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Exploring the reservoir of potential fungal plant pathogens in agricultural soil

Maaike van Agtmaal[a,b], Angela Straathof[c,d], Aad Termorshuizen[e], Sven Teurlincx[f], Maria Hundscheid[a], Stefan Ruyters[g], Pieter Busschaert[g], Bart Lievens[g], Wietse de Boer[a,c,*]

[a] Netherlands Institute of Ecology, Department of Microbial Ecology, NIOO-KNAW, Wageningen, The Netherlands
[b] Imperial College London, Division of Ecology and Evolution, Ascot, United Kingdom
[c] Wageningen UR, Department of Soil Quality, Wageningen, The Netherlands
[d] University of Manchester, Faculty of Life Sciences, Manchester, United Kingdom
[e] Bodemplant.nl, Wageningen, The Netherlands
[f] Netherlands Institute of Ecology, Department of Aquatic Ecology, NIOO-KNAW, Wageningen, The Netherlands
[g] KU Leuven, Laboratory for Process Microbial Ecology and Bioinspirational Management, Department of Microbial and Molecular Systems, Sint-Katelijne-Waver, Belgium

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ABSTRACT

Soil-borne pathogens cause great crop losses in agriculture. Because of their resilience in the soil, these pathogens persist in a population reservoir, causing future outbreaks of crop diseases. Management focus is usually on the most common pathogens occurring, but it is likely that a mixed population of pathogens together affect crops. Next generation sequencing of DNA from environmental samples can provide information on the presence of potential pathogens. The aim of this study was to obtain insight into the factors that drive the composition of potential plant pathogen populations in agricultural soils. To this end, the alpha and beta diversity of fungal OTUs that were assigned as potential plant pathogens for 42 agricultural soils were assessed. The presented study is the first inventory of the pool of pathogens and its correlating factors. The results of this inventory indicate that the composition of pathogens in soil is driven by pH, soil type, crop history, litter saprotrophic fungi and spatial patterns. The major driving factors differed between potential root- and shoot-infecting fungi, suggesting interactions among environmental factors and pathogen traits like reproduction, survival and dispersal. This information is important to understand risks for disease outbreaks and to recommend management strategies to prevent such outbreaks.

1. Introduction

As part of the total soil microbial community, soils harbour a reservoir of plant pathogenic fungal propagules, forming a 'plant pathogenic seedbank' (Gilbert, 2002; Oerke, 2006). The presence of these pathogens in a given agricultural field is determined by the crops grown and the persistence of pathogens in the absence of hosts. While the focus of research and management recommendations is often only on the most abundant or damaging pathogens, combinations of major and minor pathogens may have synergistic or additive effects on disease development (Lamichhane and Venturi, 2015). Metabarcoding technology allows to obtain an overview of the composition of the plant pathogenic reservoir.

In the absence of living hosts, most pathogens can survive actively as saprotrophs or enter a dormant state in the form of resting propagules (Lennon and Jones, 2011; Termorshuizen and Jeger, 2008). This pool of surviving propagules forms an important source of future disease outbreaks. Abiotic factors including pH, various nutrients, organic matter and clay content have been shown to influence pathogen survival (Kühn et al., 2009; Mondal and Hyakumachi, 1998; Peng et al., 1999). Yet, little is known about the impact of environmental factors on abundance, diversity and species composition of the fungal pathogenic reservoir in agricultural soils. This lack of knowledge is hampering predictions of disease outbreaks and the ability to make management recommendations.

The soil biotic community strongly influences the dynamics of pathogens (Garbeva et al., 2011, 2006; Perez-Piqueres et al., 2006). Competition for resources or withdrawal of nutrients from survival structures by indigenous microbes reduces the viability of pathogens (Hoitink and Boehm, 1999). On the other hand, competitive interactions can also trigger the formation of resting propagules or prevent their outgrowth, enhancing survival (Garbeva et al., 2011; Lennon and

* Corresponding author at: P.O. Box 50, 6700 AB Wageningen, The Netherlands.
E-mail address: W.deboer@nioo.knaw.nl (W.d. Boer).

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Jones, 2011; Lockwood, 1977; Romine and Baker, 1972).

Arable fields represent a highly dynamic habitat in which external inputs and disturbances by management practices influence the presence, activity and interactions of pathogens and other soil microbes (Bockus and Shroyer, 1998; Campos et al., 2016; Sturz et al., 1997). In addition, buildup of pathogens in agricultural soils is related to the availability of suitable host plants. Consequently, cropping history can determine the survival of plant pathogens in the soil (Bennett et al., 2012).

Root pathogens infect plants belowground and generally do not form airborne spores. These pathogens are therefore limited in dispersal ability compared to shoot-infecting pathogens. The shoot infecting pathogens often form dispersal spores, which allows them to spread more easily over large areas (Termorshuizen, 2014). Hence, life history characteristics may be an additional factor influencing the spatial distribution and diversity of the pool of plant pathogens.

For a long time, studies on distribution of pathogen propagules in agricultural soils relied on classical cultivation techniques, despite clearly recognized inherent biases of these methods, e.g. the restriction to only detect culturable microorganisms or the inability to detect dormant propagules (Filion et al., 2003). Furthermore, most studies were limited to either one or a few pathogen species or a limited number of study sites. Detection of pathogen DNA in soil can overcome these limitations (Lievens and Thomma, 2005) and the advent of next generation sequencing technologies (Goodwin et al., 2016; Margulies et al., 2005) has enabled expansion of the range of detection of pathogens in soils (Vettraino et al., 2012). Since, DNA sequences can indicate close kinship with known pathogenic species but do not prove a direct relationship with disease symptoms it is more appropriate to refer to such sequences as “potential pathogens”. The aim of this study was to get a comprehensive overview of the potential pathogens present in the fungal reservoir in a range of agricultural soils using 454 amplicon pyrosequencing. Furthermore, it was aimed to determine key environmental biotic and abiotic parameters that shape the potentially pathogenic fungal community.

2. Materials and methods

2.1. Study sites, soil sampling and handling

A total of 42 agricultural sites representative for the edaphic variation in Dutch soils were selected for this study (Fig. 1). These sites covered a wide range of soil textures, pH values and organic matter content, as well as differences in crops, fertilizers and tillage practices (Supplementary Table 1a,b). Since the interest was in the pool of surviving pathogens after former crop harvest, sites were sampled in early spring 2013 (February-March), before the start of the next growing season. Soil sampling (0–20 cm cores) was performed by Eurofins Agro (Wageningen, the Netherlands), a commercial laboratory for soil and plant analysis, according to their standard method, which comprises taking 60 subsamples in a double W-pattern over approx. 2 ha and pooling the sub-samples. This resulted in roughly 3 kg soil per site. Upon arrival in the lab, samples were homogenized and split in subsamples that were used for analysis of physico-chemical soil properties as well as microbial community analysis. Regarding the first, a broad set of soil physical and chemical parameters was determined by Eurofins Agro using standard procedures (Supplementary Table 1). For the microbial analysis, directly after sampling two 1 g subsamples per site were taken for DNA extraction and stored at −20 °C until processing, which happened within a month after sampling. All handling steps after sampling were done one by one in a sterile environment using sterile materials to avoid cross contamination.

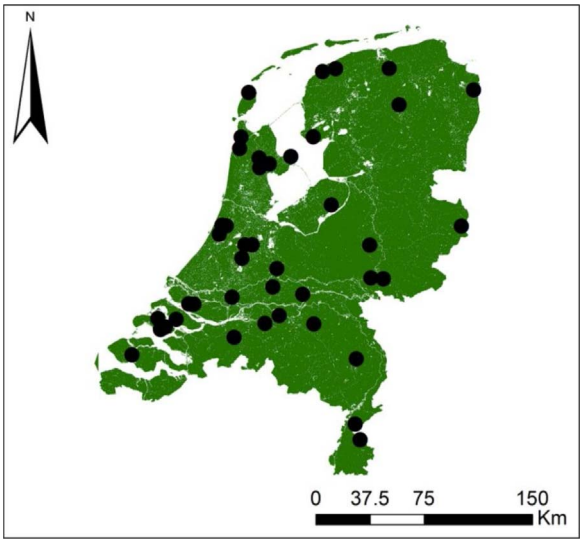

Fig. 1. Map of the agricultural sites in the Netherlands that have been sampled for this study.

2.2. DNA extraction, library preparation, 454-pyrosequencing and data processing

2.2.1. Sequencing preparation

For each of the selected fields, genomic DNA was extracted in duplicate from 2 × 1 g soil using the Mobio 96 well Powersoil® extraction kit (Mobio Laboratories Inc., Carlsbad, CA, USA). Subsequently, amplicon libraries were created using two PCR primer sets, targeting part of the bacterial 16S ribosomal RNA (rRNA) genes and the fungal internal transcribed spacer (ITS) 2 region. Primer pairs used included 577F (5’-AYTGGGYDTAAAGNG-3’) and 926R (5’-CCGTCAATTCMTTTRAGT-3’) for bacteria (Rosenzweig et al., 2012) and ITS86F (5’-GTGAATCATCGAATCTTTGAA-3’) and ITS4 (5’-TCCTCCGCTTATTGATATGC-3’) for fungi (Turenne et al., 1999; White et al., 1990). All samples were assigned unique MID (Multiplex Identifier) barcode sequences according to the guidelines for 454 GS-FLX Titanium Lib-L sequencing (Supplementary Table 2). PCR amplification was performed in a 25 µl reaction volume containing 0.15 mM of each dNTP, 0.5 µM of each primer, 1 unit Titanium *Taq* DNA polymerase, 1X Titanium *Taq* PCR buffer (Clontech Laboratories, Palo Alto, CA, USA), and 5 ng genomic DNA (measured using a Nanodrop instrument (Thermo Scientific Nanodrop Products Inc., Wilmington, DE, USA)). PCR conditions were as follows: initial denaturing (2 min at 94 °C), followed by 30 cycles of denaturation (45 s at 94 °C), annealing (45 s at 59 °C,) and extension (60 s at 72 °C), and a final extension step to ensure full length amplicons (10 min at 72 °C). After resolving the amplicons by agarose gel electrophoresis, amplicons within the expected size range were excised and extracted from the gel using the QIAquick® gel extraction kit (Qiagen, Hilden, Germany). Purified dsDNA amplicons were then quantified using the Qubit fluorometer with the high-sensitivity DNA reagent kit (Invitrogen, Carlsbad, CA, USA). Next, for each primer pair, samples were pooled at equimolar concentrations, resulting in two amplicon libraries. Each library was sequenced (Macrogen Inc., South Korea) on a separate 1/2th Pico Titer Plate (PTP) section using the Roche GS-FLX instrument with Titanium chemistry according to the manufacturer’s instructions (Roche Applied Science, Mannheim, Germany).

2.2.2. Data processing 454 pyrosequencing

Sequences obtained from the 454 pyrosequencing run were assigned to the appropriate sample (sequences from both DNA duplicates combined) based on barcode and primer sequences allowing zero discrepancies using Mothur version 1.32.1 (Schloss et al., 2009). Sequences were trimmed using Mothur based on a minimum Phred score of 30 (base call accuracy of 99.9%) averaged over a 50 bp moving window and sequences with ambiguous base calls or homopolymers