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Tag-encoded pyrosequencing analysis of the effects of fungicide application and plant genotype on rhizobacterial communities

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ABSTRACT

The application of fungicide on field crops may have unexpected non-target effects on the agro-ecosystem. We analyzed the effects of four foliar fungicide application programs and sequenced the rhizobacterial community of two chickpea cultivars over the course of two years using the 454 GS FLX amplicon pyrosequencing technology. Fungicide treatments modified the composition of the rhizobacterial communities without affecting its richness level. Correspondence analyses showed that the treatments differentially affected the rhizobacterial communities associated with different chickpea cultivars. The effects of fungicide treatment were particularly pronounced in the dry summer of 2009, when rhizobacterial richness was reduced. The influences of chickpea genotype on the fungicide effects suggest an indirect influence of the fungicide treatments through the plant secondary metabolism. In addition, the detection of boscalid residues in rhizosphere soil suggests a direct effect of fungicide. We conclude that the application of foliar fungicide influences the composition of rhizobacterial communities and this influence can be modified by plant genotype and environmental conditions.

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1. Introduction

Non-target effects of fungicidal compounds on soil bacteria have been reported (Yang et al., 2011). These effects could be important because bacteria are the most abundant soil microbial group (Gans et al., 2005) and their uniquely diverse metabolic functions support a wide range of processes essential to good soil performance (Gan et al., 2011; Nannipieri et al., 2008; Van Elsas et al., 2002). The negative non-target impact of fungicides on important functional groups of soil bacteria, such as N_2-fixers, may have consequences on the productivity of crops (Yang et al., in press). Despite the key roles of bacteria in soil processes, the influence of fungicide use on the soil bacterial community remains largely unknown. Metabolism of these chemicals are involved in complex biotic and abiotic interactions, making the impact of fungicides on soil bacterial community difficult to predict (Lo, 2010). Furthermore, the multiplicity of modes of action from different fungicides used in combination increases the difficulty of evaluating the risk associated with fungicide use. Therefore, determining these non-target effects of fungicide applications will generate scientific knowledge that can be utilized in well managed agricultural production systems.

Culture-dependent methods provide a partial picture of the soil microbial community (Nannipieri et al., 2008; Nautiyal et al., 2008). Cloning, polymerase chain reaction (PCR), denaturing gradient gel electrophoresis (DGGE) and temperature gradient gel electrophoresis (TGGE) of microbial gene markers have been used to describe soil bacterial communities at a finer scale (Bürgmann et al., 2005, 2004). However, these technologies lack sensitivity and often underestimate diversity when the number of species is high. Novel tag-encoded 454 GS FLX amplicon pyrosequencing (Roche Diagnostics Corporation) can provide a much more detailed picture of microbial communities than traditional methods (Margulies et al., 2005) and was proven very useful in microbial ecology (Qian et al., 2011; Vishnivetskaya et al., 2011). Thus, 454 GS FLX amplicon pyrosequencing technology was used here as the core method to determine the non-target effects of foliar fungicide application on the rhizobacterial community associated with field-grown chickpeas.

2. Materials and methods

2.1. Experimental design and site description

The experiment was conducted at the South Farm of the Semiarid Prairie Agricultural Research Centre in Swift Current,

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Table 1
Timing and combinations of foliar fungicide treatments in the chickpea field.

Treatment	Growing stage				
	Seedling	Vegetative	Early flower	Mid-flower	Podding
Control (C)	–	–	–	–	–
I	Headline® Duo	–	Headline® Duo	–	–
II	Headline® Duo	Bravo®	Headline® Duo	–	–
III	Headline® Duo	Bravo®	Headline® Duo	Bravo®	Bravo®

Note: Bravo® was applied at a rate of 1.0 kg a.i. ha^{-1} chlorothalonil; Headline® Duo was applied at a rate of 100 g a.i. ha^{-1} pyraclostrobin and 240 mg a.i. ha^{-1} boscalid.

Saskatchewan, Canada (50°25′N, 107°44′W), in 2008 and 2009. Two factors were arranged in a split-plot design with four replicates. Two chickpea cultivars (CDC Luna and CDC Vanguard) were randomized within main plots and four fungicide treatments (three application strategies and a no-fungicide control) were randomized within subplots. Two commercial fungicidal products commonly used for disease control in chickpea, Bravo® (Syngenta Crop Protection Canada Inc., Guelph, Ontario, with chlorothalonil as active ingredient) and Headline® Duo (BASF Canada Inc., Mississauga, ON, with pyraclostrobin and boscalid as active ingredients), were used following three different practical application strategies (Table 1).

Adjacent sites were used in 2008 and 2009. The soil was an Orthic Brown Chernozem (Campbell et al., 2000) with an organic C content of 20 g kg^{-1}, pH ($CaCl_2$) of 6.5 in the top 0–15 cm, and a silt loam texture with 28% sand, 49% silt and 23% clay. The soil contained 3.6 kg ha^{-1} N, 21.8 kg ha^{-1} P and 283 kg ha^{-1} K in 2008 and 3.1 kg ha^{-1} N, 12.6 kg ha^{-1} P and 210 kg ha^{-1} K in 2009. The average monthly precipitation during the growing seasons (1 April–30 September) differed: 59.3 mm in 2008 and 35.6 mm in 2009.

2.2. Soil sampling

Rhizosphere soil samples were taken during the chickpea harvest. The first 1 cm of surface soil was removed to eliminate plant debris, and five chickpea plants were dug out from the top 25 cm soil layer of each plot. The bulk of the soil aggregate was removed, and five samples of each plot were collected, pooled and carefully labeled. Samples were taken back to the lab where the rhizosphere soil was brushed from chickpea roots and then filtered through 2 mm sieves. The soil was well labeled and stored in small plastic bags at −20 °C for further molecular analysis. The level of fungicide residues in the soil was measured on a 200-g soil sample made by pooling 50 g of soil from each replicate of a treatment, by ALS Edmonton Environmental Lab (www.alsglobal.com). The remainder of the soil samples from each plot were kept in separate sealed plastic bags and stored at −20 °C to await further molecular analyses.

2.3. Soil bacterial DNA extraction and PCR using tag-encoded 454 GS FLX amplicon pyrosequencing

DNA was extracted from the soil using the UltraClean Soil DNA Isolation Kit (MO BIO Laboratories, Inc., Carlsbad, CA, USA) and diluted 20 times. The DNA extracts were subjected to PCR using 16S rDNA-targeting primers 968f/1401b to amplify an approximately 450 bp fragment. Primers (Table 2) had a 454 Life Science's A or B sequencing adaptor fused to the 5′ end of both the forward and reverse primers. One of 16 unique multiplex identifiers (MIDs) was added between the A sequencing adaptor and the forward primer. Platinum® PCR SuperMix (Cat. No. 11306-016, Invitrogen™) was used as the PCR mix. Thermal cycling was conducted in an Veriti™ 96-well fast Thermal Cycler (Applied Biosystems) under the following conditions: 4 min initial denaturation at 94 °C; 30 cycles of 45 s denaturation at 94 °C, 45 s annealing at 56 °C and 1 min elongation at 72 °C; and a 15 min final elongation at 72 °C. All PCR products were submitted to gel electrophoresis (1% agarose gel) under 65 V for 1 h, and the bands migrating at the target location were excised with a sterile scalpel blade. The excised bands were placed in sterile centrifuge tubes with 30 μl TE buffer (1× dilution), vortexed for 1 min and stored overnight at 4 °C. Each sample of purified PCR product was transferred into a new tube, and its concentration was measured using a Nano Drop-1000 spectrophotometer (Thermo scientific®). The concentration of each sample was adjusted to 20 ng DNA/μl. Four pools of equimolar amounts of 16 samples with different MIDs were prepared and subjected to 454 pyrosequencing at Génome Québec, Montréal, Canada.

Table 2
Primers used to amplify bacterial sequences from soil DNA samples for pyrosequencing analysis to verify population differences.

Name	Primer sequence (5′–3′)
Forward primer	
Lib-L PrimerA1-F	**CCATCTCATCCCTGCGTGTCTCCGACTCAG**ACGAGTGCGTAACGCGAAGAACCTTAC
Lib-L PrimerA2-F	**CCATCTCATCCCTGCGTGTCTCCGACTCAG**ACGCTCGACAAACGCGAAGAACCTTAC
Lib-L PrimerA3-F	**CCATCTCATCCCTGCGTGTCTCCGACTCAG**AGACGCACTCAACGCGAAGAACCTTAC
Lib-L PrimerA4-F	**CCATCTCATCCCTGCGTGTCTCCGACTCAG**AGCACTGTAGAACGCGAAGAACCTTAC
Lib-L PrimerA5-F	**CCATCTCATCCCTGCGTGTCTCCGACTCAG**ATCAGACACGAACGCGAAGAACCTTAC
Lib-L PrimerA6-F	**CCATCTCATCCCTGCGTGTCTCCGACTCAG**ATATCGCGAGAACGCGAAGAACCTTAC
Lib-L PrimerA7-F	**CCATCTCATCCCTGCGTGTCTCCGACTCAG**CGTGTCTCTAAACGCGAAGAACCTTAC
Lib-L PrimerA8-F	**CCATCTCATCCCTGCGTGTCTCCGACTCAG**CTCGCGTGTCAACGCGAAGAACCTTAC
Lib-L PrimerA10-F	**CCATCTCATCCCTGCGTGTCTCCGACTCAG**TCTCTATGCGAACGCGAAGAACCTTAC
Lib-L PrimerA11-F	**CCATCTCATCCCTGCGTGTCTCCGACTCAG**TGATACGTCTAACGCGAAGAACCTTAC
Lib-L PrimerA13-F	**CCATCTCATCCCTGCGTGTCTCCGACTCAG**CATAGTAGTGAACGCGAAGAACCTTAC
Lib-L PrimerA14-F	**CCATCTCATCCCTGCGTGTCTCCGACTCAG**CGAGAGATACAACGCGAAGAACCTTAC
Lib-L PrimerA15-F	**CCATCTCATCCCTGCGTGTCTCCGACTCAG**ATACGACGTAAACGCGAAGAACCTTAC
Lib-L PrimerA16-F	**CCATCTCATCCCTGCGTGTCTCCGACTCAG**TCACGTACTAAACGCGAAGAACCTTAC
Lib-L PrimerA17-F	**CCATCTCATCCCTGCGTGTCTCCGACTCAG**CGTCTAGTACAACGCGAAGAACCTTAC
Lib-L PrimerA18-F	**CCATCTCATCCCTGCGTGTCTCCGACTCAG**TCTACGTAGCAACGCGAAGAACCTTAC
Reverse primer	
Lib-L PrimerB-R	**CCTATCCCCTGTGTGCCTTGGCAGTCTCAG**CGGTGTGTACAAGACCCGGGAACG

Note: Adaptor A for forward primers and adaptor B for the reverse primer are shown in bold letters; 16 unique multiplex identifiers (MID) connected with forward primers are underlined.

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