to both forward and reverse primer. The primers and the PCR conditions were listed in Supplementary Table 1. PCR amplifications were performed under the following conditions: the reaction mix (25 μL) contained 10 μmoles of each primer (1 μL), 1 μL template DNA (20 ng/μL), 2.5 mmoles of dNTPs (2 μL), 5 × Q5 reaction buffer (5 μL) and 5 × Q5 GC high enhancer (5 μL), 5 U/μL of Q5 polymerase (0.25 μL). After PCR amplification, agarose gel and bands were excised and purified using the MinElute PCR Purification Kit (Qiagen, Germany), then subjected to quantification using a Qubit® 2.0 Fluorometer (Invitrogen, USA). The amplicons were subjected to unidirectional sequencing on the Illumina MiSeq sequencing platform at Personal Biotechnology Co., Ltd (Shanghai, China).