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Impact of foliar fungicides on target and non-target soil microbial communities in cucumber crops

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ABSTRACT

The application of foliar fungicides to horticultural crops has raised public concerns worldwide. In fact, it has been demonstrated that such fungicides have an impact on non-target microorganisms in the rhizosphere. Fluopyram, triadimenol and penthiopyrad are three broad-spectrum fungicides recommended to control foliar diseases. In our experiment, these fungicides were applied to a cucumber crop to mainly control downy mildew caused by *Pseudoperonospora cubensis* and grey mold caused by *Botrytis cinerea.* At the same time, we found that these treatments also controlled other fungal pathogens affecting cucumber crops, particularly penthiopyrad, which was more effective. Once the fungicide application period was over, the effect decreased, although fungicide traces remained in the soil. Furthermore, microbial soil community analysis indicated that both fungicide treatments affect fungal communities to a greater extent than bacterial communities.

1. Introduction

Fungicides are routinely used in conventional agriculture to control fungal diseases. In Europe, the pesticide use has increased in recent decades, and Spain is the European country in which the highest amounts of pesticides have been used (Eurostat, 2015). Fungicides are bioactive and toxic substances that directly or indirectly influence soil productivity and agroecosystem quality (Jørgensen and Thygesen, 2006). Fungicides have different modes of action, and they can have a broad range or target a specific group of fungi (Morton and Staub, 2008). Moreover, the fungicide type and method of use vary for different crops.

The application of fungicides before the appearance of a disease, instead of once symptoms have appeared, is the most common practice for farmers, despite the fact that this practice is 50% less effective (Holmes et al., 2015). The excessive use of fungicides has produced pathogen resistance (Bellón-Gómez et al., 2014). Furthermore, fungicides have an effect not only on fungal pathogens, but also on non-target fungi. It is important to understand the effect of fungicides on non-target microorganisms, because some of these microorganisms have antagonistic activity against different pathogens and can help to optimize fungicide application strategies.

Fluopyram, triadimenol and penthiopyrad are foliar fungicides with broad-spectrum activity for controlling fungal pathogens (including those causing downy mildew and grey mold in cucurbits) (Labourdette et al., 2010; Yanase et al., 2013). Fluopyram and penthiopyrad inhibit succinate dehydrogenase (SDHI), which is part of the tricarboxylic acid cycle associated with mitochondrial electron transportation, affecting fungal respiration (Proffer et al., 2013). Triadimenol is a rapid systemic fungicide that acts on the formation of ergosterol, preventing the formation of the cell membrane of fungi. Beyond the effect of these fungicides against aerial plant pathogens, it is also being reasonable to assume that foliar applications could seep into the soil and accumulate in it, affecting both target and non-target soil microorganisms. Studies related to the possible impact of fluopyram-triadimenol and especially penthiopyrad on soil microorganisms are however limited.

Nowadays, the use of molecular tools to study soil microbial communities is widespread (Sugiyama et al., 2014; Sylla et al., 2013; Sułowicz et al., 2016). The information obtained from high-throughput sequencing platforms allows us to evaluate the possible cause-effect relationship between fungicide application and soil community composition. Quantitative PCR analysis has proved to be highly efficient in detecting and quantifying bacterial and fungal pathogens, which are the causal agents of plant diseases in both above-and belowground (Lievens

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et al., 2006; Schaad et al., 2002). This technique makes it possible to monitor the presence of pathogens in both plants and the soil, and it could thus be a good approach to optimizing pesticide use.

The accessibility of a fast, sensitive, and accurate method for detecting pathogens to improve disease control is of increasing importance. Quantitative PCR has significant potential in terms of quantifying low disease levels with high sensitivity and speed (López-Mondéjar et al., 2012, Blaya et al., 2016). Furthermore, simultaneous detection of more than one pathogen provides significant benefits, particularly for diagnostic programs dealing with a lot of samples and using quantitative PCR. In this study, we used a Vegalert quantitative PCR kit (Microgaia Biotech), which made it possible to detect and quantify different fungal pathogens in cucurbits in the soil and on leaves.

The main aim of this study was therefore to analyze the effect of fluopyram-triadimenol and penthiopyrad foliar fungicide treatments on a) fungal diseases in cucumber crops, especially *Pseudoperonospora cubensis* and *Botrytis cinerea*, and on b) non-target soil bacterial and fungal communities.

2. Materials and methods

2.1. Experiment design and sample collection

In November 2015, the experiment was set up in sandy soil in a greenhouse (550 m^2) located in Almería (Spain). Twelve (6 × 6 m) plots were selected (four plots per treatment). The following treatments were applied: the two fungicide treatments Luna Devotion [(Fluopyram and Triadimenol, Bayer CropScience) (136 L ha^{-1})] (**FL**) and Fontelis [Penthiopyrad, Dupont (182 L ha^{-1})] (**PE**) (Fungicide treatments) and one non-fungicide treatment (**Control**). We used N-P-K (100-40-120) fertilization. A total of 25 cucumber plants were sown per plot. Fungicides were aerial sprayed on leaves following the manufacturers´ recommendations (6 times with intervals of 7 days between each application).

The rhizosphere soil and plant leaves from 8 plants per plot were sampled just after the first fungicide application (T1, December 2015); the last fungicide application (T2, February 2016); and two months after the last fungicide application (T3, April 2016). Soil samples were sieved (< 2 mm) and maintained at − 20 °C. Leaf samples were maintained at − 80 °C until measurements.

2.2. DNA extraction

For each plot, DNA samples from 8 plant leaves and from 8 rhizosphere soil samples were obtained and pooled in a composite sample for each plot. The total DNA from the soil samples (500 mg) was extracted using the Mo Bio PowerSoil DNA Isolation Kit (Mo Bio Laboratories, Carlsbad, CA) following the modification described by Taskin et al. (2011). Total DNA was extracted from leaves (100 mg) using a Mo Bio PowerPlant DNA extraction kit (Mo Bio Laboratories, Carlsbad, CA) following the manufacturer's protocol.

2.3. Quantitative PCR analyses (qPCR)

Pathogen detection and quantification was performed using the Vegalert quantitative PCR kit for cucurbits (Microgaia Biotech S.L, Murcia, Spain). The pathogens analyzed are indicated in Table 1. The qPCR amplifications were performed in leaves and soil samples using a 7500 Fast Real-Time PCR System (Applied Biosystems) in a total volume of 15 µL. The reaction mixtures contained a final concentration of 1 × TaqMan Universal Master Mix II no UNG (Applied Biosystems), 0.3 µm of each primer, 0.1 µm of TaqMan probe, 0.1 mg mL^{-1} of BSA, 3 µL of DNA template and nuclease-free water. Samples were run in triplicate. The thermal cycling conditions were as follows: 95 °C for 10 min, 95 °C for 10 s and 60 °C for 40 s (40 cycles) and a final step at 50 °C for 2 min. The amplification results were analyzed with 7500 Fast Real-Time PCR Software v.2.0 (Applied Biosystems).

Table 1
Fungal and bacterial microorganisms measured by Vegalert quantitative PCR kits for cucurbits.

FUNGI		BACTERIA
Acremonium cucurbitacearum	*Phytophthora* spp.	*Acidovorax avenae* subsp. *citrulli*
Alternaria spp.	*Podosphaera fuliginea*	*Erwinia carotovora* subsp. *atroseptica*
Botrytis cinerea	*Pseudoperonospora cubensis*	*Erwinia carotovora* subsp. *carotovora*
Colletotrichum spp.	*Pyrenochaeta lycopersici race 1*	
Dydimella bryoniae	*Pyrenochaeta lycopersici race 2*	
Erysiphe cichoracearum	*Pythium aphanidermatum*	
Fusarium oxysporum	*Pythium irregulare*	
Fusarium oxysporum f. sp. *cucumerinum*	*Pythium* spp. *(I)*	
Fusarium oxysporum f. sp. *melonis*	*Pythium* spp. *(II)*	
Fusarium oxysporum f. sp. *radicis-cucumerinum*	*Pythium ultimum*	
Fusarium solani	*Rhizoctonia solani*	
Fusarium spp.	*Rhizopycnis vagum*	
Macrophomina phaseolina	*Sclerotinia minor*	
Monosporascus cannonballus	*Sclerotinia sclerotiorum*	
Olpidium bornovanus	*Sclerotium rolfsii*	
Olpidium brassicae	*Verticillium albo-atrum*	
Phytophthora capsici	*Verticillium dahliae*	
Phytophthora cryptogea/ Phytophthora erythroseptica		
Alternaria spp.		
Botrytis cinerea		
Erysiphe cichoracearum		
Sphaerotheca fuliginea		
Dydimella bryoniae		
Pseudoperonospora cubensis		

2.4. Sequencing

For bacteria, the V4 region of bacterial 16S rDNA was amplified using the barcoded primers 515F and 806R (Caporaso et al., 2012). For fungi, the ITS2 region was amplified with the primer pair gITS7/ITS4 (Ihrmark et al., 2012). Each sample was amplified in triplicate as described previously by Žifčáková et al. (2016). Amplicons were purified using the QIAquick PCR Purification Kit (Qiagen, Hilden, Germany), and the DNA concentration was measured by Qubit (Thermo Fisher, Ca). After amplification and purification of the amplicons, a TruSeq PCR-Free kit was used for library preparation. Sequencing of bacterial and fungal amplicons was performed on Illumina MiSeq, and the sequences were generated with the MiSeq Reagent Kit v2 on a Paired-end mode with sizes of 251 basepairs (Institute of Microbiology of the CAS, Czech Republic).

The amplicon sequencing data were processed using the SEED 1.2.3 program (Větrovský and Baldrian, 2013). Pair-end reads were merged using fastq-join (Aronesty, 2013), and whole amplicons were processed. Chimeric sequences were detected using Usearch 7.0.1090 (Edgar, 2010) and removed. Non-chimeric sequences were clustered to 97% similarity using UPARSE implemented within Usearch (Edgar, 2013). Consensus sequences were constructed for each cluster, and the closest hits both at the genus and species level were identified using BLASTn against the RDP (Cole et al., 2014) and GenBank databases for bacteria or UNITE and GenBank databases for fungi (Kõljalg et al., 2013). Sequences identified as non-bacterial or non-fungal were excluded from subsequent analyses. The Shannon–Wiener index (H) was calculated for 4500 randomly chosen sequences per sample. The pipeline SEED 2.0.4