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Effect of fumigation with chloropicrin on soil bacterial communities and genes encoding key enzymes involved in nitrogen cycling☆

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ABSTRACT

Chloropicrin (CP) is a potential alternative for methyl bromide as a soil fumigant given that the use of methyl bromide has become limited. However, little is known about how fumigation with CP affects the condition of the soil microbial community. In this study, 16S rRNA amplicon sequencing and quantitative PCR were combined to investigate the effect of CP on soil bacterial community. In total, 938,922 effective reads were obtained from 18 samples and clustered into 58,662 operational taxonomic units at a similarity cut-off of 97%. Both approaches showed that the primary structure of bacterial community in soil did not significantly change at the phylum level after fumigation, but CP had a significant impact on the abundance of the bacterial microbiome that was recovered and identified. Additionally, bacterial community diversity decreased significantly, and there was a shift in the predominant populations. *Staphylococcus*, *Actinomadura*, *Acinetobacter* and *Streptomyces* significantly decreased in number or disappeared, and *Bacteroides*, *Lachnoclostridium*, *Pseudoalteromonas*, *Colwellia*, *Idiomarina* and *Cobetia* became the new predominant populations. In addition, some species associated with biodegradation, such as *Sphingomonas* spp. and *Rhodococcus* spp., significantly increased in number. The abundance of ammonia-oxidizing archaea (AOA) were significantly inhibited, yet the abundance of ammonia-oxidizing bacteria (AOB) significantly increased, and denitrification was significantly promoted. These changes in bacterial flora can considerably impact soil function and health and lead to negative effects on the environment surrounding fumigated soils, indicating the need for proactive risk management. Our study provides useful information for environmental safety assessments of CP in China.

1. Introduction

Soil fumigation has been extensively used to control pathogenic bacteria, fungi, nematodes and insects present in soil. Methyl bromide (MB) has been the most widely used fumigant since 1940 because of its broad-spectrum activity. However, MB has been gradually phased out in developing countries due to its detrimental effects on the stratospheric ozone layer (Bell, 2000). In recent years, many researchers have attempted to identify effective and economically acceptable alternatives to MB, and which leads to the development of a variety of alternatives. As a recently developed soil fumigant, chloropicrin (CP) has shown good activity against pathogenic bacteria, fungi, and nematodes present in soil and was selected as one of the most promising alternatives to MB by the Methyl Bromide Technical Options Committee of the United Nations (Mao et al., 2014). CP has been registered in many countries and is widely used in the production of tomatoes, cucumbers, strawberries, gingers and other high-value crops (Ajwa and Trout, 2004; Desaeger et al., 2008; Mao et al., 2014; Xie et al., 2015).

Most previous studies on CP main focused on its efficacy for different crops, as well as its distribution, emission and degradation characteristics (Wade et al., 2015; Wang et al., 2015; Yates et al., 2015). However, the environmental effects of CP have rarely been reported. As one of the best alternatives to MB, CP also has broad-spectrum activity. As a result, however, non-target organisms will inevitably be impacted when CP is used to control pathogenic microorganisms. As fumigant biotechnology has advanced, various

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effects of fumigants on soil microbiology communities have been published. Approaches such as Biolog (culture-dependent), phospholipid fatty acid (PLFA, culture-independent) analyses and denaturing gradient gel electrophoresis (DGGE, culture-independent) have been used to examine the effects of fumigants on soil microbial communities (Ibekwe et al., 2001; Wang et al., 2011; Dangi et al., 2015). However, these techniques have limitations and may not reflect the overall community of various microorganisms in soil. This situation has been improved by next-generation sequencing (NGS). The NGS exhibit overwhelming superiority on profiling complex bacteria community, because the generated sequencing data can give very detailed information on microbial community structure qualitatively and quantitatively. This innovative approach has renewed interest in the study of environmental microbial ecology.

NGS technology has been applied in many areas, such as in the analysis of soil, water, and sewage systems (Poulsen et al., 2013; Gołębiewski et al., 2014; Shu et al., 2015). However, NGS has rarely been used to study the effects of fumigants on soil microbial communities. Using 16S ribosomal RNA (rRNA) amplicon 454 sequencing, Liu et al. found that the application of 1,3-Dichloropropene (1,3-D) had only transient suppressive effects on soil bacterial communities (Liu et al., 2015). Feld et al. combined 16S rRNA amplicon sequencing and quantification of *amoA* expression to study the effects of dazomet and observed that large shifts in the dominant populations of bacterial community occurred on day twelve of treatment (Feld et al., 2015). However, few studies have examined the effect of CP on soil microbial communities, and few have assessed the ecological safety of CP using NGS.

In this study, in addition to using 16S rRNA amplicon sequencing to investigate the effects of CP on soil microbial communities, real-time quantitative PCR was performed to examine the effects of CP on genes encoding key enzymes involved in soil nitrogen cycling. The aim of the current study was to evaluate the effects of CP on the soil bacterial community under laboratory conditions.

2. Materials and methods

2.1. Soil and microcosm set-up

Soil samples were taken from the top 20 cm of greenhouse soil at Beijing Yanqing (GPS coordinates 40°03′24.6″N, 116°56′14.6″E). In the same greenhouse, soil fumigated with CP for successive years (expressed as CP) and never-fumigated soil (expressed as CK) were sampled. The soil was composed of 5% coarse sand, 64% fine sand, 25% silt, and 6% clay; the detailed characteristics are listed in Table S1. The soil was homogenized by sieving (2 mm mesh).

Microcosms were prepared with 600 g of sieved CK soil in a desiccator. We added CP (CP 99.5LD (Dalian Dyestuffs & Chemicals Co., Dalian, Liaoning, China), a commercial liquid product containing 99.5% CP) and sterile distilled H_2O to a final soil moisture content of 60% WHC. High and low doses of CP were set to 20 mg kg^{-1} and 10 mg kg^{-1}, respectively (Mao et al., 2014). All of the microcosms were incubated at 25 °C until sampling at the 15th day (the first sampling time point) and at the 55th day (the second sampling time point) after application of CP. The first set of samples collected post-fumigation with high and low dosage of CP are expressed as H1 and L1, respectively; the second set of samples collected post-fumigation with high and low dosage of CP are expressed as H2 and L2, respectively. Three microcosms were processed for each treatment.

2.2. DNA extraction and quantitative PCR

Total genomic DNA was extracted from each sample using a MoBio Power Extraction kit (MoBio, Carlsbad, CA, USA) according to the manufacturer's instructions. The quality of the extracted DNA was verified using a Nanodrop 1000 spectrophotometer (Thermo Scientific, Hudson, NH, USA) and on 0.5% agarose gels.

The primers and thermal conditions used for gene detection are listed in Table S2. The PCR reactions were performed in a 20 μL volume containing approximately 20 ng of soil genomic DNA, 0.5 μM of each primer and 10 μL of 2 × Taq Mix (TSINGKE Biotech, Beijing, China). The PCR products were verified by standard 2% agarose gel electrophoresis. The environmental target gene products were recovered and purified with a DNA purification kit (Transgen Biotech, Beijing, China) and were subsequently cloned using a pEASY T1 cloning kit according to the manufacturer's instructions (Transgen Biotech, Beijing, China). Recombinant *Escherichia coli* Trans1-T1 strains (Transgen Biotech, Beijing, China) were used to carry the target-gene recombinant plasmids. Inserted fragments were verified by sequencing (BGI, Shenzhen, Guangdong, China), and plasmid concentration was determined using a Nanodrop 1000 spectrophotometer (Thermo Scientific, Hudson, NH, USA). qPCR standards were prepared from serial dilutions of a linearized plasmid and then used to generate standard curves.

Quantitative PCR was performed using a CFX96 real-time PCR system (Bio-Rad, Hercules, CA, USA). Target genes were quantified with the fluorescent dye EvaGreen. Each reaction was performed in a 20 μL volume containing 10 ng soil genomic DNA, 0.2 mg ml^{-1} BSA, 0.5 μM of each primer and 10 μL of 2 × Sso Fast EvaGreen Supermix (Bio-Rad, Hercules, CA, USA). The thermal program was as follows: 2 min at 98 °C, 40 cycles of 5 s at 98 °C, and 5 s at annealing temperature, followed by a plate read. Product specificity was verified by melting curve analysis and 2% agarose gel electrophoresis. All reactions were run in triplicate.

2.3. Amplicon library preparation and Illumina sequencing

Genomic DNA samples were diluted to 1 ng/μL to reduce the effects of PCR inhibitors. The V4 region of the bacterial 16S rRNA gene was amplified using universal primers 515F [5′-GTGCCAGCMGCCGCGGTAA-3'] and 806R [5′-GGACTACHVGGGTWTCTAAT-3'] with barcodes (Liu et al., 2016). Each PCR reaction contained 2 μL dd H_2O, 15 μL of 2 × Phusion® High-Fidelity PCR Master Mix (New England Biolabs, Beverly, MA, USA), 3 μL of 6 μM 515F and 806R, and 10 μL genomic DNA. The PCR conditions were 98 °C for 1 min, followed by 30 cycles of 98 °C for 10 s, 50 °C for 30 s, 72 °C for 30 s, and a final extension at 72 °C for 5 min. The same volume of 1× loading buffer (containing SYBR green) was mixed with the PCR products and electrophoresed on 2% agarose gels for detection. Samples with a bright primary band between 400 and 450 bp were chosen for further experiments. The PCR products were mixed in ratios of equal density. Then, the mixed PCR products were purified using a GeneJET Gel Extraction Kit (Thermo Scientific, Hudson, NH, USA).

Sequencing libraries were generated using a NEB Next® Ultra™ DNA Library Prep Kit for Illumina (New England Biolabs, Beverly, MA, USA) following the manufacturer's recommendations, and index codes were added. Library quality was assessed using a Qubit@ 2.0 Fluorometer (Thermo Scientific, Hudson, NH, USA) and an Agilent Bioanalyzer 2100 system. Finally, the library was sequenced on an Illumina HiSeq platform, and 250 bp paired-end reads were generated. The 16S rRNA gene amplicon sequencing was conducted at Novogene Bioinformatics Technology Co., Ltd., Beijing, China.

2.4. Data analysis

The effects of different CP treatments on the abundance of genes