Suppression of soil-borne plant pathogens

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“What is essential is invisible to the eye”
Antoine de Saint Exupéry, Le petit prince

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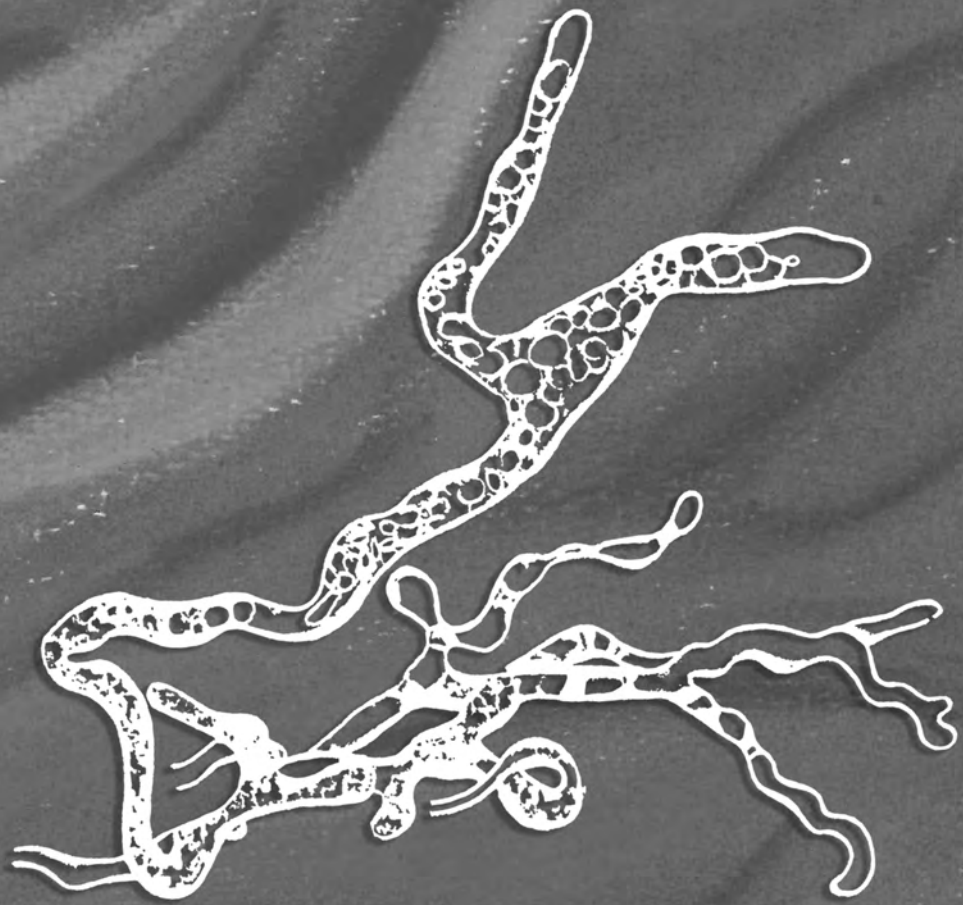
Summary



Summary

Soil borne plant pathogens considerably reduce crop yields worldwide and are difficult to control due to their ″masked″ occurrence in the heterogeneous soil environment. This hampers the efficacy of chemical - and microbiological control agents. Outbreaks of crop diseases are not only dependent on the presence of pathogen propagules in the soil, but are also influenced by soil-related properties like physico-chemical characteristics, microbial activity and community composition. Strong competition for limited available carbon substrates, restricts or prevent germination and pre-infective growth of pathogens. This competition can occur directly by rapid exploitation of substrates, so called resource competition, or indirectly via inhibitory secondary metabolites, called interference competition

The overall effect of all competition based mechanisms and the abiotic envrironment on disease development is known as *"general disease suppression"* and is the sum of all factors that reduce disease. The aim of this thesis was to study different aspects of general disease suppression, in order to get more insight into the interplay between microbial communities, pathogen dynamics, and substrate availability in different agricultural soils.

The first objective was to study the role of microbial volatile organic compounds in natural disease suppression in agricultural soils. In chapter 2 a series of simultaneous experiments were performed on a agricultural soil that received different management practises. We showed a strong correlation between root infection and -biomass production in a bioassay and the suppressive effects of microbial volatiles on the *in vitro* growth of the pathogen *Pythium intermedium*. No or weak volatile suppression coincided with significant lower root biomass and a higher disease index, whereas a strong volatile suppression related to high biomass and a low disease index . Furthermore, the composition of the original soil bacterial community showed a drastic shift due to the legacy effects of management practices, coiniciding with the loss of volatile suppression. By comparing the emission profiles and the bacterial community composition of the differently managed soils, candidate inhibitory compounds and volatile producing bacterial groups could be identified. Altogether these results indicate that volatile organic compounds can have an important role in general disease suppression.

To follow up on volatile suppression chapter 3 investigates the influence of soil-related (abiotic and biotic) variables on volatile mediated *in vitro* growth inhibition of different plant pathogens via an extensive soil survey including 50 Dutch arable agricultural fields. The volatile mediated suppression of three phylogenetic different soil borne pathogens (*Rhizoctonia solani, Fusarium oxysporum* and *Pythium intermedium*) was linked to a wide range of soil-related variables with univariate and multivariate regression models. The overall suppression of different pathogens was

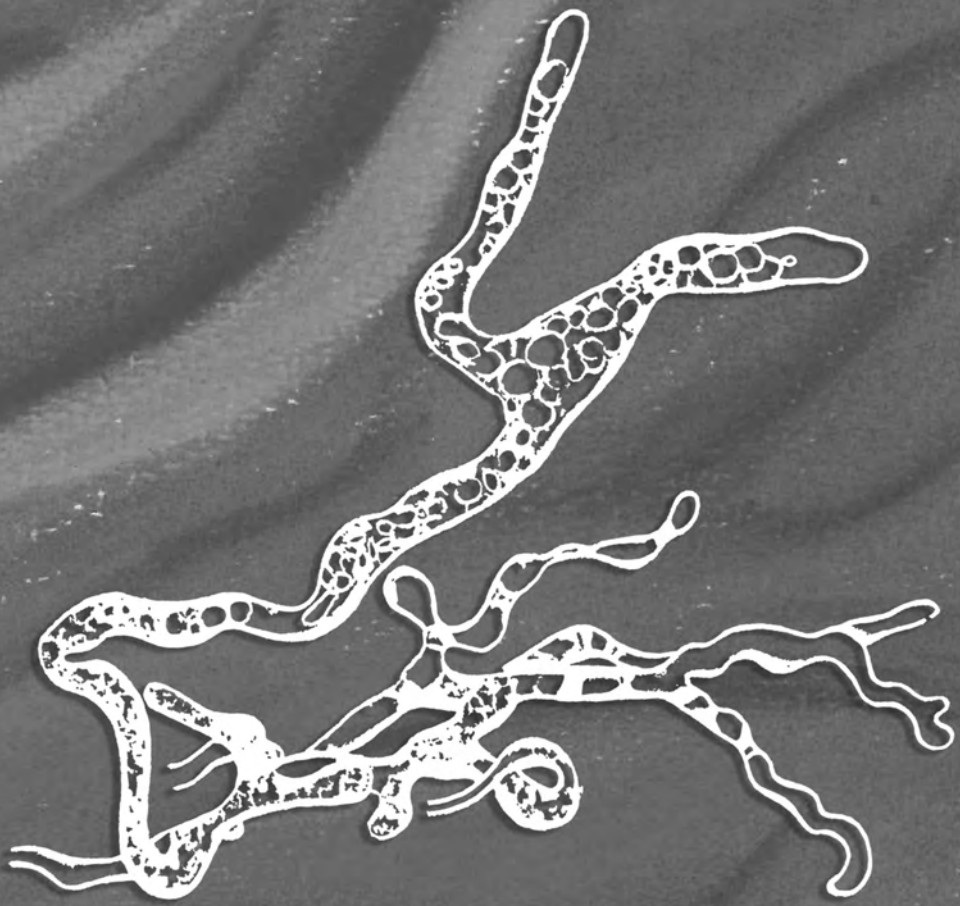
linked to microbial activity and organic substrates. However, different pathogens showed different sensitivity to volatile suppression. Furthermore, the soil-related factors corresponding to volatile mediated suppression were pathogen specific. In total, the results described in this chapter show that part of volatile suppression for a particular pathogen is based on general microbial activity, but our data shows as well that the individual response is pathogen specific.

Chapter 4 explores the reservoir of potential plant pathogens harboring agricultural soils before the start of the growth season, together with the environmental drivers of this pool of pathogens. By investigating the pathogenic seedbank in relation to its environment we assessed which soil-related variables could explain differences among site pathogen community composition. Pathogens differing in phylogeny or mode of infection were related to different soil variables. For example the among-site differences in the presence of oomycetes could not be related to their environmental context. On the other hand the variation in root and shoot fungal pathogen community composition was linked to soil physico-chemical properties and non-pathogen microbial community composition, with potentially a significant role of litter saprophytes therein.

As the presence of pathogen propagules in soil is not necessarily related to disease incidence, chapter 5 investigates the dynamics of root pathogens in the presence of a root in a model rhizosphere. We developed a qPCR based assay to test the growth response of a pathogen (*Pythium intermedium*) to the presence of root exudates over time. This exposure to root exudates showed soil specific pathogen dynamics. This finding may indicate that *in situ* (microbial) processes can successfully prevent pathogen development in some of the soils but not in others. Thus this method has the potential to provide an alternative way to assess the susceptibility of a soil to certain soil-borne diseases.

The results of this thesis gave new insights into different aspects of disease suppression in agricultural soils which could serve as a fundament to develop environmentally-friendly control methods based on natural occurring ecological processes. Ideas for the implementation of this study and future research are discussed in chapter 6.

CHAPTER 1.
INTRODUCTION



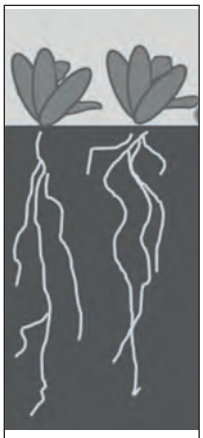


Figure 1.1
Plant roots growing in soil

Soil

noun

1: The upper layer of earth in which plants grow, a black or dark brown material typically consisting of a mixture of organic remains, clay, and rock particles (Oxford dictionary).

Soil is, besides the 'earth layer' where plant roots take up nutrients and water, also the highly diverse and heterogeneous habitat harboring the majority of the earth's biodiversity. In this complex environment microbes play a key role in ecosystem processes, - functions and -services. Amongst others they are involved in mineralization, decomposition and turnover of organic matter and soil aggregate formation, all contributing to plant growth and crop productivity. Plants interact with soil microbes living in close proximity of the plant roots, the rhizosphere, in which rhizodeposition provides the microbes with a source of easily degradable organic substrates (Bais *et al.*, 2006; de Boer *et al.*, 2006; Dennis *et al.*, 2010). In this highly competitive soil zone plant-microbe associations are established. Next to interactions with free-living rhizosphere microbes, plants do also establish mutualistic relationships with root-entering microbes such as mycorrhiza fungi that colonize plant roots and exchange essential nutrients with their host (Bonfante and Anca, 2009). However, besides beneficial effects, plant-microbe interactions can be detrimental for the plant. Plant infection by pathogenic soil microbes considerably reduce crop yields worldwide (Oerke, 2006).

Outbreaks of crop disease are not only dependent on the presence of pathogens in agricultural soil (inoculum density) but are also influenced by physico-chemical soil properties and microbial activity and community composition (Benson, 1994). Abiotic factors including pH, organic matter and clay content have been shown to influence pathogen survival (Mondal and Hyakumachi, 1998; Peng *et al.*, 1999; Kühn *et al.*, 2009). Limited availability of easily degradable organic compounds (carbon-limitation) in many soils restricts or prevents activation (germination and growth) of plant pathogenic propagules (Lockwood, 1977). Indigenous microbes contribute to this "pathogen dormancy" by depleting carbon sources resulting in decreased possibilities for pre-infective growth and, consequently, root infection (Hoitink and Boehm, 1999). This competition-based mechanism of control of pathogens is also known as 'general disease suppression' (Cook and Baker, 1983; Hoitink and Boehm, 1999) and is the overall effect of all biotic and abiotic factors resulting in reduced disease in the presence of the pathogen (Huber and Schneider, 1982).

This thesis focusses on the impact of the biotic and abiotic soil environment on different aspects of dynamics of soil-borne plant pathogens: occurrence of survival structures, (pre-infective) hyphal growth and root infection. This research was performed in close collaboration with a study on the role of dissolved organic carbon as microbial substrate in soil, presented in the thesis "Explorations of soil microbial processes driven by dissolved organic carbon", by Angela Straathof.

The microbial soil habitat

Soil ecosystems

The soil harbors a community of living organisms of which the vast majority is microbial. One gram of soil can contain several thousands of bacterial species (Curtis *et al.*, 2002) and up to 10 billion microbial cells (Torsvik and Øvreås, 2002). The soil ecosystem encompasses the network of all interactions; among microbes, between microbes and plants (and other organisms) and microbial interactions with the abiotic environment. The latter is important in shaping the growth conditions for microbes and consequently the microbial community composition. For example various abiotic factors including pH, inorganic nutrients, organic matter and clay content are important factors in structuring microbial communities (Garbeva *et al.*, 2004; Schutter *et al.*, 2001; Fierer and Jackson, 2006; Lauber *et al.*, 2008; Rousk *et al.*, 2010).

Plant pathogens in soil

The complexity of the soil environment in which pathogen propagules reside is hampering effective control of soil-borne plant diseases. Many pathogens form survival structures adapted to harsh conditions that can survive for years and thus remain present as a source of infection for a very long time (Bruehl, 1987). However, biotic and abiotic factors can contribute to irreversible loss of viability of pathogenic propagules. This thesis focusses on factors determining the composition of the pathogen seedbank, the growth dynamics and infection ability of the oomycete *Pythium intermedium as well as the in vitro* growth of the fungi *Rhizoctonia solani* and *Fusarium oxysporum. P. intermedium*, *R. solani* and *F. oxysporum* have been selected to serve as model organisms for soil-borne plant pathogen in agriculture. This choice is based on the common presence in arable fields, previous reported sensitivity to natural suppression (Ghini and Morandi, 2006; Van Bruggen and Semenov, 2000), economic relevance of the pathogen, previous developed tools to assess pathogen dynamics and on the availability of reliable bioassays for these host pathogen systems. The characteristics of the plant pathogens included in this study are briefly summarized in box 1.1.

Box 1.1 The pathogens used as model organism in this study

Rhizoctonia

Members of the fungal genus *Rhizoctonia* belong to the basidiomycetes and are responsible for crop losses in a broad range of plants, including economically important agriculture crops like rice, potato sugar beet maize and many vegetables. The fungus *R. solani* is considered as a species complex; subdivided in different anastomosis groups (AGs) representing genetic differences. Typical symptoms are damping off of seedlings, wilting and root. *Rhizoctonia* does not produce asexual spores and thus exist primary as mycelium. The primary inocula are either basidiospores or mycelia, for short time survival, or sclerotia, a compact mass of hyphae that can remain in the soil for several years.

Fusarium

The genus Fusarium belongs to the ascomycetes and habors several notorious pathogenic species such as *F. culmorum, F. solani* and *F. oxysporum*. The latter is a globally distributed fungal species causing vascular wilt, stunting, foot and root rot. The fungus has a broad host range; different *formae specialis*, each pathogenic to a specific crop, can infect more than 120 plant species. *Fusarium spp.* are important plant pathogens worldwide, causing severe losses in many agricultural systems, including wheat, flowers and vegetables. During its life cycle *F. oxysporum* produces three kinds of (asexual) spores, microconidia, macroconidia, and chlamydospores, thick-walled spore that enables a fungus to survive unfavorable conditions and acts as the primary inoculum. These survival spores can persist for a long time in the soil.

Pythium

The genus *Pythium* belongs, in contrast to the other two model pathogens, not to the kingdom of fungi but to the oomycetes. Many *Pythium* species are plant pathogens of economic importance in agriculture. They infect a broad range of crop species. *Pythium* is causing root rot, the symptoms are damping of in seedlings and wilting in older plants, generally under moist conditions. During the sexual phase anteridia and oogonia are formed, that fuse and produce the thick walled oospores which are able to survive harsh conditions (Agrios,2005). During the asexual phase sporangium production is initiated and gives rise to the zoospores (Carlile *et al*., 2003; van West *et al.*, 2003). The zoospores are attracted by root exudates. Both oospores and sporangia can serve as inocula for *P. ultimum* infections. Survival propagules of *Pythium* can persist in the soil for a long time (Stanghellini and Hancock, 1971).

Microbial competition

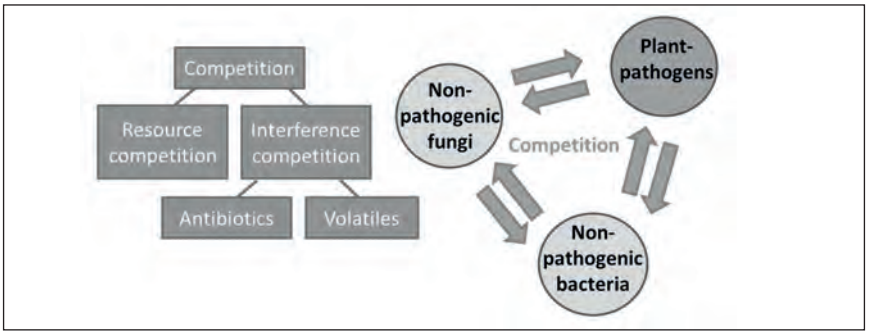


Figure 1.2 Graphical representation of competitive microbe-microbe interactions in soil

Soil is a heterogeneous environment in which microbial growth is mostly limited by the availability of organic substrates. Consequently microbes aggregate at nutrient rich hotspots in which fungi and bacteria have to compete for the same resources. In this highly competitive habitat a broad range of strategies are used. Microbes can be outcompeted by others either due to direct rapid exploitation of substrates, so called resource competition, or by the exposure to inhibitory secondary metabolites, interference competition. In the bulk soil substrate utilization is strongly linked to the ability to degrade recalcitrant organic matter. It is therefore an unfavorable habitat for plant pathogens, since most of the pathogens have limited saprophytic abilities and are thus weaker competitors for the limited carbon sources as compared to organotrophic bacteria and fungi (Garrett, 1970). Most pathogens survive in the absence of a host as resting structures that can persist in the soil for a long time.

A major hotspot in soil is the rhizosphere, where carbon limitation is (temporary) relieved by the release of simple organic compounds (exudates) by the root. Those root derived resources can function as germination trigger for dormant pathogenic propagules and can induce the start of an infection cycle. However, the availability of simple degradable compounds does not only reactivate plant pathogens, other soil dwelling microbes are triggered as well, resulting in strong competitive pressure to obtain "food" in which rapid carbon uptake by other rhizosphere microbes or the release of antagonistic metabolites can outcompete plant pathogens.

Fungistasis

Fungistasis, Dobbs and Hinson (1953) is the restricted ability of the main proportion fungal propagules to germinate or grow in soils. This restriction is either caused by the presence of inhibitory compounds, mainly of microbiological origin (Dobbs and Hinson, 1953; Hora and Baker, 1970; Watson and Ford, 1972) or via withdrawal of nutrients from fungal propagules by the soil microbial community (Lockwood, 1977). Likely, both nutrient-deficiency and the presence of inhibitory compounds

are involved, as they are proposed to be mechanistically linked (Garbeva *et al.*, 2011). Several studies identified soil microbial diversity, community, structure and activity to be involved in soil fungistasis (reviewed by Garbeva, 2011). Germination and growth of pathogenic propagules is affected by fungistasis and may have two potential effects; 1) restriction of growth and germination with ultimately loss of viability (Lockwood, 1977); 2) prevention of germination and growth under unfavorable conditions, which is beneficial for the pathogen in the absence of hosts. Yet, the intensity of fungistasis has been found to be linked with the strength of pathogen suppression (Knudsen *et al.*, 1999; Termorshuizen and Jeger, 2008) which would imply that unfavorable effects for the pathogen prevail.

Volatile organic compounds

Volatile organic compounds (VOCs) can play important roles in the network of all interactions in soil. Microorganisms from diverse ecosystems produce a wide range of volatile compounds (VOCs) with low molecular mass, high vapor pressure and a low boiling point. These properties enable the easy diffusion of volatiles in air and water filled pores leading to a large potential activity range (Insam and Seewald, 2010; Effmert *et al.*, 2012). Thus, volatile compounds might be important components of the natural surroundings of belowground microorganisms; by facilitating distant interactions they enable competition between physically separated soil microorganisms (Effmert *et al.*, 2012; Garbeva *et al.*, 2014a; Kai *et al.*, 2009). The amount and composition of volatiles produced by microorganisms differs due to differences in substrate/medium composition (Garbeva *et al.*, 2014b). Some VOCs are commonly produced by different (unrelated) microorganisms, while other volatiles are strain specific (Schulz and Dickschat, 2007; Garbeva *et al.*, 2014a). Volatiles emitted by microbes serve various functions; they stimulate or reduce spore germination, can inhibit or stimulate mycelial growth and can cause stimulation or reduction of sporulation (Wheatley, 2002; Kai *et al.*, 2007; Minerdi *et al.*, 2009; Garbeva *et al.*, 2011). They are reported to have inhibitory effects on of several plant pathogenic fungi and oomycetes including *Rhizoctonia solani* (Garbeva *et al.*, 2014b; Kai *et al.*, 2007*)*, *Fusarium oxysporum* and *Pythium spp* (Chaurasia *et al.*, 2005).

Abiotic soil properties, organic matter and field management practices

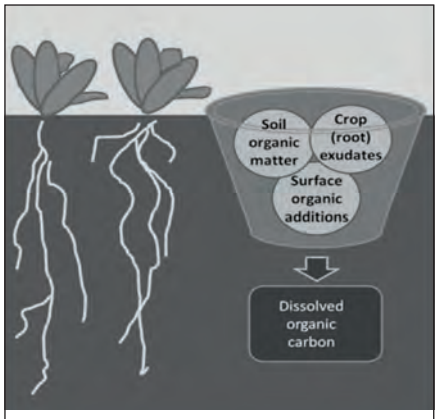


Figure 1.3 Summary of organic matter sources that contribute to the pool of dissolved organic carbon

Abiotic soil properties

The activities and composition of soil microbes are strongly linked with physico-chemical soil components (Girvan *et al.*, 2003). It is commonly accepted that physico-chemical properties of soils influence microbes but also the other way around. Janvier et al., (2007) reviewed biotic and abiotic factors influencing soil health. Some soil properties, like clay content and pH are factors that directly influence growth and spread of pathogens (Hoper and Alabouvette, 1996).

(Dissolved) organic matter

The major source of energy for soil microbes is organic matter, therefore the metabolic activity of the microbial community is strongly related to the quantity and quality of the organic substrates. Likely the most bioavailable fraction of organic matter is dissolved organic carbon (DOC), Kalbitz (2000) reported a tight mechanistic link between dissolved organic carbon and microbes in the soil. DOC originates from litter decomposition and root exudates. Growing plants release a complex mixture of organic compounds containing amino acids, organic acids, sugars, vitamins, enzymes and other molecules (Dakora and Phillips, 2002; Dennis *et al.*, 2010). These compounds can be detected by microbes and act as source of energy for growth or as signal to germinate. Root exudates have two main impacts on soil microbes: the metabolic activity is increased and the microbial composition is modified (Bais *et al.*, 2006). Therefore exudates are important in structuring rhizosphere microbial communities, as reviewed in (Berg and Smalla, 2009). As different plant have an unique set of exuded compounds, host plants as well exude specific triggers for pathogen germination (Nelson, 1991).

Agricultural practices/compost amendment

Management of the biotic and abiotic properties of a soil can influence disease incidence (Janvier *et al.*, 2007). Some practices can reduce pathogen survival or decrease the number of viable fungal propagules. Crop rotation is a practice known for centuries and associated with reducing plant diseases and pathogen population

decline (Cook, 2006). However this method is less successful against pathogens with a wide host range or with long-term survival propagules. Compost addition to agricultural soils is applied to supply the crops with inorganic nutrients (released after mineralization) and to improve disease suppression (Lozano *et al.*, 2009; Lozano *et al.*, 2005; Termorshuizen *et al.*, 2006). The pathogen suppressing effect of composts is attributed to increased soil microbial numbers and activity thereby enhancing competition for root exudates and reducing the possibilities for plant pathogens to respond to the presence of crops. This is a control practise that does not directly eliminate pathogens, but instead modifies the microbial balance in a positive direction for pathogen control (Mazzola, 2004).

General disease suppression

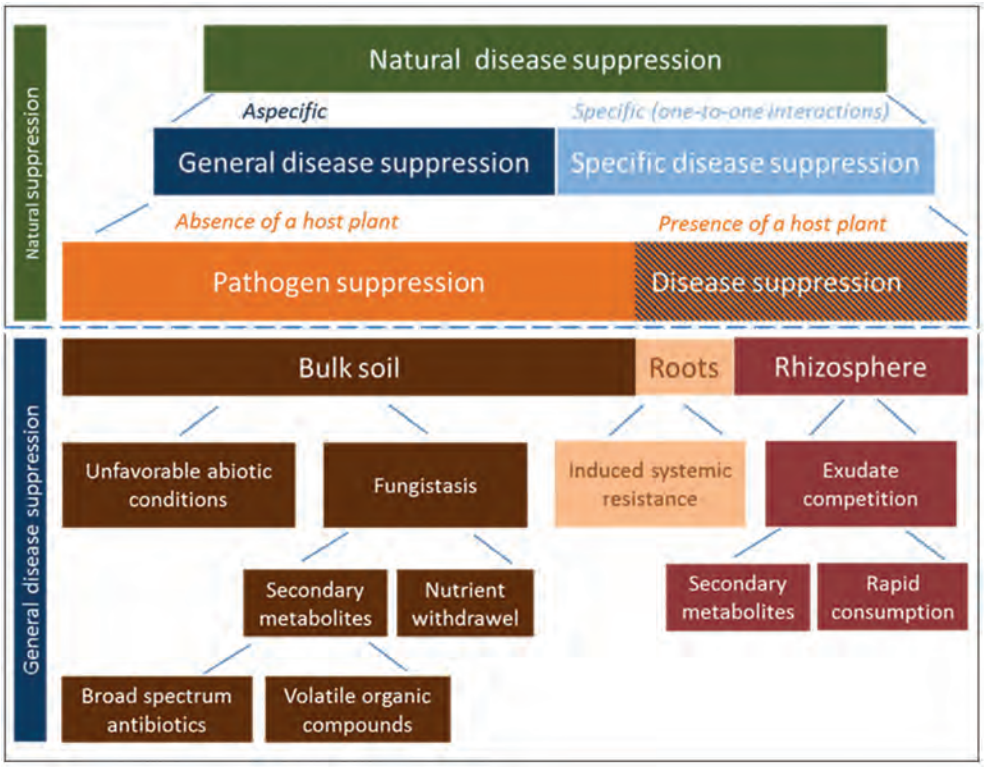


Figure 1.4 Summary of natural disease suppression and the mechanisms involved in general disease suppression (GDS).

The ability of a soil to suppress soil-borne plant pathogens is an important soil ecosystem function. Abiotic soil properties, soil microbial biomass and activity as well as soil microbial community composition and presence of suppressive compounds determine whether a soil is more conducive or suppressive to pathogens. Two types

of disease suppression are usually distinguished: specific and general (Baker and Cook, 1974) of which the latter is the focus of this thesis. Specific disease suppression (reviewed by Weller et al., 2002) is based on one-to-one antagonistic interactions by one or a few specific microbes. Mechanisms involved include parasitism and competition. The occurrence of specific disease suppression can occur anywhere in the soil but seems to be most dominant in the rhizosphere soil, which distinguishes specific interactions from pathogen suppression, generally occurring in absence of a host plant. The umbrella term for all aspecific inhibitory effects on soil-pathogens in the bulk soil and rhizosphere is named 'general disease suppression' (GDS). GDS acts against multiple pathogens and is the 1st buffer against soil-borne plant diseases. GDS, as a result of pathogen suppression encompasses a combination of multiple effects, including competition for or withdrawal of nutrients, fungistasis (partly comprising inhibitory volatile compounds) and antibiosis (Noble and Coventry, 2005). Natural occurring disease suppression, with special emphasis on general disease suppression, is summarized in figure 1.4.

Whether a soil is conducive or suppressive is depending on different components (Alabouvette *et al.*, 1982; Höper and Alabouvette, 1996), including soil factors that influence the inoculum density and pathogenic infection capacity. Important factors of pathogen sensitivity to GDS are related to the biology of the pathogen e.g. the saprophytic capability of the given pathogen, to soil type, environmental factors (pH, nutrients) and agricultural factors, e.g. crop sensitivity, field history and agricultural practices (van Diepeningen *et al.*, 2006).

Hence, disease development depends on abiotic, biotic and pathogen characteristics. (Hoper and Alabouvette, 1996) indicated 5 characteristics of pathogens related to disease incidence:

1) host range; host sensitivity; host species
2) bulk soil activity: sensitivity to fungistasis, ability to compete for organic matter
3) rhizosphere selective presence and activity
4) sensitivity to abiotic conditions, including temperature, moisture content, pH
5) survival capability.

All together these characteristics determine, in interaction with the pathogen inoculum, disease incidence and development in soil. The involvement of multiple mechanisms, factors and conditions that determine whether or not pathogens are suppressed makes soil suppression a complex phenomenon. Different aspects and mechanisms of pathogen suppression will be investigated in this thesis.

The need for novel approaches and for disease control

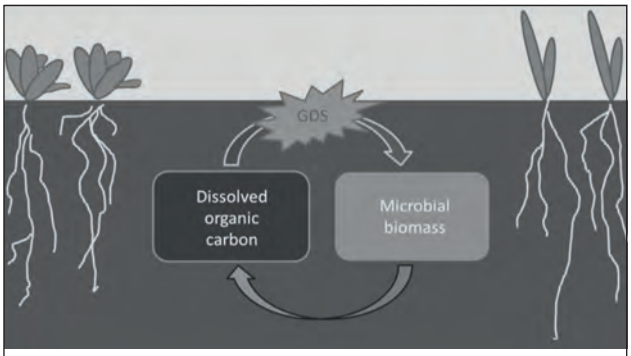


Figure 1.5 The mechanistic link dissolved organic carbon and the microbes in the soil with a proposed link to general disease suppression (GDS)

Novel environmentally-friendly approaches to control diseases by soil-borne pathogens are urgently needed. Inadequate control by chemical fungicides due to resistance of pathogens and the negative impacts pesticides have on the environment motivates the search for alternative control strategies. These alternative control strategies should focus on natural occurring ecological processes in soil. Yet, it is commonly accepted that all soils exhibit a degree of suppression against soil-borne pathogens, primary due to *in situ* competition for substrates. The availability of substrates for metabolism and biosynthesis is a prerequisite for germination and successful pre-infective growth of soil-borne pathogens. Strong competition for substrate compounds and production of inhibitory secondary compounds by competing soil microbes will prevent the start of an infection cycle and therefore increase GDS.
Control strategies based on the management practices that "boost" these natural occurring processes may lead to sustainable control strategies. Current management of soil-borne plant disease is based on the presence of the pathogen in the soil. However, the suppressive characteristics of a soil are not included, although it is emphasized that natural suppression contributes significantly to suppression of crop diseases.

The suppressive properties of a soil and the underlying ecological processes have not been sufficiently identified so far. To accomplish this, the relationship between substrate availability, microbial activity and the production of inhibitory compounds and their effect on soil-borne pathogens needs to be studied. Indicators of soil health and suppressive capacity of a soil deduced from such research could lead to novel, environmentally-friendly strategies to control soil-borne diseases.

Aims and research questions

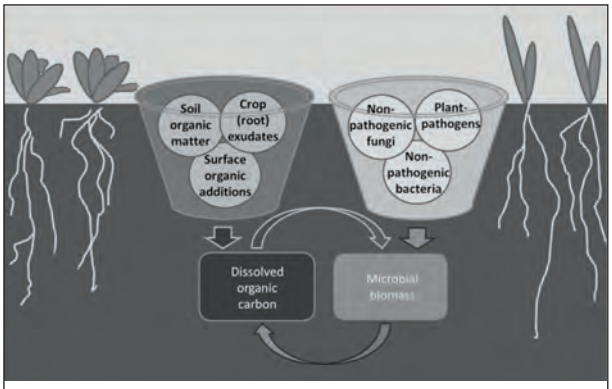


Figure 1.6 Overview of the link between resource availability and microbial interactions in the soil

In the light of sustainable agriculture and the call for reducing pesticide use, insights in the mechanisms of natural suppression of soil-borne pathogens are essential. Therefore, understanding the relationships between soil microbes and plant pathogens is needed to develop procedures for effective and consistent control. The presence of bio-available carbon substrates is a prerequisite for successful pathogen growth and infection. Strong competition for these substrates either through resource competition or interference competition by indigenous soil microbes reduces the viability and pre infective growth of soil-borne pathogens and, ultimately, leads to reduced disease pressure. The aim of the work presented in this thesis is to get more insight in the interplay between microbial communities, pathogen dynamics and substrate availability in different agricultural soils.

The specific research objectives, which each are addressed in one of the chapters of this thesis, are:

I. Obtain insight in the role of microbial volatile organic compounds in the natural suppression of pathogen growth and disease development in agricultural soils
II. Assess which abiotic and biotic variables influence the volatile mediated growth inhibition of different plant pathogens
III. Explore the reservoir of potential plant pathogens harbouring agricultural soils and assess which environmental variables can explain between field variation of potential pathogens
IV. Investigate the role of exudate competition on dynamics of plant pathogens in soil

Outline

Different aspects of pathogen suppression in relation with their environmental context have been investigated and are described in this thesis.

Chapter *two* and *three* address the role volatile organic compounds emitted from agricultural soil in soil-borne pathogen suppression.

Chapter 2

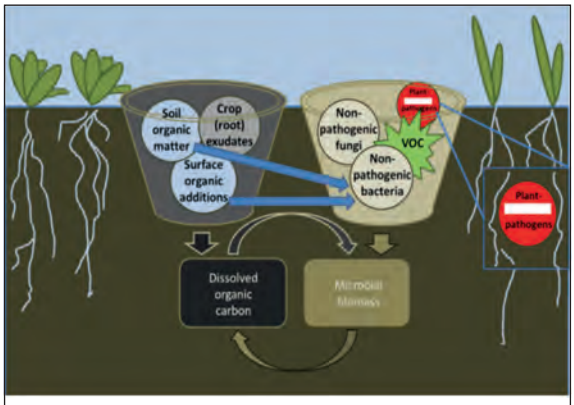


Figure 1.7 Graphical representation of Chapter 2, the link between volatile (VOC) -mediated pathogen suppression and root infection in differently managed soils.

The main subject of chapter *two* is the relation between VOC-mediated pathogen suppression and root infection in an agricultural soil that received different management practises. Furthermore in this chapter volatile suppression is linked to microbial community composition and emission of inhibitory volatiles. Lastly the legacy effects of the management practises on volatile suppression, root infection and community structure are addressed.

Chapter 3

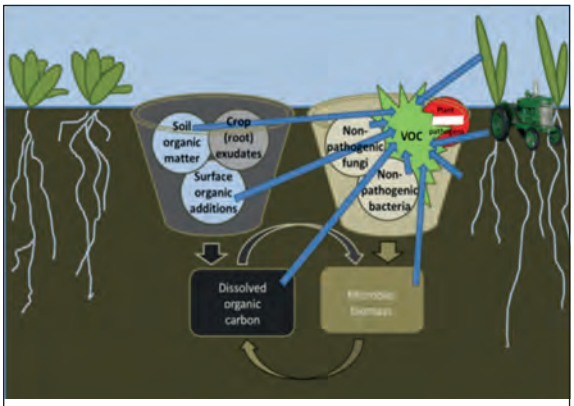


Figure 1.8 Graphical representation of Chapter 3, the identification of soil-related variables indicative for volatile (VOC) suppression.

The results of chapter two lead to new questions about how widespread the volatile mediated suppression is, how it influences phylogenetic different soil borne pathogens and which soil variables influence the outcome of volatile mediated suppression.

The *third* chapter describes a 50 soils survey to statistically relate a large set of soil-related variables to the extent of growth suppressive effects of volatiles emitted from this 50 sites on three soil borne pathogens, *Rhizoctonia solani, Fusarium oxysporum* and *Pythium intermedium*. Both univariate and multivariate analyses are used to identify indicative properties for volatile suppression.

Chapter *four* and *five* zoom in on the pool of plant pathogens s in agricultural soils.

Chapter 4

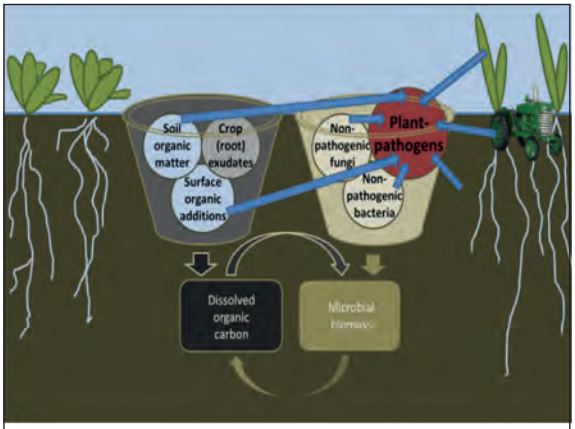


Figure 1.9 Graphical representation of Chapter 4, an overview of the pool of pathogens in the soil and the soil-related variables related to variation in pathogens among sites.

(Dormant) pathogenic propagules in soil are the source of many diseases. We wanted to investigate the composition of the pool of pathogens (pathogen seedbank) in agricultural soils and the environmental drivers of this composition.

Chapter *four* provides an overview of potential pathogens in 42 agricultural fields and uses a multivariate analysis of the potential pathogenic community, abiotic and biotic soil environment, field history and spatial patterns to relate among site pathogen community differences to soil-related properties.

Chapter 5

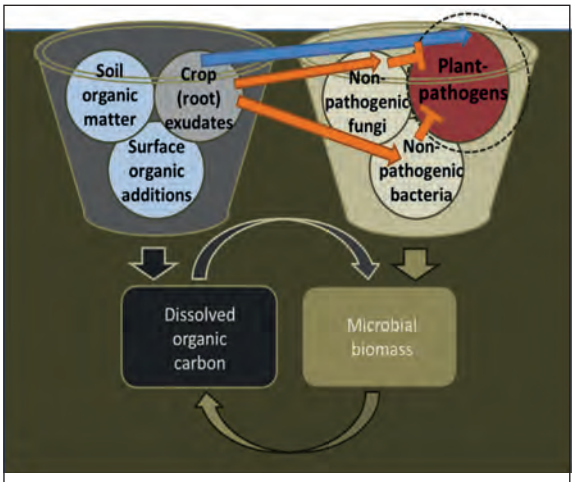


Figure 1.10 Graphical representation of Chapter 5, development of a model system to assess pathogen dynamics upon root exudate exposure.

Presence of pathogens does not always lead to crop infection which may be due to strong microbial competition for root exudates. Therefore we investigated pathogens dynamics in a competitive setting.

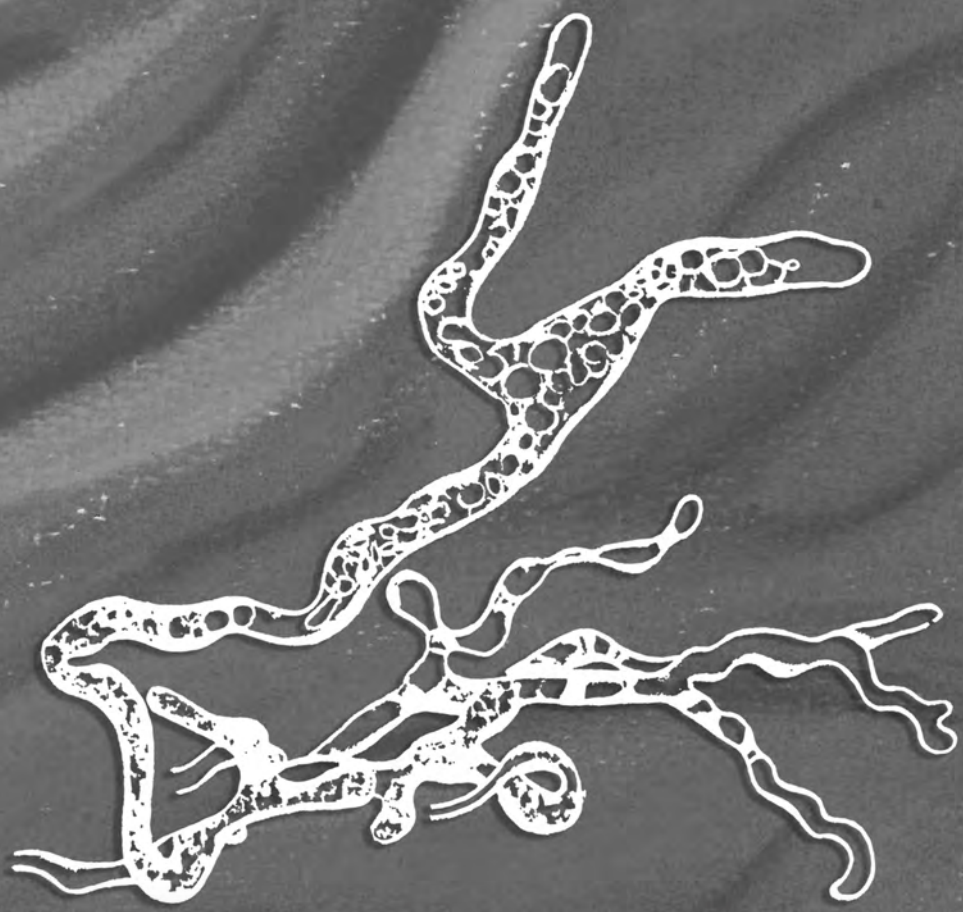
Chapter *five* describes a bilayer model rhizosphere in which pathogen growth upon root exudate exposure is quantified over time.

Chapter 6

In chapter *six* I will discuss the contributions of the research presented in this thesis to the knowledge on pathogen suppression and general disease suppression. I will finish this discussion with future research priorities and how the results obtained in this research can contribute to sustainable agricultural management strategies.

CHAPTER 2.

LEGACY EFFECTS OF ANAEROBIC SOIL DISINFESTATION ON SOIL BACTERIAL COMMUNITY COMPOSITION AND PRODUCTION OF PATHOGEN-SUPPRESSING VOLATILES



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Abstract

There is increasing evidence that microbial volatiles (VOCs) play an important role in natural suppression of soil-borne diseases, but little is known on the factors that influence production of suppressing VOCs. In the current study we examined whether a stress-induced change in soil microbial community composition would affect the production by soils of VOCs suppressing the plant-pathogenic oomycete *Pythium*. Using pyrosequencing of 16S ribosomal gene fragments we compared the composition of bacterial communities in sandy soils that had been exposed to anaerobic disinfestation (AD), a treatment used to kill harmful soil organisms, with the composition in untreated soils. Three months after the AD treatment had been finished, there was still a clear legacy effect of the former anaerobic stress on bacterial community composition with a strong increase in relative abundance of the phylum *Bacteroidetes* and a significant decrease of the phyla *Acidobacteria, Planctomycetes, Nitrospirae, Chloroflexi* and *Chlorobi*. This change in bacterial community composition coincided with loss of production of *Pythium* suppressing soil volatiles (VOCs) and of suppression of *Pythium* impacts on Hyacinth root development. One year later, the composition of the bacterial community in the AD soils was reflecting that of the untreated soils. In addition, both production of *Pythium*-suppressing VOCs and suppression of *Pythium* in Hyacinth bioassays had returned to the levels of the untreated soil. GC/MS analysis identified several VOCs, among which compounds known to be antifungal, that were produced in the untreated soils but not in the AD soils. These compounds were again produced 15 months after the AD treatment. Our data indicate that soils exposed to a drastic stress can temporarily lose pathogen suppressive characteristics and that both loss and return of these suppressive characteristics coincides with shifts in the soil bacterial community composition. Our data are supporting the suggested importance of microbial VOCs in the natural buffer of soils against diseases caused by soil-borne pathogens.

Introduction

In the light of sustainable agriculture and the call for reduction of pesticide use, insights in the mechanisms of natural suppression of soil-borne pathogens are essential. Therefore, understanding the interactions of plant pathogens with other members of soil microbial communities is needed to develop strategies for effective and consistent control (Chaparro *et al.*, 2012). In general, depletion of carbon sources by indigenous microbes hampers the pre-infective growth of soil-borne pathogens resulting in lower infection rates (Hoitink and Boehm, 1999). This competition-related mechanism of pathogen control is also known as 'general disease suppression' (Hoitink and Boehm, 1999). Disease suppression is closely related to soil fungistasis, the restricted ability of most fungal propagules to germinate or grow in most soils (Dobbs and Hinson, 1953). As for general suppression, it has been hypothesized to be caused by soil microbial withdrawal of nutrients from soil or even from fungal propagules (Lockwood, 1977). However, besides substrate competition, also inhibitory compounds, released by microbes, have been indicated to contribute to fungistasis (Romine and Baker, 1972; de Boer *et al.*, 2003). This implies that not only the carbon-withdrawing activity of the total soil microbial community is involved in fungistasis but also the secondary metabolite production of certain groups within the soil microbial community. Based on this, Garbeva and co-authors (2011) argued that the composition of soil microbial communities is more important in fungistasis than previously has been appreciated.

Most soil-borne pathogens are poor competitors and have limited saprotrophic capacities in their pre-infective stages. Therefore, they are sensitive to general disease suppression. Among them is the oomycete genus *Pythium,* which includes many plant pathogenic species. They infect roots of seedlings generally resulting in damping-off and, consequently, reduced yield in a broad range of crops (Martin and Loper, 1999). In flower bulb crops, several species of *Pythium* cause severe root rot, leading to considerable losses in bulb yield (van Os *et al.*, 1998). Infection can occur by zoospores and is initiated by a chemotactic response to compounds exuded by roots. Yet, *Pythium* is considered to be a poor competitor for these root exudates and, therefore, natural control of *Pythium* infection is attributed to high competitive pressure exerted by other exudate-consuming soil microbes (Chen *et al.*, 1988; Van Os and Van Ginkel, 2001). Hence, the current view on the cause of natural buffering of soils against *Pythium* infection is mainly pointing at resource competition rather than at interference competition (involvement of inhibitory secondary metabolites).

Antimicrobial volatile organic compounds (VOCs), emitted by soil microbes, may be an important factor in causing fungistasis facilitated by their ability to diffuse through the porous soil matrix (Wheatley, 2002; Garbeva *et al.*, 2011; Effmert *et al.*, 2012). The potential role of VOCs in suppression of soil-borne plant pathogenic organisms was already reviewed in 1976 by Stotzky *et al.* but regained interest recently (Effmert *et*

al., 2012; Garbeva *et al.*, 2011; Weisskopf and Bailly, 2013). Production of antifungal volatiles has been shown for a broad range of bacterial phyla: it has been estimated that 30-60% of the soil bacterial species can produce fungistatic volatiles (Wheatley, 2002; Zou *et al.*, 2007)2007. Further support for the role of volatiles in fungistasis came from an extensive inventory by Chuankun *et al.* (2004), who observed a significant positive correlation between fungistatic activity (inhibition of spore germination) and production of VOCs by 146 soils. The inhibition of pathogen growth by bacterial VOCs has been shown in several studies (Alström, 2001; Wheatley, 2002; Kai *et al.*, 2007; Kai *et al.*, 2009; Effmert *et al.*, 2012; Zou *et al.*, 2007) indicating the potential of microbial volatiles in disease reduction. Inhibition of *Pythium* mycelial growth by bacterial volatiles has been shown, albeit under *in vitro* conditions and not in soils (Garbeva *et al.*, 2014a). Hence, possible involvement of volatiles in natural soil suppression of *Pythium* is unknown.

Agricultural management practices may influence the composition of soil microbial communities and, therefore, also the production of pathogen-suppressing secondary metabolites. Different management practices are in use to reduce pathogen pressure. Anaerobic soil disinfestation (AD) uses crop residues and airtight covering of the soil with plastic foil to stimulate the development of anaerobic microbes producing toxic substances that eliminate harmful nematodes and fungi (Blok *et al.*, 2000). Although AD is used as an environmentally-friendly alternative for chemical disinfestation it is expected to have a tremendous effect on microbial community composition and functioning as aerobic soil microbes face a period of anaerobiosis. Little is known on the possible legacy that AD may have on the composition and functioning of soil microbial communities after the treatment has been finished and cultivation of new crops are started. It has been shown that stress-induced shifts in soil microbial community composition can cause a drastic reduction of fungistasis (de Boer *et al.*, 2003). Hence, there is a potential risk that AD and other disinfestation treatments have similar effects on the pathogen-suppressing activities of soil microbial communities. The current study was aimed to address possible legacy effects of AD of sandy bulb soils on bacterial community composition and soil suppressive characteristics, with special emphasis on the production of pathogen-suppressing volatiles. To this end measurements were done at the start of the flower bulb season (planting of bulbs in autumn) in the year that AD had been applied (3 months after AD) and one year later. The oomycete *Pythium intermedium,* a notorious pathogen of flower bulbs, was used to test soil suppressiveness as *Pythium* species are opportunistic pathogens that can rapidly cause problems under conditions where general suppressiveness has been reduced (Postma *et al.*, 2000). Simultaniously he production of *Pythium*-suppressing volatiles by AD-treated and control soils were tested and compared with results of bioassays (root development of Hyacinth bulbs in the presence of *P. intermedium*) to determine the role of volatiles in natural suppression. Bacterial community composition was determined using 454 sequencing of 16S rDNA fragments.

The tightly linked series of analyses and experiments lend strong support to the importance of bacterial community composition and - volatile production in natural suppression.

Materials & Methods

Soil treatments and sampling

Experiments were performed with soil samples from the experimental fields of Applied Plant Research (Wageningen UR) in Lisse, The Netherlands (coordinates: N 52.25. 52; E 4.54. 77). At this location the alluvial sandy soil has a low organic matter content ranging between 1.0% and 1.5%, which is representative of the soil type used in cultivation of flower bulbs along the dunes of the coastal area of the North Sea. In 2010, a field trial was initiated to examine the effect of soil organic matter content and management practices on disease suppression against several soil-borne pathogens. From the current experiment, plots of four soil treatments (Table 2.1) with four replicates per treatment (60 m^2 per replicate) were included. In May 2010, organic matter (OM) content was elevated by incorporating a mixture of peat (95%) and cattle manure (5%) (504 tonnes ha^{-1}, 0-30 cm deep), resulting in an increase of the soil OM content from 1.2% to 3.0%. In August 2011, anaerobic soil disinfestation (AD) was applied to a subset of the plots according to the method of Blok *et al.* (2000) using 'Herbie 7025' (van Overbeek *et al.*, 2014), a defined protein-rich vegetal by-product of food processing industry (Thatchtec B.V., Wageningen, The Netherlands). Herbie was applied 24 tonnes ha^{-1}, was incorporated 0-30 cm deep and anaerobic conditions were created by watering followed by airtight covering of the soil with plastic for six weeks. Three and fifteen months after removal of the plastic cover (November 2011 and November 2012 respectively), soil samples were taken from each field plot (22 kg per plot, randomly collected from 0-20 cm depth) and kept at 4^0C until use. In between sampling dates, *Gladiolus* was cultivated on all field plots (April-November 2012).

Table 2.1 Overview of soil treatments, properties and application- and sampling dates.

Code	Treatment	Organic Matter	Date of application	Plots	Sampling dates
U	Untreated	1.2%	-	4	Nov. 2011, 2012
P	Peat	3.0%	May 2010	4	Nov.2011, 2012
AD	Disinfested	1.2%	Aug. 2011	4	Nov. 2011, 2012
ADP	Disinfested + Peat	3.0%	Aug. 2011, May 2010	4	Nov. 2011, 2012

Bioassay for assessment of root rot

From each of the 16 field plots, soil samples were artificially infested with a three-week-old oatmeal culture (1% v/v) of *Pythium intermedium* (isolate P52, Applied Plant Research Flowerbulbs, Nursery Stock & Fruit, Lisse). Non-infested and pasteurized soils (2h at ≥ 70 °C) were used as controls. Soil moisture content was adjusted to 20% (w/w). Five bulbs from *Hyacinthus orientalis* cultivar 'Pink Pearl' were planted in pots (3 L) and incubated during 8 weeks at 9°C. Impact of *Pythium* on Hyacinth root development was assessed by measuring root weight and by rating root-rot disease symptoms. At the end of the growing period, bulbs were removed from the soil and roots were washed with tap water. Root-rot ratings of infested treatments were related to the healthy root systems of non-infested control treatments. Roots were visually examined for root rot severity according to Van Os *et al.* (1998) using an arbitrary disease index ranging from 0-5, where 0 = no root rot, 1 = 1-20%, 2 = 21-40%, 3 = 41-60%, 4 = 61-80%, and 5 = >80% root rot, i.e., relative loss of healthy root mass induced by infection, compared to the corresponding healthy root systems. Roots were scored for each plant individually and a mean root rot index for each pot was calculated. After the scoring of the disease index, roots were removed from the bulbs and excess water was removed by blotting the roots on filter paper and total fresh root weight per pot (5 bulbs) was determined. Means of four pots soil treatment were used in statistical analysis. Separate bioassays were performed for both years.

In vitro tests for production of *Pythium*-suppressing volatiles from soil

An experimental set-up was designed to enable exposure of *Pythium intermedium* to volatiles produced by the soils, without direct contact between *Pythium* and soil. Soil samples equal to 20 g dry weight (20% (w/w) soil moisture content) were spread evenly on the bottom of 90 mm Petri dishes and incubated for 1 week at 10°C. A 4 mm layer of water yeast agar (WYA, 20g agar, 1g KH_2O_4, 0.1g $(NH_4)_2SO_4$, 0.1g yeast extract (Difco) L^1 pH 6.5) was poured in lids of Petri dishes. Agar plugs of 6 mm potato dextrose agar (PDA 19,5 g L^{-1} (Oxoid) with CMN agar 7,5 g L^{-1} (Boom) colonized by *P. intermedium* (incubated 5-10 days at 20 °C) were transferred to WYA plates and kept at 10°C. After 48 h, a WYA agar disc (Ø 6 mm) containing *Pythium* mycelium was placed in the center of the lid. The mycelium-containing lid was carefully placed on top of the bottom compartment containing soil and sealed using Parafilm (Figure S2.1). Plates were incubated for 10 days at 10°C. Petri dishes without soil and with gamma-radiated soil (untreated 2012) (> 25 kGray, Isotron, Ede, the Netherlands) were used as controls for conditions without microbially produced volatiles. Before the start of the experiment the gamma-radiated soil was left for 4 days in a sterile flow cabinet to remove all residual volatiles. Mycelial biomass determination was done according to the method of Garbeva *et al.* (2014b) with some modifications.

Briefly, *Pythium* mycelia were harvested by melting the colonized agar from the lids of the Petri-dishes in a beaker glass with water in a microwave oven (c. 100°C), followed by sieving with a tea strainer and three washing steps with water (c. 90°C) in order to remove agar residues. For measurements of dry biomass weight, mycelia were frozen at -20°C and freeze-dried during 24 h. Pictures of *Pythium* hyphae were taken before harvest 1 cm from the edge of the plate with a stereo microscope (Olympus, SZX12, Tokyo, Japan) connected to a AxioCam MRC5 camera (Zeiss, Jena, Germany) under a 90x magnification.

Trapping and GC/MS analysis of microbial volatiles

For collection and analysis of released volatiles from soil the method of Garbeva *et al.* (2014b) was used with some modifications. Soil from two plots per treatment was randomly selected for GC/MS analysis. Briefly, soil samples were plated in special designed glass petri dishes, with an exit to which a steel trap could be connected with as trapping material 150 mg Tenax TA and 150 mg Carbopack B (Markes International Ltd., Llantrisant, UK) which could fix VOCs released from the soil. VOCs were collected after 168 hours of incubation at 10°C. Then, traps were removed, sealed and stored at 4°C until further analysis. Volatiles were desorbed from the traps using an automated thermodesorption unit (model Unity, Markes International Ltd., Llantrisant, UK) at 200°C for 12 min (He flow 30ml/min). Each trap was heated for 3 min up to 270°C to introduce the volatiles into the GC-MS (model Trace, ThermoFinnigan, Austin, TX, USA). Split ratio was set to 1:4, and the used column was a 30 mm × 0.32 mm ID RTX-5 Silms, film thickness 0.33 μm (Restek, Bellefonte, PA, USA). The used temperature program was: from 40°C to 95°C at 3°C min^{-1}, then to 165°C at 2°C min^{-1}, and to 250°C at 15°C min^{-1}. The VOCs were detected by the MS operating at 70 eV in EI mode. Mass spectra were acquired in full scan mode (33–300 AMU, 0.4 scans s^{-1}). Compounds were identified by their mass spectra using deconvolution software (AMDIS) in combination with NIST 2008 (National Institute of Standards and Technology, USA,), Wiley 7th edition spectral libraries and by their linear retention indices (lri). The lri values were compared with those found in the NIST and the NIOO lri database. Candidate compounds possibly related to volatile inhibition of *Pythium* growth were identified by screening for volatiles that were absent in disinfested soils (AD and ADP) in 2011 and present one year later (2012) and in non-disinfested soils (U and P) 2011 and 2012.

Pyrosequencing of soil bacterial communities

DNA was extracted directly after sampling from three randomly selected plots per treatment using Mobio 96 well Powersoil® extraction kit according to the manual. Amplicons for barcoded 16S pyrosequencing were generated using PCR reactions (5 minutes 95°C followed by 25 cycles 95°C 30s, 53°C 60s, 72°C 60s + 1 s per cycle finishing with 10 minutes 72°C and 10°C soak) performed in triplicate for each

sample using the primerset 515F (5'-GTGCCAGCMGCCGCGGTAA-3') and 806R (5'-GGACTACVSGGGTATCTAAT-3') (Caporaso *et al.*, 2011). The 515F primer included the Roche 454-pyrosequencing adapter and a GT linker, while 806R included the Roche 454- sequencing adapter, a 12-bp barcode (unique to each sample), and a GG linker. PCR products were cleaned (Qiagen Pcr purification kit) pooled and were sequenced (Macrogen Inc. Company, South Korea) on a Roche 454 automated sequencer and GS FLX system using titanium chemistry (454 Life Sciences, Branford, CT, USA). The obtained 454 sequences were filtered and analyzed using QIIME (Caporaso *et al.*, 2010) in the Galaxy interface. Sequences were denoised (DENOISER, (Reeder and Knight, 2010)) and chimeras were removed using UCHIME (Edgar *et al.*, 2011) followed by trimming of low quality reads (<200bp, quality score 20). The remaining high quality sequences were clustered into operational taxonomic units (OTU's) using UCLUST (Edgar, 2010) with a minimal sequence identity cut off of 97% using the most abundant unique sequence as cluster representative. Sequences were deposited in the European Nucleotide Archive under accession number PRJEB6155 (http://www.ebi.ac.uk/ena/data/view/PRJEB6155).

Data analysis

In the bioassay, mean disease indices per pot were converted to percentages. The assumption of normality was tested with Shapiro–Wilk statistics and Levene's test was used to confirm homogeneity of variances. An analysis of variances, a 3-way ANOVA, was performed to test the effects of soil treatment, *Pythium* addition, organic matter level and their interactions on root weight or percentage root rot.

To test the effects of VOC produced in soil on *Pythium* biomass the average hyphal weight per Petri dish per soil treatment was determined in 2011 (n=16) and 2012 (n=8). Data were calculated as percentage of the growth of the control. Normality was tested with Shapiro-Wilk test and homogeneity of variances was assessed with Levene's test. A two-way ANOVA was performed to determine differences between soil treatments.

A 3-way ANOVA was performed on the data from the pyrosequencing analysis in order to test the effects of peat addition, soil treatment and sampling year and their interactions on number of reads. Pyrosequencing data were rarefied to the lowest number of obtained reads, 2047 reads per sample. Per phylum all soil treatments in the two seasons were tested for a change in relative abundance, based on the number of reads per phylum. The average number of reads per phylum per soil treatment was calculated and tested with one-way ANOVA. The average number of OTUs per treatment was used to express OTU richness. Although the OTU richness data did not meet the assumption of normality in the analysis these data were also analyzed with a one- way ANOVA to determine differences between treatments. Statistical analyses were done in R3.0.2 and PAST (Hammer *et al.*, 2001).

Results

Impact of *Pythium* on Hyacinth root rot (bioassays)

Management practises strongly affected root biomass and root rot severity. Addition of *Pythium* to the soils showed an overall effect of the pathogen: the root weight was significantly reduced in all soils in the consecutive years. However, the magnitude of the effect of *Pythium* on root biomass reduction was different depending of the management regime the soil had received (Figure 2.1a,b; Figure 2.2c). *Pythium*-induced root biomass reduction was most strong in recently (2011) anaerobic disinfested (AD) soils. This was also indicated by the significant interaction between soil disinfestation and *Pythium* addition in 2011 (Table 2.2). In contrast to 2011, the effect of *Pythium* addition to the soil in 2012 was independent of the former AD treatment and did not show differences between the differently managed soils (Figure 2.1b). In all pasteurized soil samples, inoculation with *Pythium* resulted in a severe loss of root biomass, average root weight was reduced by >60% (Figure 2e).

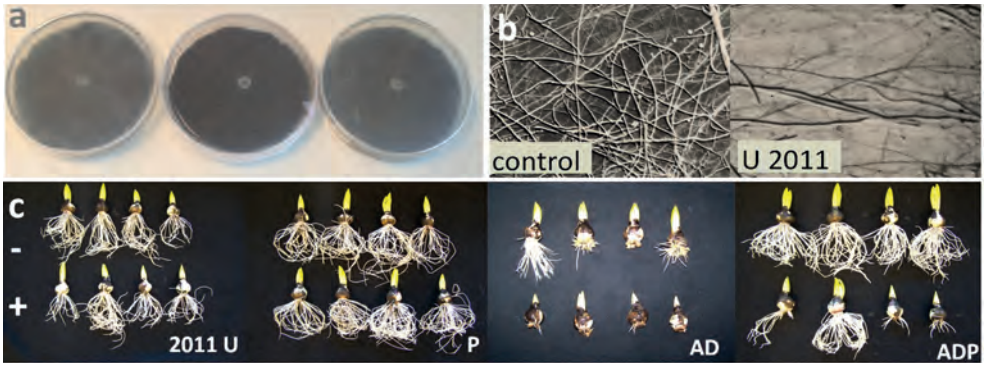


Figure 2.2 *Pythium* volatile exposure assays and Hyacinth bioassays. **a**, Differences in *Pythium* hyphal density upon volatile exposure sterilized soil (control) and untreated and disinfested soil in 2011. **b** Detailed pictures taken from agar plates after volatile exposure. **c**, Results of Hyacinth bioassays in soil from differently managed fields without (-) and with (+) *Pythium intermedium* addition. U= untreated, P = peat addition, AD = anaerobic disinfestation.

Root biomass in the bioassays was not only affected by *Pythium* but also by the different management practises as became apparent from the control bioassays, i.e. the pots without *Pythium* addition. Both peat amendment and soil disinfestation significantly affected root weight in both years of sampling (see Figure 2.1a,b; Figure 2.2c; Table 2.2). Addition of organic matter significantly increased the root weight in both years; the root biomass was significantly higher in peat-amended soils (2011, 21% for P and 43% for ADP; 2012 19% for P and 16% for ADP) than in the comparable soils without peat addition. Soils that had received a recent AD treatment had a significantly reduced root weight, 39% (AD) and 14% (ADP), as compared to the untreated (U) and peat-amended soil (P) respectively. One year later this effect was

reversed. In 2012, plants in formerly anaerobic disinfested soils had a higher root biomass, 10% and 7% more, compared to the non-disinfested soils with the same organic matter level. Similar to the effects of *Pythium* on root weight, anaerobic disinfestation enhanced the effects of *Pythium* on root rot symptoms in 2011 (Figure S2.2a; Table 2.2). This interaction was no longer apparent in 2012 (Figure S2.2b; Table 2.2). Even without addition of *Pythium*, an increase of root rot symptoms was found for recently disinfested soils (Figure S2a; Table 2.2). Peat addition reduced the severity of root-rot symptoms significantly (Figure S2.2b, Table 2.2). The infective ability of the applied *Pythium* inoculum was confirmed, as a strong increase of root rot symptoms was seen in pasteurized soil (Figure 2.1e).

Table 1.2 Analysis of variance for root biomass and disease indexes of hyacinth bulbs in the soil bioassays and hyphal biomass in the volatile exposure assays.

	Df	F 2011	p 2011	Df	F 2012	p 2012
Root weight						
anaerobic soil disinfestation	1	26,24	**3,1E-05**	1	22,77	**7,4E-05**
peat addition	1	8,23	**8,5E-03**	1	57,63	**7,9E-08**
Pythium addition	1	21,64	**1,0E-04**	1	107,4	**2,4E-10**
disinfestation: peat amendment	1	0,18	0,68	1	3,92	0,06
disinfestation: *Pythium* addition	1	5,19	**0,03**	1	1,60	0,22
peat amendment: *Pythium* addition	1	0,76	0,39	1	0,18	0,68
disinfestation: peat : *Pythium* addition	1	0,45	0,51	1	2,42	0,13
Volatile assay						
anaerobic soil disinfestation	1	85	**4.0E-13**	1	0,58	0,45
peat addition	1	4	0,05	1	0,43	0,52
disinfestation: peat amendment	1	3	0,11	1	1,17	0,29
Disease index						
anaerobic soil disinfestation	1	54,62	**1,3E-07**	1	2,74	0,11
peat addition	1	4,52	**0,04**	1	17,38	**3,4E-04**
Pythium addition	1	5,81	**0,02**	1	125,09	**5,3E-11**
disinfestation: peat amendment	1	5,07	**0,03**	1	1,27	0,27
disinfestation: *Pythium* addition	1	5,51	**0,03**	1	1,93	0,18
peat amendment: *Pythium* addition	1	0,20	0,66	1	6,00	**0,02**
disinfestation: peat : *Pythium* addition	1	0,91	0,35	1	0,22	0,65

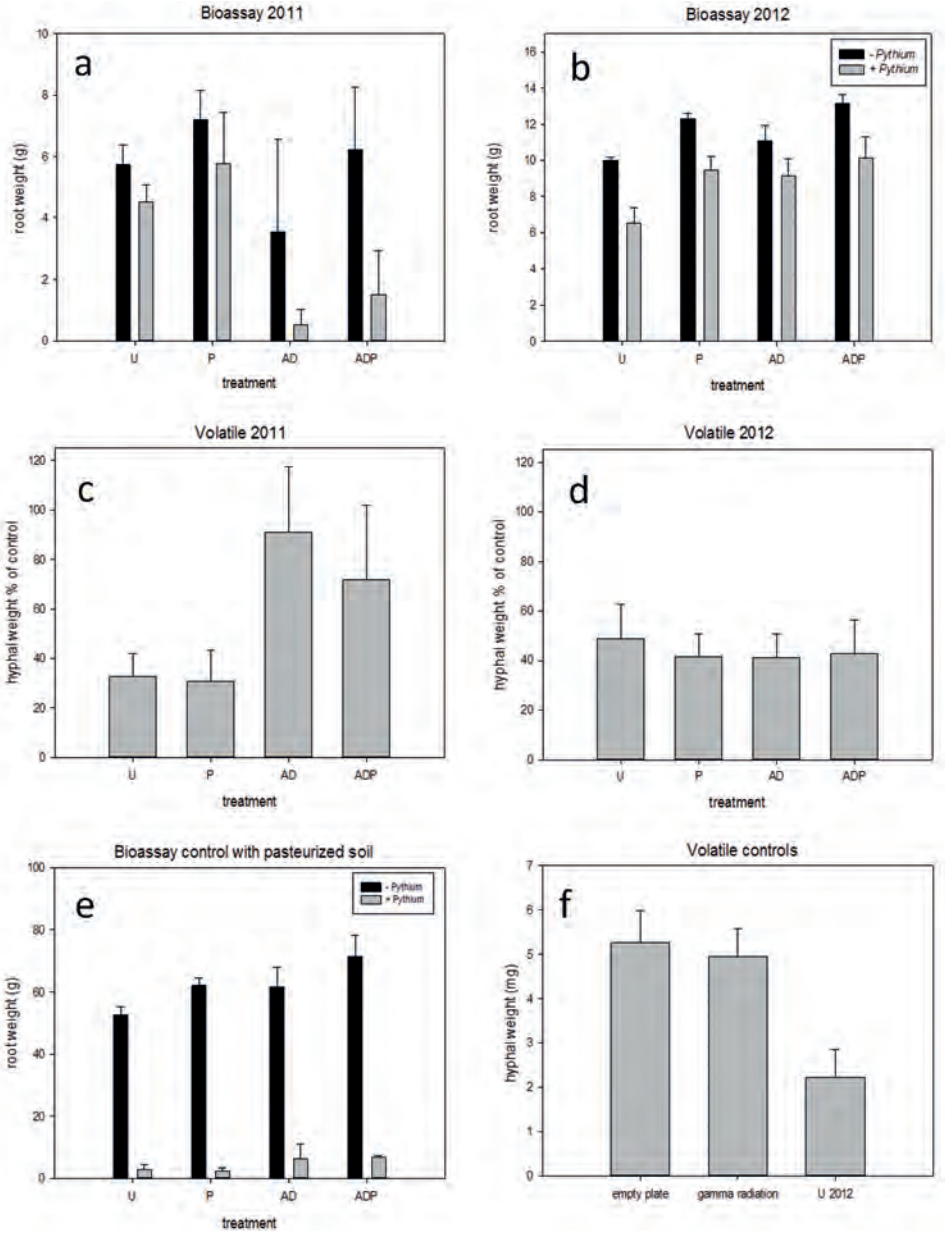


Figure 2.1 Root biomass of Hyacinth bulbs in soils with and without addition of *Pythium intermedium* and production of hyphal biomass by *P. intermedium* during exposure to soil volatiles. **a** and **b**, Average weight of roots extending from Hyacinth bulbs grown in differently managed soils (U= untreated, P = peat addition, AD = anaerobic disinfestation) with and without *Pythium* addition. **c** and **d**, Average hyphal weight of *P. intermedium* hyphae that had been exposed to volatiles produced by differently managed soils. *Pythium* biomass is presented as percentage of the empty plate control. e and **f**, control experiments: e, Bulb root weight in pasteurized soils with and without addition of P. intermedium; f,Average hyphal weight of empty plates, gamma irradiated soil and the untreated soil in 2012. Significant changes and interactions are presented in Table 2. Error bars represent standard deviation.

Emission of *Pythium*-inhibiting volatiles by soils and soil microbes

Exposure of *Pythium* to volatiles released from the soils resulted in a strong reduction of *Pythium* biomass production (Figure 2.1c,d; Figure 2.2a,b). There were, however, differences between treatments and sampling years. Compared to the empty plate control, both the untreated soil and soil with peat amendment gave a 3-fold reduction in mycelial biomass in 2011 ($p < 0.0001$) (Figure 2.1c,d; Table 2.2). In contrast, exposure of *Pythium* to volatiles released from the anaerobic disinfested soils did not (AD) or only slightly (ADP) result in reduction of *Pythium* biomass (Figure 2.1c,d; Figure 2.2a,b; Table 2.2). In 2012, this lack of volatile suppression in disinfested soils was no longer apparent as volatiles from all soils significantly reduced *Pythium* growth by at least 50% compared to soils (Figure 2.1d; Figure 2.2a,b). The impact of soil-derived volatiles on *Pythium* growth was not significantly affected by peat addition. Volatile-suppression of *Pythium* growth was not seen when exposed to gamma-radiated soils (Figure 2.1f), indicating that no growth-reducing volatiles were produced in soil without microflora.

Trapping and GC/MS analysis of bacterial volatiles

GC/MS analysis identified > 700 different volatile compounds that were released from the soil of which fifteen compounds were found to be absent in the anaerobic disinfested soil in 2011 (Table 2.3), mostly ketones. Some of these compounds, namely 2-octanone, 2-undecanone and 2-nonanone, are known to be inhibitors of eukaryotic pathogenic soil organisms (Table 2.3). Besides ketones the fifteen potential suppressive compounds included glycol ethers, alkanes, a fatty acid and two yet unidentified compounds with retention indices of 1692 and 1743. One year later, the 15 volatile compounds were again released by the previously disinfested soils.

Pyrosequencing of soil bacterial communities

454 Pyrosequencing identified over 3000 different OTUs from 31 bacterial and 2 archaeal phyla. There was no soil treatment effect on the number of reads obtained per sample, average numbers of reads were not different between soil treatments or sampling years. In 2011, anaerobic soil disinfestation had resulted in reduction of OTU richness. At higher organic matter level the reduction was significantly less (Figure 2.3a). In 2012 no differences in OTU richness were seen between soil treatments. Most abundant (36 – 63 %) in all samples were OTUs assigned to *Proteobacteria.* Sub-phyla within the *Proteobacteria, i.e. Alpha- Beta- Gamma-* and *Deltaproteobacteria,* did not change significantly between different soil treatments (Figure S2.3). In 2011, six phyla showed significant differences between plots with and without anaerobic soil disinfestation (Figure 2.3c-h). At the start of the bulb planting season anaerobic soil disinfestation had still clear effects on the bacterial community composition. Relative abundances of OTUs

assigned to *Acidobacteria, Chloroflexi, Nitrospirae, Chlorobi* and *Planctomycetes* were significantly lower in the disinfested soils, whereas relative abundance of OTUs assigned to *Bacteroidetes* was higher compared to untreated soils. In 2012, 15 months after the disinfestation treatment, the relative abundance of these phyla was restored to the same levels as occurred in untreated soils for *Acidobacteria, Chlorobi* and *Planctomycetes* ($p<0.05$), with the same tendency for *Chloroflexi* and *Nitrospirae* ($p<0.1$) (Figure 2.3, Table S2.1).

Table 2.3 Volatile organic compounds of which the production appeared to be negatively affected by the anaerobic disinfestation treatment in 2011. Bold compounds have been previously identified as fungus suppressing compounds.

2011					2012			
	U	P	AD	ADP	U	P	AD	ADP
2-octanone	-	+	-	-	+	+	+	+
2-nonanone	+	+	-	-	+	+	+	+
2-undecanone	+	-	-	-	-	+	+	+
2-hexanone	-	+	-	-	+	-	+	+
2-tetradecanone	+	-	-	-	-	+	-	+
2,5hexadione	-	+	-	-	+	-	+	-
1-octen3-one	+	+	-	-	+	+	-	+
2-propanol, 1-butoxy-	+	+	-	-	+	+	+	+
ethanol, 2 butoxy-	-	+	-	-	+	+	+	+
hexadecane	+	+	-	-	+	+	+	+
nonanol	+	+	-	-	+	+	+	-
cyclohexane nonyl	-	+	-	-	+	+	+	+
heptanoic acid	-	+	-	-	+	+	+	+
unknown 1692a*	+	+	-	-	+	+	+	+
unknown 1743a*	+	+	-	-	+	+	+	+

*Unknown 1692a Lri: 1692; EI: 88 (100), 121 (75), 174 (10)
*Unknown 1743a Lri: 1743; EI: 104(100), 78(11), 208(3)

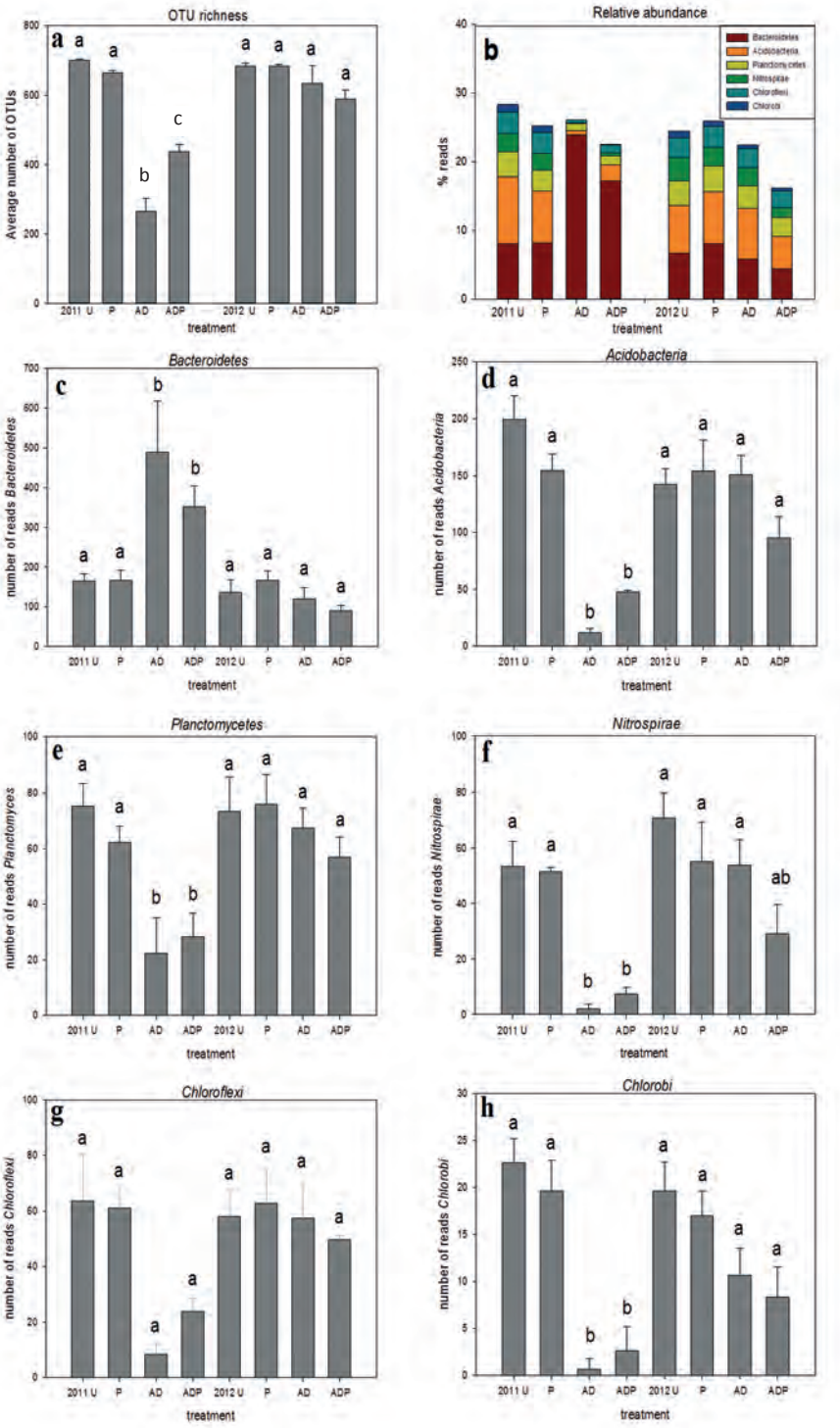

a
OTU richness
Average number of OTUs
treatment
2011 U
P
AD
ADP
2012 U
b
Relative abundance
% reads
Bacteroidetes
Acidobacteria
Planctomycetes
Nitrospirae
Chloroflexi
Chlorobi
c
Bacteroidetes
number of reads Bacteroidetes
d
Acidobacteria
number of reads Acidobacteria
e
Planctomycetes
number of reads Planctomyces
f
Nitrospirae
number of reads Nitrospirae
g
Chloroflexi
number of reads Chloroflexi
h
Chlorobi
number of reads Chlorobi

Figure 2.3 OTU richness and average relative abundance of selected bacterial phyla. **a**, average number of OTUs in differently managed soil (n=3, error bars represent stdev, U = untreated, P = peat addition, AD = disinfestation). **b-h**, average relative abundance of phyla that differ significantly between the disinfested soils in 2011 (AD and ADP) and all other treatments in 2011 and 2012.

Discussion

Volatile organic compounds form an important part of the underground chemical communication network between plants, fungi and bacteria (Ryu *et al.*, 2003; Vespermann *et al.*, 2007; Insam and Seewald, 2010) (Effmert *et al.*, 2012; Bitas *et al.*, 2013; Fiers *et al.*, 2013). They can have different roles in the soil including plant growth promotion and signalling (Vespermann *et al.*, 2007). There are also indications that VOCs produced by soil micro-organisms can have an important contribution to the restriction of growth and germination of pathogenic fungi (fungistasis) that occurs in most soils (Garbeva *et al.*, 2011). However, despite the ability of several soil microbial VOCs to reduce pathogenic growth; little attention has been paid to the role of these VOCs in suppression of plant diseases caused by soil-borne pathogens.

Our results provide an indication about the involvement of VOCs in natural disease suppression of a soil-borne pathogen. Our study revealed interesting co-incidences of severe *Pythium*-induced root weight loss, absence of production of *Pythium* suppressing soil volatiles and shifts in bacterial community composition shortly after an anaerobic disinfestation treatment. One year later these effects of the disinfestation treatment had largely disappeared. The coinciding dynamics of root biomass and production of suppressing volatiles suggests that microbial volatiles can have an important contribution in the natural control of *Pythium intermedium*. Furthermore our results point at the importance of microbial community composition as disinfestation-induced shifts in community composition which coincided with the loss in suppressiveness by volatiles.

Induced changes in microbial community composition can yield important information on the functioning of the original soil microbial communities (Griffiths and Philippot, 2013). Management practices can alter the abundance of microbial groups that are thought to be involved in disease suppression (Garbeva *et al.*, 2004; Mazzola, 2004; Chaparro *et al.*, 2012; Sipilä *et al.*, 2012). In the current investigation we observed that anaerobic soil disinfestation had a dramatic effect on soil microbial diversity and community composition. This is not surprising as the microbes in the well-drained sandy soils were confronted with a long period of oxygen-depletion resulting in a shift from aerobic metabolism to predominant anaerobic metabolism. The impact of this period of anaerobis was still clearly visible in the bacterial community composition at the time that flower bulbs were planted i.e. three months after the soil disinfestation treatment had been ended (removal of cover plastic). Most striking was the high abundance of *Bacteriodetes,* a phylum that has been

shown to strongly respond to fluctuating redox conditions (DeAngelis *et al.*, 2010).

In contrast, the phyla *Acidobacteria, Planctomycetes, Nitrospirae, Chloroflexi* and *Chlorobi* decreased significantly after disinfestation. So far, these groups have rarely been studied in the context of disease suppression, due to limitations to obtain cultivable representatives. Strongest reduction in relative abundance of OTUs after soil disinfestation was seen for *Acidobacteria*. A recent study showed that *Acidobacteria* were one of the most sensitive groups to a strongly disturbing soil treatment (fumigation)(Domínguez-Mendoza *et al.*, 2014). Earlier studies have shown that changes in land use, fertilization and management caused shifts in relative abundance of *Acidobacteria* and its different subgroups (Barnard *et al.*, 2013; Navarrete *et al.*, 2013; Jones *et al.*, 2009). However, the impact of such shifts in abundance of *Acidobacteria* on disease suppressiveness has not been examined. Yet, next to our results a study by Hunter *et al.* (2006) provides indications for a possible role of *Acidobacteria* in disease suppressiveness. In that study it was observed that *Acidobacteria* were present in the peat suppressive to *P. sylvaticum* whereas they were absent in peats that were conducive for *P. sylvaticum* damping-off. No other documentation is available on antagonistic roles of *Acidobacteria,* nor on production of antimicrobial VOCs. Our data suggest a potential role in the production of suppressing volatiles by *Acidobacteria.* However, the actual role of *Acidobacteria* in volatile production and disease suppression, as well as that of the other phyla that showed similar dynamics upon disinfestations remains to be established.

Remarkably, the main classes of *Proteobacteria*, which contain many known potential biocontrol bacteria (Weller *et al.*, 2002; Haas and Défago, 2005) did not change significantly as a result of the disinfestation treatment (Figure S2.3). However, this does not necessarily imply that *Proteobacteria* did not contribute to *Pythium* disease suppression. Mendes *et al.* (2011) compared a soil suppressive against the plant pathogen *Rhizoctonia solani* with another, similar soil that was conducive for disease caused by this fungus and found similar abundances for all classes of *Proteobacteria* in both soils. However, at species level, e.g. within the genus *Pseudomonas*, differences were observed with higher abundance of antibiotic-producing species in the suppressive soil.

Comparison of the volatiles produced by differently treated soils revealed potential *Pythium*-inhibiting compounds, mostly methyl ketones. These VOCs were present in the untreated and peat-amended soils that exhibited a high level of natural suppression against *Pythium*, but absent in the recently disinfested soils that were susceptible to infection of bulbs by *Pythium*. Among these VOCs, there were compounds like 2-octanone, 2-nonanone and 2-undecanone that were previously found to be suppressive against soil fungi and nematodes (Chen *et al.*, 1988; Alström, 2001; Wheatley, 2002; Gu *et al.*, 2007; Kai *et al.*, 2007; Effmert *et al.*, 2012). Thus

these findings support the potential suppressive role of the VOCs identified in this study. However, the antimicrobial role of the other potential suppressive compounds identified in this study remains to be assessed.

Anaerobic soil disinfestation is applied to kill a broad range of pathogens (Blok *et al.*, 2000). The demonstrated reduction of disease suppression shows that such a drastic treatment of the soil has the risk of a (partial) elimination of the natural suppressive microflora. After one growing season, fifteen months after application, disease suppression against *Pythium* was restored to the level of the non-disinfested plots. Similar loss of suppressing activity of the indigenous microflora has been found with pathogen-eliminating measures like flooding (8 weeks) and chemical soil disinfestation with cis-dichloropropene or methylisothiocyanate (van Os *et al.*, 1999). Postma *et al.* (2000) observed enhanced *Pythium* outbreaks in cucumber grown on rockwool after sterilisation of the rockwool and recolonisation by a microbial community which lacked the suppressive properties of the original community. Hence, (temporal) changes in the suppressive community, by reducing the competition pressure or elimination of useful microbes, can enhance disease outbreaks of opportunistic pathogens such as *Pythium* (van Os *et al.*, 1999; Van Os and Van Ginkel, 2001). Our study included two consecutive years of bulb planting to determine the longer term effect of the management treatments on *Pythium* suppression. Fifteen months after the disinfestation treatment, the bacterial community composition resembled the composition of the non-disinfested soils. This is in agreement with the results of Mowlick *et al.* (2013) who found a restoration of the original microbial community composition in the biological disinfestation treatment after plant growth. Anaerobic disinfestation had impact on the taxonomic composition of the soil microbial community but also on an important function, namely disease suppression. In our study, after the cultivation of the summer crop *Gladiolus* the suppression of *Pythium* in both volatile assay and bioassay also returned to the level of that in non-disinfested soils. This recovery of the natural suppression against *Pythium* indicates resilience of the soil to re-establish this essential ecosystem function after a strong disturbance (Griffiths and Philippot, 2013).

In the year of application (2011), the anaerobic soil disinfestation had a negative effect on Hyacinth root development even without addition of *Pythium*. This may be the result of phytotoxic effects of compounds that have been produced during anaerobic decomposition and were still present. It is known that decomposition of crop residues during the period of oxygen depletion can produce phytotoxic compounds (Bonanomi *et al.*, 2007b). Since this reduced root growth was only significant in the disinfested soil without peat addition, the increased organic matter levels in peat-amended soils may have absorbed possible phytotoxic compounds.

Organic amendments do influence soil physical-chemical properties as well as soil microbial activity and composition (Hoper and Alabouvette, 1996; Bonanomi *et*

al., 2007a). Therefore, we expected to find an effect of peat addition on volatile suppression. The addition of peat increased root weight and reduced root rot symptoms in 2012 as compared to the untreated soil. However, the volatile suppression of peat-amended soils was not different from that of the unamended soils. This is in line with the microbial community composition which was not strongly affected by peat amendment but does imply that other mechanisms of disease suppression, besides volatiles, contribute to disease suppression after peat addition. It is clear that organic amendments and disease control measures can have long-term effects on both the soil microflora and on disease suppression, although the effects of the amendments might depend on the nature and maturity of the organic additions (Hoitink and Boehm, 1999; Termorshuizen *et al.*, 2006). In order to get more understanding of time-related changes it is necessary to monitor these soil characteristics during longer periods of time. Since flower bulb production, and more general arable agriculture, are not only seriously threatened by *Pythium*, but also by several other soil-borne fungi and nematodes, is would be recommendable to extend these studies to include also other pathogens like *Rhizoctonia solani*, *Pratylenchus penetrans* or *Meloidogyne hapla*. In conclusion, our study indicates that the production of suppressing volatiles by soil microbes may be an important factor in the natural suppression of root-infection by *Pythium*. More general, this indicates that microbial volatiles may be an essential part of the natural buffering of soils against soil-borne diseases, the so-called general disease suppression. This would open new perspectives and insights for the control of soil-borne pathogens. Volatile-inhibition tests as well as the presence of certain VOCs and microbial groups could be an indicator of the susceptibility of a given soil to soil-borne pathogens. Obviously, more research is needed to find support for this. In depth studies are needed to further assess the role of volatiles in disease suppression and should also consider the dynamics of production of VOCs in soils, as well as the conditions that affect the sensitivity of the pathogens for VOCs.

Acknowledgements

We are grateful to Mattias de Hollander for assistance with the bioinformatics. Furthermore we would like to thank Marjan de Boer for her role in setting up the field experiment the soils originated from. Thanks to Suzanne Breeuwsma and Jan van de Bent for technical assistance. Sequences were deposited in the European Nucleotide Archive under accession number PRJEB6155 (http://www.ebi.ac.uk/ena/data/view/PRJEB6155). The data (bioassays, *Pythium* inhibition assays, OTU table and volatiles data) have been archived in the Data and Information Portal of the NIOO-KNAW http://data.nioo.knaw.nl/index.php and is available on request. This research is supported by the Dutch Technology Foundation STW, which is part of the Netherlands Organisation for Scientific Research (NWO), and which is partly funded by the Ministry of Economic Affairs.

Supplementary data

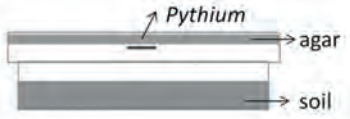


Figure S2.1 Illustration of the petri dish set-up used to determine the effect of soil-derived volatiles on *Pythium* biomass. Bottom petri dish compartments contain soil. Lid compartments contain water yeast agar with in the middle a *Pythium* plug as inoculum.

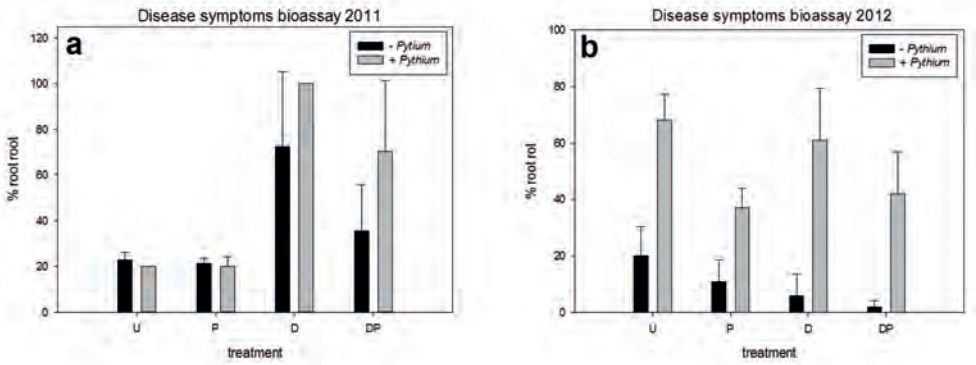


Figure S2.2 Percentage root rot of Hyacinth roots in soils with and without addition of *Pythium intermedium*. **a** and **b**, Average percentage of roots extending from Hyacinth with root rot symptoms with and without *Pythium* addition in differently managed soils (U= untreated, P = peat addition, AD = disinfestation) Significant changes and interactions are presented in Table 2. Error bars represent standard deviation.

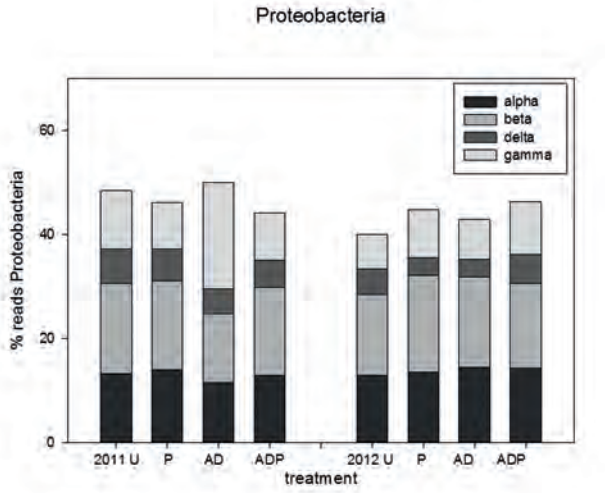
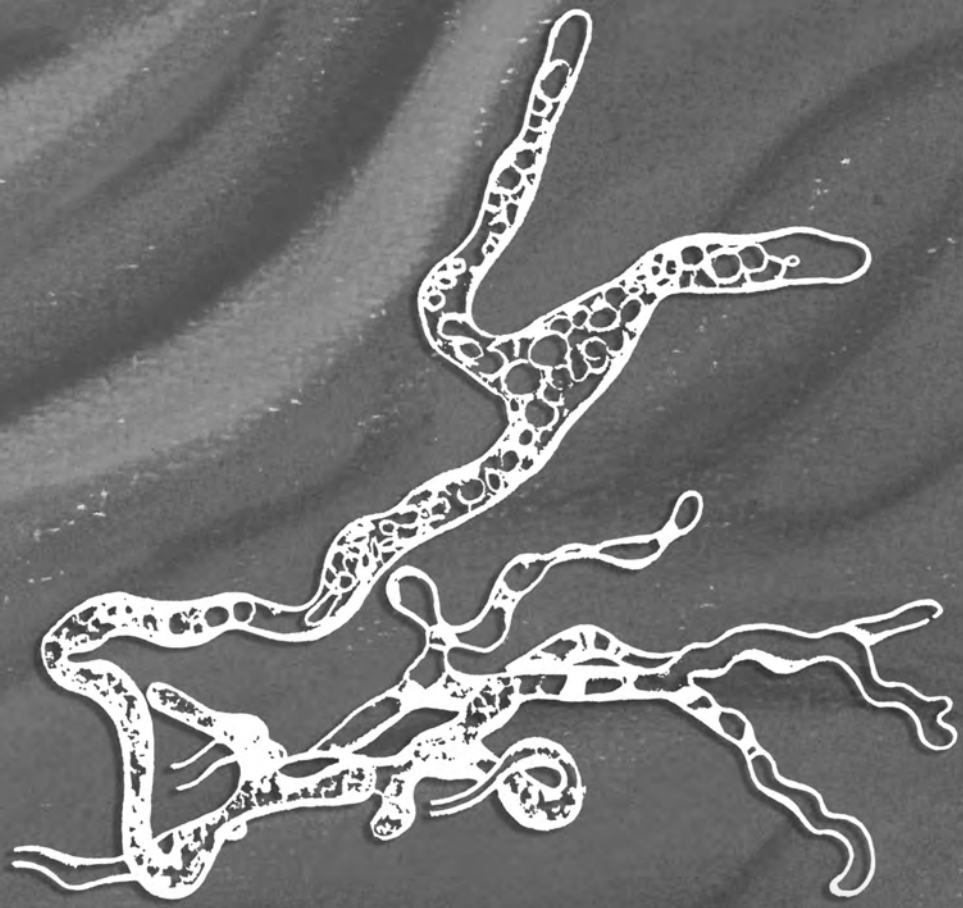


Figure S2.3 Average relative abundance of *Proteobacterial* OTUs (U= untreated, P = peat addition, AD = disinfestation).

Table S2.1 *p* values of the analysis of variance for different bacterial phyla that significantly differ between the management treatments and sampling years (U= untreated, P = peat addition, AD = disinfestation).

		U 2011	P 2011	AD 2011	ADP 2011	U 2012	P 2012	AD 2012	ADP 2012
Acidobacteria	AD 2011	0.000175	0.000175	X	0.2119	0.000175	0.000175	0.000175	0.000441
	ADP 2011	0.000175	0.000181	0.2119	X	0.00022	0.000182	0.000186	0.04657
Bacteroidetes	AD 2011	0.000194	0.000196	X	0.09146	0.000179	0.000196	0.000177	0.000175
	ADP 2011	0.01058	0.01155	0.09146	X	0.003094	0.01138	0.001548	0.000555
Chloroflexi	AD 2011	0.000255	0.000328	X	0.586	0.000483	0.000275	0.000535	0.002456
	ADP 2011	0.003313	0.006124	0.586	X	0.0124	0.004161	0.01453	0.08498
Nitrospirae	AD 2011	0.000193	0.000208	X	0.9924	0.000175	0.000185	0.000191	0.0213
	ADP 2011	0.00027	0.000345	0.9924	X	0.000175	0.000235	0.000261	0.09054
Chlorobi	AD 2011	0.000175	0.000176	X	0.9819	0.000179	0.000196	0.000177	0.000175
	ADP 2011	0.000175	0.000186	0.9819	X	0.003094	0.01138	0.001548	0.000555
Planctomycetes	AD 2011	0.000227	0.001769	X	0.9917	0.00027	0.000219	0.000615	0.006472
	ADP 2011	0.00044	0..,007649	0.9917	X	0.000615	0.0004	0.002245	0.02931

CHAPTER 3.
SOIL PARAMETERS LINKED TO VOLATILE-MEDIATED SUPPRESSION OF PLANT PATHOGENS



Maaike van Agtmaal*, Angela Straathof*, Aad Termorshuizen, Wietse de Boer, Ellis Hoffland

*Authors contributed equally to this work

Abstract

There is increasing evidence that volatile organic compounds have a role in suppressing soil-borne plant pathogens but it is thus far unknown which edaphic properties are indicative of a soil's capacity to produce pathogen-suppressing volatiles. We measured the growth-suppressive effects of volatiles emitted from a broad range of agricultural soils on the agronomically important pathogens *Rhizoctonia solani, Fusarium oxysporum* and *Pythium intermedium. In vitro* growth suppression caused by exposure to soil volatileswas linked to edaphic properties, microbial community composition and field history using a multivariate statistical approach. Our results show volatile-mediated suppression of mycelial development for all pathogens; however, the range of effects and the significant edaphic variables differ per pathogen. Suppression of *R. solani* by volatiles was positively correlated with organic matter content, microbial biomass and amount of litter saprophytes but negatively correlated with pH, Shannon diversity and amount of *Acidobacteria.* Suppression of *F. oxysporum* and *P. intermedium*, however, was more affected by field history. *P. intermedium* suppression was also negatively correlated with soil sulphur content. Regression modelling of the three pathogens' overall suppression rate identified microbial activity, dissolved organic carbon (substrate availability) and crop history as the most influential variables. This study identified the overall and pathogen-specific drivers of growth-suppressive volatiles of soil-borne pathogens, which may be valuable components of a soil's potential for natural disease suppression.

Introduction

Soil-borne plant pathogens cause crop loss world-wide and there is a need for enhancing natural control mechanisms as a component of sustainable agriculture. In soils, one natural phenomenon that decreases disease manifestation of such pathogens is called general disease suppression (GDS), which is proposed to be related to substrate-driven microbial activity (Hoitink and Boehm, 1999). The edaphic properties that determine a soil's capacity for GDS, however, remain largely unidentified (Van Bruggen and Semenov, 2000), but an indicator of this capacity would be valuable for moving towards prediction of natural suppression and risk of disease outbreak. One aspect of soil that is positively correlated with GDS is pathogen suppression (Termorshuizen and Jeger, 2008) which occurs when a pathogen's germination and/or hyphal extension is restricted by the soil microbial community through either resource competition or the production of antifungal compounds (Watson and Ford, 1972). The latter may be in the form of volatile organic compounds (VOCs), which have recently been proposed as important agents in pathogen suppression (as an outcome of fungistasis) and, thus, may contribute to GDS (Garbeva *et al.*, 2011). The diffusive nature of VOCs facilitates their permeation of the soil matrix, resulting in a greater effective range relative to other suppressive compounds or organisms.

The effect of exposure to volatiles on growth of several phylogenetically different, agronomically important soil-borne plant pathogens has been tested, including *Rhizoctonia solani., Fusarium* spp. (Kai *et al.*, 2009; Garbeva *et al.*, 2014b), and *Pythium* spp. (Chaurasia *et al.*, 2005; Garbeva *et al.*, 2014b). The growth of these pathogens is reported to be inhibited by VOCs released from various bacteria and fungi (Weisskopf and Bailly, 2013), including soil-dwelling *Bacillus* spp., *Burkholderia* spp., *Pseudomonas* spp., *Serratia* spp., and *Stenotrophomonas* spp. (Fiddaman and Rossall, 1994; Pandey *et al.*, 1997; Kai *et al.*, 2007). Growth stimulation, however, has also been reported, including observations of volatiles that are suppressive against some pathogenic species but promote the growth of others (Wheatley, 2002). Most volatile-pathogen interaction studies have been performed with bacterial isolates (Campos *et al.*, 2010) on artificial media, outside the indigenous environment of both the volatile-producers and the pathogens. This limits the conclusions that can be made about volatiles emitted by the collective soil community, or volatiles produced as a result of microbe-microbe or edaphic-microbial interactions in the soil.

The effects of edaphic-microbial interactions on volatile production may be two-fold: first, the management and/or inherent properties of a soil may influence the composition of the microbial community (Ettema and Wardle, 2002; Marschner, 2003; Rousk *et al.*, 2010), which is responsible for volatile production. Secondly, the quality and availability of substrates for microorganisms may influence the rate

and profile of VOCs produced (Fiddaman and Rossall, 1994; Gray *et al.*, 2010). Leff and Fierer (2008) measured both higher emission rates and higher diversity of VOCs emitted from litter than from mineral soils, and found organic C quality and microbial biomass were, respectively, the most influential edaphic properties of those sources. The soil environment may further confound volatile emissions, because of absorptive properties of the soil matrix; for instance, differences in recovery rates of polar, aromatic and aliphatic VOCs were significant when compounds were forced through either sand or clay soil types (Ruiz *et al.*, 1998). Because of the myriad of influences the soil environment can have on both production and release of VOCs (Peñuelas *et al.*, 2014), it is important to consider pathogen-suppressing VOCs as they are emitted from a variety of soils. Chuankun *et al.* (2004) found a widespread suppressive effect of volatiles from 146 soils on fungal spore germination. Furthermore, Campos et. al (2010) suggested that volatile-mediated pathogen suppression is far more extensive than currently known; however, no studies have investigated volatile-mediated suppression from multiple soils for multiple plant pathogens thus far.

We conducted a large-scale survey on agricultural soils to measure a broad range of soil properties and potentially relevant parameters never before measured in the context of volatile-mediated pathogen growth. The objectives of this survey were to 1) measure the effect of volatiles emitted from agricultural soils on *in vitro* biomass production of the soil-borne plant pathogens *Rhizoctonia solani*, *Fusarium oxysporum* and *Pythium intermedium*, and 2) identify the most statistically informative soil properties, microbial community structure, or field management parameters relevant for an agricultural soil's production of pathogen-suppressing volatiles. This study will therefore contribute to the development of hypothesis-driven testing of volatile-mediated pathogen suppression in soils.

Materials & Methods

Field selection, soil sampling and pre-treatment

A total of 50 arable fields were selected from across the Netherlands (Figure S3.1), covering a wide range of soil properties, e.g. texture, pH and organic matter content. Fields were sampled in February-March 2013, before the start of the growing season. Soil sampling (0-20 cm cores) was performed by taking 60 subsamples in a double W-pattern from an area of about 2 ha in each field. These subsamples were pooled and manually homogenized, resulting in a 3 kg sample per field, which was kept at 4°C during transportation.

Upon arrival in the lab, soils were processed directly; they were split in two parts used for determining (1) chemical soil properties, (2) dissolved organic carbon (DOC) fractions, microbial biomass N, respiration, microbial community composition, and the *in vitro* suppression of pathogen growth by volatiles released by the soil. Part (1)

was oven-dried (40°C), ground, and processed by BLGG (Wageningen, The Netherlands) according to standard procedures (Table 3.1). Part (2) was sieved to 4 mm. Per soil, two 1 g subsamples were taken and stored at -20°C for DNA extractions. Soil moisture was determined from the mass differential measured by drying the soil at 105°C for 24 h. Part (2) was then separated into three equal-sized samples. The three samples per field were then pre-incubated for 3 days at 9°C and 60% water-holding capacity (WHC). By equilibrating each soil under the same conditions, the effects of variable temperature and moisture conditions between different fields over the four weeks of sampling were minimized. After separation and pre-incubation, measurements of soil properties were performed in triplicate and the averages of the three samples were used as the input data.

Determination of soil properties

A brief summary of all measured soil properties and the respective methodological references can be found in Table 3.1.

Soil properties

The soil dissolved organic carbon (DOC) was suspended in a 1:2 (w:w) extraction, where one part fresh soil (mass of dry-weight equivalent (DWE) was suspended in two parts ultra-pure water (UPW). Samples were equilibrated for 1 h on a horizontal shaker, centrifuged 20 min at 3000 g, and ultra-centrifuged 10 min at 11700 g. The supernatant was filtered through a 0.45 mm cellulose nitrate membrane and a subsample of the filtrate was analysed for total DOC with a TOC-5050A analyzer (Shimadzu Corporation, Kyoto, Japan). The remaining filtrate was fractionated into four DOC fractions (Table 3.1) based on their hydrophobicity (Thurman and Malcolm, 1981) using a batch fractionation procedure (Van Zomeren and Comans, 2007). The aromaticity of total DOC, humic acids, fulvic acids and the hydrophilic fraction was also measured by each solution's absorption of UV light at 254 nm (Genesys 10S UVeVIS, Thermo Fisher Scientific Inc., Waltham MA, USA).

Basal respiration rates were determined by CO_2 emission from incubated soils. The DWE of 100 g fresh soil was weighed into a 335 ml glass bottle and incubated in the dark at 20°C. Soil moisture in each bottle was maintained at starting levels (60% WHC) by adding UPW as needed, at least once per week. After the start of incubation (T0), emissions were measured nine days after T0 (T1), three weeks after T1 (T2), and 11 weeks after T2 (T3). At the start of a measurement period, each glass bottle was flushed with N_2 gas, and then sealed with a rubber septum for 4 h (T1), 6 h (T2), or 12 h (T3). Accumulated CO_2 concentrations in the bottle's headspace were then measured through the septum using an INNOVA 1412 Photoacoustic field gas-monitor (LumaSense Technologies, Ballerup Denmark). Cumulative CO_2 emissions for the 14 weeks of incubation (T1-T3) were calculated by linear interpolation of T1, T2 and T3, which produced a better fit than exponential interpolation.

Microbial biomass N was determined using the chloroform fumigation-extraction method (Brookes *et al.*, 1985) on 20 g DWE fresh soil. Before and after a 24 h fumigation, a soil subsample was equilibrated for 1 h in 80 ml 0.5 M K_2SO_4, and the solution was filtered through a 0.45 mm cellulose nitrate membrane. Total soluble N in the filtrate was measured on a San^{++} 6 channel segmented flow analyser (Skalar, The Netherlands).

Community analysis

Microbial community composition was assessed using 454 pyrosequencing. Two DNA extractions per soil were performed using Mobio 96-well Powersoil® extraction kit according to the manual. Amplicons for barcoded pyrosequencing (10 bp unique barcode per sample) of bacterial 16S ribosomal DNA fragments, and fungal and oomycetal ITS regions were generated using PCR reactions (primers, sequencing adapters and PCR conditions are listed in Table S1). PCR product quality was examined on a 1% agarose gel and subsequently purified using gel electrophoresis, followed by gel extraction (QIAGEN Inc., Valencia, CA). Concentrations of amplified DNA were measured by the Qubit® 2.0 Fluorometer (Life Technologies) and samples were pooled equimolar. Sequencing was performed by Macrogen (Macrogen Inc., South Korea) on a Roche 454 automated sequencer and GS FLX system using titanium chemistry (454 Life Sciences, Branford, CT, USA).

The obtained 454 sequences were filtered and analyzed using Mothur version 1.32.1. Briefly, primer and barcode information was identified in sequences allowing 0 errors. 16S sequences were trimmed based on a Phred score of 30, or 400 bases. Chimeras were identified by Uchime (Edgar *et al.*, 2011)especially when sequencing single regions (e.g. 16S rRNA or fungal Internal Transcribed Spacer. Sequences were aligned to the Silva reference alignment (Pruesse *et al.*, 2007) followed by a classification (Wang *et al.*, 2007). A distance matrix was calculated (distance cutoff 0.10), and clustered using average neighbour clustering. Operational taxonomic units (OTUs) were determined at 97% similarity. A representative sequence was taken for each OTU and blasted against the NCBI database. An indication of taxonomy was based on the first five blast hits. ITS sequences were similarly filtered and analyzed using a minimum sequence length of 180 and a maximum length of 400 bases. ITS sequences (after chimera removal) were aligned and clustered using cd-hit-est (Li and Godzik, 2006) version 4.5.4 (parameters: word size 9, compare both strands, cluster sequences into most similar clusters instead of first cluster). The same workflow was used for 18S sequences, but a minimum length of 200 bases and a maximum length of 450 bases was applied.

rDNA sequences obtained from the two DNA extractions of each soil were pooled and rarefied to the minimum number of reads. The bacterial sequences were grouped on phylum level. Phyla were included in further analysis if a phylum contained over

0.05% of the total reads. The OTUs of the fungal dataset that could be assigned at the species level were each classified into one of eight potential functional groups (pathogenic, arbuscular mycorrhizal, coprophilic, endophytic, hyperparasitic (i.e. parasitizing on fungi), nematophagous, saprophytic on wood, saprophytic on litter), based on literature screening. For 40-50% of the total reads, a potential function could be indicated. The oomycete sequencing data were also classified into functional groups. However, in contrast to the other two datasets, the data were only pooled and not rarefied, due to the apparent absence or low number of reads in several soils. Shannon diversity index was calculated from the fungal, bacterial and combined dataset.

Field management survey

For each of the 50 soils, an interview with the farmers was conducted to document management practices, including questions on tillage, fertilization, previous crop and crop rotation. Of the 50 fields sampled, 46 fields had successfully completed management surveys; four fields were not included in subsequent analysis where field management variables were input. Results were grouped according to different management practices: different tillage practices were categorized into three groups, (1) conventional tillage, (2) reduced tillage, or (3) no tillage; fertilizer application was categorized into four groups, (1) artificial fertilizer, (2) liquid manure, (3) solid manure, or (4) compost; cover crop was grouped based on presence or absence of a cover crop during the previous field season.

Volatile Assay

An experimental set-up was designed to determine the growth response of three different soil-borne plant pathogens to volatile organic compounds (VOC) released from soils. The three pathogens selected were the basidiomycete *Rhizoctonia solani* AG2-2-IIIB (strain 02-337, IRS, isolated from *Beta vulgaris*), the ascomycete *Fusarium oxysporum* f. sp. *tulipae* (strain TuA, Applied Plant Research Wageningen University and Research Centre, PPO Lisse isolated from *Tulipa* bulbs) and the oomycete *Pythium intermedium* (strain P52, Applied Plant Research, Wageningen University and Research Centre, isolated from *Narcissus* bulbs). The experiment was designed to ensure enough airspace between pathogen and soil so the exposure to volatiles produced by the soils was enabled without physical contact between the pathogens and soil, an assay modified from Garbeva *et al.*, 2014b (Figure S2). For each pathogen, 20 g DWE soil (60% WHC) was spread evenly on the bottom of a 90 mm Petri dish and incubated for 1 week at 10°C before the start of the experiment. For each Petri dish, a 4 mm layer of Water Yeast Agar (WYA; 20 g agar, 1 g KH_2PO_4, 0.1g $(NH_4)_2SO_4$, 0.1 g yeast extract (Difco) L^1, pH 6.5) was poured into the lid. Agar plugs of 6 mm diameter Potato Dextrose Agar (PDA; 19.5 g L^{-1} (Oxoid)) colonized by *R. solani, F. oxysporum* or *P. intermedium*, incubated 5-10 days at 20°C, taken

from the growing front, were transferred to WYA plates and incubated at 10°C. After 48 h, a WYA agar disc (Ø 6 mm) containing the pathogen mycelium was placed in the center of the (agar-filled) lid. The lid was then carefully placed on top of the bottom (soil-containing) compartment and sealed using Parafilm (Figure S3.2). Plates were incubated for 10 days at 10°C. Petri dishes without soil were used as controls to measure the development of mycelium under conditions without soil-released volatiles. The assay was performed with six replicates. Mycelial biomass determination was done according to the method of Garbeva *et al.* (2014b), with some modifications. Briefly, pathogen mycelia were harvested by melting and dissolving the colonized agar from the lids of the Petri dishes in a glass beaker with water in a microwave oven (c. 100°C), followed by sieving the mycelium with a tea strainer and three washing steps with water (c. 90°C) to remove agar residues. For measurements of dry biomass weight, mycelia were frozen at -20°C and freeze-dried for 24 h.

Data Analysis

Statistics

All statistical analyses were performed in R (3.0.0) with the R packages vegan, packfor, ade4, leaps, car, and ape. For the soil properties data, normality and homogeneity of variances were examined using the Shapiro-Wilk test and Levene's test, respectively. Variables that did not meet these assumptions were log-transformed or square root-transformed (Table 3.1). To study the community composition of the fungal and oomycete community, the OTUs from the sequencing that could be assigned to species were grouped by function. The bacterial community was grouped by taxa. The sequencing datasets were Hellinger-transformed to minimize the effects of large abundances and zero values in the community dataset (Legendre and Gallagher, 2001). The field management data were dummy-coded because the data were categorically either absent or present. Pathogen suppression by volatiles was converted to the proportion of reduction of mycelial biomass in comparison to the soil-free control (control = 0 (no suppression), 1 = maximum suppression, <0 = stimulation). The *F. oxysporum* and *P. intermedium* suppression datasets were arcsin-square-root-transformed and three growth-promoting outlier soils were removed from the *P. intermedium* dataset to meet the basic assumptions of normality and homoscedasticity. These soils prevented normal distribution of suppression rate data and were excluded from subsequent analysis. They were not outliers with regards to any other measured parameters.

Table 3.1 Edaphic parameters measured in this study. Transformation of raw data was performed only on the parameters indicated. Parameters in **bold** were included as independent variables in subsequent multivariate analysis where the *in vitro* suppression of *Rhizoctonia solani, Fusarium oxysporum,* and *Pythium intermedium* by soil volatiles were the dependent variables. All units indicated are per unit of soil dry weight equivalent.

Parameters	Assigned dataset	Method of determination	Raw data transformation	Methodological reference
Total and available **N,** P, **K,** C, **S**, Ca (mg kg^{-1}); subsequent **C:S and C:N ratios**; **Organic Matter**, sand, silt, **clay**, organic C, **$CaCO_3$** (%); **CEC** (mmol kg^{-1});	Soil properties	Near-infrared spectroscopy on soil dried at 40°C and ground	log (P, K, N, S, CEC); square root ($CaCO_3$)	Malley et al., 1999
pH; **P-, Na-**, Mg-, K- (mg kg^{-1}), Ca-available (kg ha^{-1})	Soil properties	0.01 M $CaCl_2$ extraction on soil dried at 40°C and ground	log (Na)	Houba et al., 1990
Total DOC; humic acid, fulvic acid, hydrophobic neutral and **hydrophilic DOC fractions** (mg kg^{-1})	Soil properties	Water extraction, 0.45 μm filtration, and subsequent rapid-batch fractionation	log (total DOC); square root (hydrophilic fraction)	Thurman and Malcolm, 1981; Van Zomeren and Comans, 2007
Aromaticity of total DOC and of humic acid, fulvic acid, and hydrophilic DOC fractions in solution (l mg^{-1} cm^{-1})	Soil properties	Specific UV absorption at 254nm	log	Weishaar et al., 2003; Amery et al., 2008
CO_2 emission ($\mu g\ g^{-1}\ h^{-1}$) after 1, 3, and 14 weeks incubation and **cumulative CO_2** ($\mu g\ g^{-1}$)	Soil properties	Headspace concentration measurements using photoacoustic gas monitor	log	Straathof et al., 2014 (modified)
Microbial biomass N (mg kg^{-1})	Soil properties	Chloroform fumigation extraction	log	Brookes et al., 1985followed by immediate extraction with 0.5 M K2SO4 and measurement of total N released by CHC13 in the soil extracts. The amounts of NH4-N and total N extracted by K2SO4 immediately after fumigation increased with fumigation time up to 5 days. Total N released by CHC13 after 1 day fumigation (1 day CHC13-N; Joergensen and Mueller, 1996

Parameters	Assigned dataset	Method of determination	Raw data transformation	Methodological reference
Bacterial community analysis	Microbial community	454 pyrosequencing	Hellinger	Legendre and Gallagher, 2001
Fungal community and functional group analysis	Microbial community	454 pyrosequencing	Hellinger	Legendre and Gallagher, 2001
Oomycete community analysis	Microbial community	454 pyrosequencing	Hellinger	Legendre and Gallagher, 2001
Tillage practice, organic matter application and source, artificial fertilizer application, crops in rotation, cover crop	Field history	Survey with farmers	Dummy-coded (presence/ absence)	
Suppression of hyphal biomass relative to soil-free control: *R. solani*, *F. oxysporum*, *P. intermedium* (control = 0 (no suppression), 1 = maximum suppression, <0 = stimulation)	N/A (dependent variables)	Petri-dish assay for measuring soil volatile effects on hyphal biomass	arcsin-square root (*F. oxysporum*, *P. intermedium)*	Garbeva et al., 2014b (modified)

Selection of parameters for regression analysis

Covariation between all measured soil properties was first examined using a principal component analysis (PCA). The resulting plots of the first two PCs (Figure S3.3) were used to make a selection of the most relevant parameters (Table 3.1), to avoid covariability and reduce the number of input parameters for downstream regression analyses. The decision-making process for selecting soil properties included: 1) if two parameters' PCA vectors had overlapping length and direction, the most biologically relevant of the two was selected (*e.g.* available nutrients from $CaCl_2$ extraction were chosen over less soluble bound elements (Houba *et al.*, 1990)), 2) if two parameters have an inverse relationship, the most biologically relevant of the two was selected (*e.g.* DOC proportion of humic acids' vector is divergent from the proportion of hydrophilic compounds, but the latter has been found to be more closely related to rates of microbial respiration (Straathof *et al.*, 2014)), and 3) parameters previously identified in the literature as being associated with disease suppression in agricultural soils (*e.g.* microbial respiration and biomass (Janvier *et al.*, 2007)). For microbial community and field history datasets, all parameters were used as input.

Multiple linear regression analysis

For the soil property and microbial community datasets, a multiple linear regression analysis was performed, followed by permutation tests to determine the contribution and significance of the selected parameters in the suppression rates of each pathogen by volatiles (the dependent variable). To create a regression model, this was followed by a forward selection procedure with double stopping criterion, adjusted R^2 and $\alpha < 0.05$ (Blanchet *et al.*, 2008). Correlations between significant ($P < 0.05$) parameters and suppression rates were tested with the Pearson correlation coefficient (*r)*. A similar approach was taken for the field history dataset, but using multiple logistic regression models (due to the binary nature of this dataset) and forward selection to create a parsimonious model (Blanchet *et al.*, 2008).

Multivariate regression analysis

Preliminary observations of pathogen growth response to volatiles revealed that no one soil or group of soils was highly suppressive to all three pathogens. Furthermore, a lack of common significant variables among the three pathogens resulting from the univariate multiple linear regression prompted analysis of multivariate regression. Redundancy analysis (RDA) was performed for each dataset to test the contribution and significance of the selected soil property, microbial community, and field history parameters on suppression by volatiles of all three pathogens in combination. Within the RDA plot, an ordination value was generated for the overall pathogen suppression rates. This three-way ordination value became the dependent variable in subsequent regression; a permutation test and then forward selection procedure with double stopping criterion, adjusted R^2 and $\alpha < 0.05$ (Blanchet *et al.*, 2008) was performed to

create parsimonious models and identify the most relevant parameters. To assess the contribution of each variable in the model, partial R^2 values were calculated. Furthermore, for each model, each significant ($P<0.05$) variable was removed one-by-one to assess their respective contribution to the significance, explanatory value, and the subsequent parameters selected in the forward selection procedure. All of the parameters found to be significant in the multiple linear regression for each pathogen, and the multivariate regression within the RDA (all models run) were combined into one dataset for a final regression analyses on all significant parameters only.

Results & Discussion

In part due to the geographical dispersion of the 50 arable fields sampled across The Netherlands (Figure S3.1), a wide variety of soil properties and management strategies was obtained. This in turn resulted in a broad range of soil properties measured: soils contained from 1-45% clay and 8-97% sand. Organic matter (OM) content ranged from 1.3-41% (mean 6.4%), although mineral soil (excluding five peat soils) mean OM content was 4.1%, which agrees with the 4.3% mean OM content of Dutch mineral arable soils found by Reijneveld *et al.* (2009). The mean pH of all 50 soils was 6.4, again similar to values previously reported (6.6 (Reijneveld *et al.*, 2009)), indicating our selection of soils is representative of arable soils in The Netherlands. Other soil properties varied broadly as well and not necessarily collinearly (Figure S3.3), and 17 were ultimately selected as input variables into the regression analysis (Table 3.1). Tillage regimes, manure applications, and the use of a cover crop varied among the selected soils as a result of the spread in edaphic properties and the variety of crops. Corn was the most frequently grown crop at sampled sites (24), followed by wheat (19). Furthermore, the crop rotation on the sampled fields often included flower bulbs, sugar beet, potato and/or onion.

The soils also varied in their compositions of indigenous bacteria, fungi and oomycetes. The 16S sequencing resulted in a minimum of 14789 bacterial reads per field, from 16 phyla. Most reads were assigned to *Proteobacteria* (19-41% of the reads per field) and *Acidobacteria* (13-34% of the reads per field). More rare phyla included *Tenericutes* and *Spirochaetes,* which each had maximum 42 reads per sampled site. Most fungi inhabiting these soils (minimum 2745 reads per field) were classified within the functional group litter saprophytes, yielding in total 23-90% of the reads per field, whereas most of the oomycetes were from the family Pythiales (>99%) in the genera *Phytophthora* or *Pythium*.

Variability among soils was measured in *in vitro* pathogen growth suppression by soil VOCs (Figure 3.1) and was determined to be a general soil phenomenon with pathogen-specific outcomes (Table 3.2). Volatile-mediated effects differed among

the tested soil pathogens (Table 3.2). However, relating these overall pathogen response effects to measured parameters identified microbial activity, previous crop type, and multiple C- and S-related parameters as the most statistically influential (Table 3.3). Each of *R. solani*, *F. oxysporum*, and *P. intermedium* exhibited a range of responses from suppression to promotion of hyphal biomass production, but 91% of pathogen-soil combinations resulted in at least some suppression relative to the soil-free control. This is similar to results showing fungal growth can be either inhibited (up to 60%) or stimulated (up to 35%) by the volatiles of bacterial isolates (Mackie and Wheatley, 1999; Wheatley, 2002). When comparing responses to soil volatiles between pathogens (Figure 3.1), patterns failed to emerge; *i.e.* volatiles from soils that were promoting the growth of *F. oxysporum* were not necessarily promoting growth of *R. solani* (Figure 3.1A) or *P. intermedium* (Figure 3.1B), and *vice versa*.

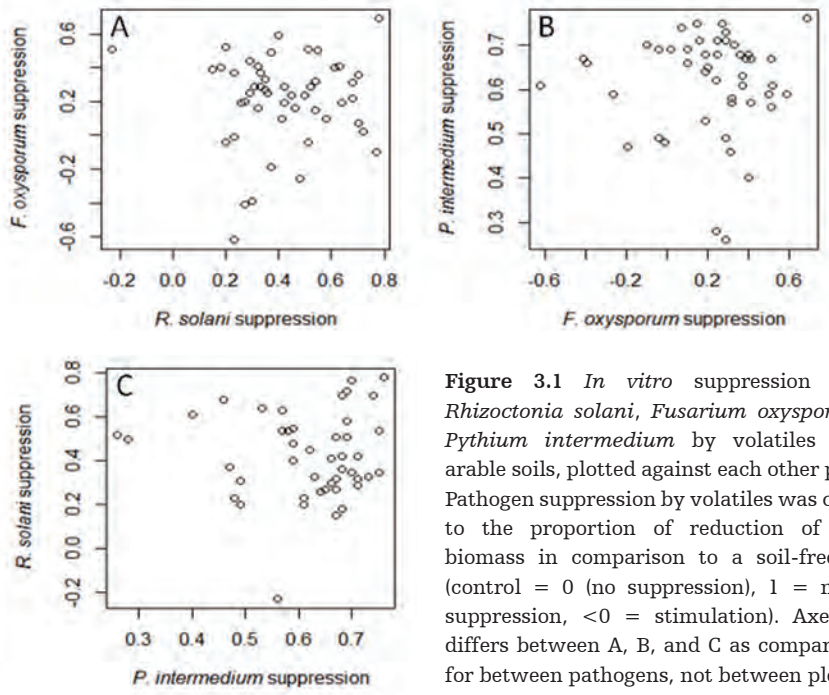


Figure 3.1 *In vitro* suppression rates of *Rhizoctonia solani*, *Fusarium oxysporum*, and *Pythium intermedium* by volatiles from 50 arable soils, plotted against each other pathogen. Pathogen suppression by volatiles was converted to the proportion of reduction of mycelial biomass in comparison to a soil-free control (control = 0 (no suppression), 1 = maximum suppression, <0 = stimulation). Axes scaling differs between A, B, and C as comparisons are for between pathogens, not between plots.

The observation of pathogen-specific response effects (Figure 3.1) to volatiles emitted from the 50 soils in our study agrees with previous research which found species-specific responses to volatiles from various bacterial and fungal isolates under laboratory conditions (Mackie and Wheatley, 1999; Bruce *et al.*, 2000; Garbeva *et al.*, 2014b). Pathogen-specific response variability may result from volatile compounds affecting different sites of action on the pathogen, or from differences between each pathogen's ability to detoxify the VOCs (Kai *et al.*, 2009). Both taxon- and genus-specific sensitivity has been reported between pathogens; generally oomycetes tend

to be very sensitive to volatiles while *Fusarium* spp. were reported to have relative tolerance towards them (Weisskopf and Bailly, 2013; Hunziker *et al.*, 2015), which is in line with our results. These observed differences in tolerance to volatile exposure may also be related to the biology and morphology of each pathogen. Hunziker *et al.* (2015) have suggested that cell wall differences between pathogens may contribute to the permeability and, consequently, the inhibitory effect of volatiles. Oomycete (*P. intermedium*) cell walls contain cellulose whereas fungal (*R. solani* and *F. oxysporum*) hyphae are built with a chitin matrix. Strong differences in lysis have been observed between *Rhizoctonia* and *Fusarium* after contact with non-volatile anti-microbial metabolites, which Potgieter and Alexander (1966) attributed to differences in cell wall structure and composition between the two fungi.

Suppression of *R. solani* by volatiles

Of the three pathogens, *R. solani* was most consistently suppressed by volatiles, with only one soil emitting volatiles that promoted *R. solani* growth (Figure 3.1A and 3.1C) by about 20% more than the soil-free control. The best multiple linear regression model determined for *R. solani* explained a proportion of 0.51 variation (Table 3.2), and was produced from the dataset of soil properties. While OM content and microbial biomass N were positively correlated with *R. solani* suppression rates (Figure 3.2A and 3.2C, respectively), pH was negatively correlated with growth suppression by volatiles (Figure 3.2B). A moderate correlation (r=0.51) was found between OM content and microbial biomass N, and OM content and pH (r=-0.52), but no relationship was found between microbial biomass N and pH. The decomposition of complex OM constituents may result in the release of VOCs (Isidorov and Jdanova, 2002) as intermediary compounds of decomposition processes (Dickschat *et al.*, 2005; Gray *et al.*, 2010). With regards to pH, while it may act as a direct determinant of a volatile's phase, the potential effects of this property so tightly link soil chemical and microbiological feedback that it is difficult to disentangle whether its effect is direct or interactive.

When considering the microbial community dataset, another significant model included the Shannon diversity index value, the fungal functional group litter saprophytes, and the taxonomic group *Acidobacteria* as significant model parameters (Table 3.2). *Acidobacteria* were present in each of the 50 soils, had about 1800-5000 reads after rarefication, and were negatively correlated with volatiles suppressing *R. solani* (Figure 3.2F). Abundance of *Acidobacteria* has been shown to positively correlate to VOC-based pathogen suppression in other experiments (Chapter 2). As the Shannon diversity index of the total microbial community increased, *R. solani* suppression decreased (Figure 3.2D). On the contrary, an increase in suppression was measured as litter saprophyte read numbers increased (Figure 3.2E).

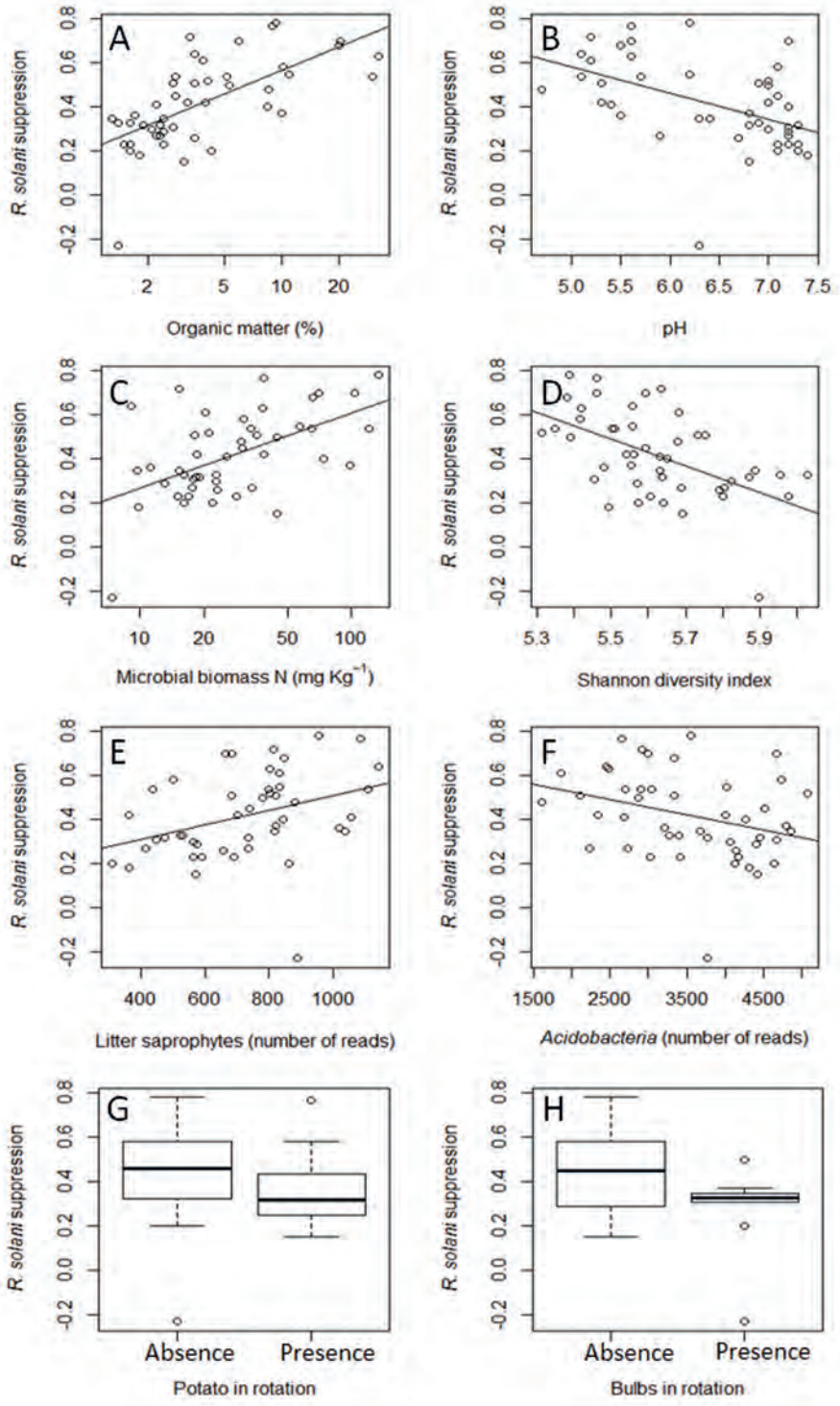

A
R. solani suppression
-0.2 0.0 0.2 0.4 0.6 0.8
2 5 10 20
Organic matter (%)
B
R. solani suppression
-0.2 0.0 0.2 0.4 0.6 0.8
5.0 5.5 6.0 6.5 7.0 7.5
pH
C
R. solani suppression
-0.2 0.0 0.2 0.4 0.6 0.8
10 20 50 100
Microbial biomass N (mg Kg^{-1})
D
R. solani suppression
-0.2 0.0 0.2 0.4 0.6 0.8
5.3 5.5 5.7 5.9
Shannon diversity index
E
R. solani suppression
-0.2 0.0 0.2 0.4 0.6 0.8
400 600 800 1000
Litter saprophytes (number of reads)
F
R. solani suppression
-0.2 0.0 0.2 0.4 0.6 0.8
1500 2500 3500 4500
Acidobacteria (number of reads)
G
R. solani suppression
-0.2 0.0 0.2 0.4 0.6 0.8
Absence Presence
Potato in rotation
H
R. solani suppression
-0.2 0.0 0.2 0.4 0.6 0.8
Absence Presence
Bulbs in rotation

Figure 3.2 Relationships between *in vitro* suppression of *Rhizoctonia solani* by soil volatiles and properties of 50 arable soils: A) Organic matter (% (log-scaled)), B) pH, C) microbial biomass N (mg kg^{-1} (log-scaled)), D) Shannon diversity index, E) *Acidobacteria* (Total OTU reads), F) Litter saprophytes (Total OTU reads), G) Potatoes in the crop rotation, and H) Bulbs in the crop rotation. Properties A-H were significant ($P<0.05$) model parameters determined by forward-step regression where suppression of *R. solani* by soil volatiles was the dependent variable. Suppression by volatiles was converted to the proportion of reduction of mycelial biomass in comparison to a soil-free control (control = 0 (no suppression), 1 = maximum suppression, <0 = stimulation).

With regards to the historical field management practices at each site, a lower (0.18) amount of variation could be accounted for by that model, although this was still significant ($P<0.05$) (Table 3.2). Field sites that included bulbs and/or potato in their crop rotation were most relevant in this model (Table 3.2) and appeared to slightly decrease *R. solani* suppression by volatiles (Figures 3.3G and 3.3H). Plant species are known to differentially alter soil microbial community composition (Berg and Smalla, 2009), which also applies to crops grown in long-term agricultural soils (Maul and Drinkwater, 2009) and may offer an explanation for this observation.

Table 3.2 Multiple linear regression models determined from soil properties, microbial community, and field history parameters measured from 50 soils; the dependent variable was *in vitro* suppression of *Rhizoctonia solani, Fusarium oxysporum,* or *Pythium intermedium* by soil volatiles. Model parameters presented explain significant ($P<0.05$) amounts of variation in volatile suppression rates. Parameters are derived from a reduced regression model after a forward selection procedure, which was only run on significant ($P<0.05$) models, and not non-significant (ns) models.

Pathogen	Dataset	Model type	R^2	Significant model parameters with respective partial R^2 (in brackets)
R. solani	Soil properties	linear	0.51	organic matter (0.40), pH (0.27), microbial biomass N (0.26)
	Microbial community	linear	0.44	Shannon diversity index (0.28), litter saprophytes (0.17), *Acidobacteria* (0.02)
	Field history	logistic	0.18	bulbs (0.10), potato (0.08)
F. oxysporum	Soil properties	linear	ns	
	Microbial community	linear	ns	
	Field history	logistic	0.08	reduced tillage (0.08)
P. intermedium	Soil properties	linear	0.16	S-total (0.18), microbial biomass N (0.08)
	Microbial community	linear	ns	
	Field history	logistic	0.39	solid manure (0.24), bulbs (0.09), corn (0.08)

Suppression of *F. oxysporum* by volatiles

Volatiles from the soils measured in this experiment were least effective in suppressing *F. oxysporum in vitro*. The only parameter from all three measured datasets which significantly contributed to a model explaining variation in suppression of *F. oxysporum* (R^2 = 0.08) was the practice of reduced tillage (Table 3.2). Field sites using reduced tillage (n=9) had slightly higher suppression levels of *F. oxysporum* than sites using conventional or no-till management (Figure 3.3). Although most soils were suppressive to some degree, the mean rate of suppression for *F. oxysporum* relative to the control (0.2) was much lower than for *R. solani* or *P. intermedium* (0.7 and 0.6, respectively). There were also nine soils from which volatiles promoted biomass production of this pathogen (Figure 3.1A and 3.1B), although the remainder were suppressive to at least some degree (<0.7).

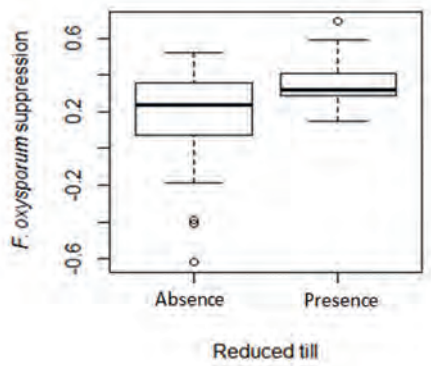


Figure 3.3 Relationship between *in vitro* suppression of *Fusarium oxysporum* by soil volatiles and the practice of reduced tillage in 50 arable soils. Reduced tillage was a significant ($P<0.05$) model parameter determined by forward-step regression where suppression of *F. oxysporum* by soil volatiles was the dependent variable. Suppression by volatiles was converted to the proportion of reduction of mycelial biomass in comparison to a soil-free control (control = 0 (no suppression), 1 = maximum suppression, <0 = stimulation).

Different *Fusarium* species have been reported to be more tolerant to VOCs, e.g. *F. solani* was resistant to bacterial VOCs (Kai *et al.*, 2007) and *F. oxysporum* has been shown to have only limited sensitivity to volatiles produced by antagonistic strains (Weisskopf and Bailly, 2013; Hunziker *et al.*, 2015). The relative tolerance of *Fusarium* to microbial volatiles and specifically bacterial volatiles may be one of the underlying reasons for the lack of edaphic variables corresponding with *F. oxysporum* volatile-mediated suppression. This may, however, be strain-specific or dependent on the volatile profile the fungus is exposed to, as strong inhibitory responses of *F. oxysporum* upon volatile exposure have also been found (Garbeva *et al.*, 2014b).

Suppression of *P. intermedium* by volatiles

Several oomycetes (*Pythium* spp. and *Phytophthora* spp.) have consistently been shown to be highly sensitive to microbial volatiles (Chapter 2; Hunziker *et al.*, 2015). From the soil properties dataset, two significant parameters contributed to a model predictive of suppression with a R^2 of 0.16: S-total and microbial biomass N (Table 3.2). When

correlated individually against suppression, relationships were slightly negative (Figure 3.4A and 3.4B, respectively), although variability between sites was high for both parameters. Sulphur-based volatile compounds like dimethyl disulphate (DMDS) and dimethyl trisulphate (DMTS) have been shown to be produced by soil microbes (Kai *et al.*, 2007), e.g. the major compound emitted (94%) by an *Achromobacter* isolate was DMDS (Minerdi *et al.*, 2011). Sulphur-containing compounds have been related to reduced *Pythium* infections in cucumber, both after direct addition of DMDS to soil or after incorporation of S-rich *Allium* crop residues (Arnault *et al.*, 2013). As production of S-containing VOCs from bacterial isolates seems dependent on nutrient availability (including S) in the growth medium (Garbeva *et al.*, 2014b), this would imply that S-availability in soils may also influence emission of S-containing VOCs.

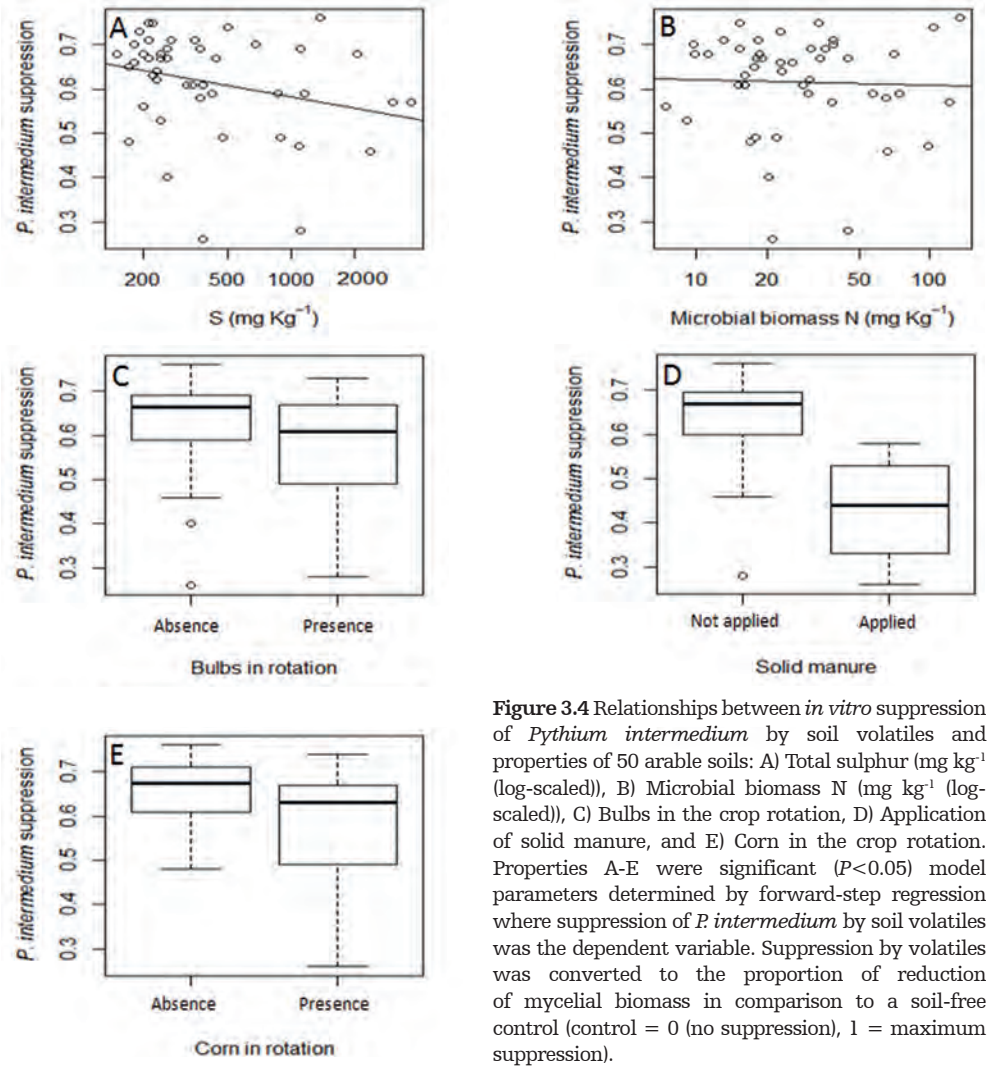


Figure 3.4 Relationships between *in vitro* suppression of *Pythium intermedium* by soil volatiles and properties of 50 arable soils: A) Total sulphur (mg kg^{-1} (log-scaled)), B) Microbial biomass N (mg kg^{-1} (log-scaled)), C) Bulbs in the crop rotation, D) Application of solid manure, and E) Corn in the crop rotation. Properties A-E were significant ($P<0.05$) model parameters determined by forward-step regression where suppression of *P. intermedium* by soil volatiles was the dependent variable. Suppression by volatiles was converted to the proportion of reduction of mycelial biomass in comparison to a soil-free control (control = 0 (no suppression), 1 = maximum suppression).

A model with a better fit (R^2 = 0.39) to the *P. intermedium* suppression rates was determined from the field history data. The presence of bulbs in the crop rotation (Figure 3.4C), the presence of corn in the crop rotation (Figure 3.4E), and the application of solid manure (Figure 3.4D) were all negatively correlated with the suppression rates of *P. intermedium*. The latter, however, was the most explanatory parameter in terms of its partial R^2 value (Table 3.2).

Multivariate analysis of overall pathogen suppression by volatiles

Multivariate analysis of the overall pathogen response to volatiles was prompted by the observation that there was no one soil or group of soils producing volatiles highly suppressive to all three pathogens (Figure 3.1). Furthermore, a lack of common significant variables among the three pathogens resulting from the univariate multiple linear regression (Table 3.2) warranted a multivariate approach to determine whether the combined response rate would have significant model parameters. Therefore, the parameters of all three datasets were combined using the overall response rate's ordination of the three pathogens as one dependent variable (Table 3.3). The ordination of each point on the RDA plot (Figure S3.4) was more spread out compared to clustering that had been seen in PCA plots (Figure S3.3), suggesting that while soils shared similar properties, this did not translate to commonalities in overall suppression of the pathogens.

Significant models were obtained from the soil properties and field history datasets, but not from the microbial community dataset (Table 3.3). Parameters in the most explanatory model of soil properties (R^2 = 0.24) were different from those identified in the univariate multiple linear regression. Instead, DOC, cumulative CO_2 production, and C:S ratio were the most significant of the 17 soil properties included. Removing each of these significant parameters did not drastically reduce the model's coefficient of variation, but it did indicate relationships between parameters beyond collinearity. Parameters removed from regression analysis are not necessarily replaced by parameters with the most similar RDA vectors (Figure S3.4A). For example, removing CO_2 as a model input parameter results in Na as a new significant parameter (Table 3.3).

Dissolved organic carbon is a known substrate for soil microorganisms (Haynes, 2005) chemical, and biological properties. However, changes in contents of organic carbon (C. Substrate quality impacts VOC composition, as resource variations have been shown to change the type, amount and suppressiveness of volatiles (Wheatley *et al.*, 1997; Ezra and Strobel, 2003; Gray *et al.*, 2010). Furthermore, resource availability and quality of DOC is reflected in microbial activity rates (CO_2) (Straathof *et al.*, 2014), which have been found to positively correlate to VOC production rates and microbial biomass in soil and litter samples (Leff and Fierer, 2008). Any combination of these effects may thus result in the significance of DOC for the overall pathogens suppression measured.

The best field history model for overall pathogen suppression yielded an R^2 of 0.18 where the presence of corn and/or potato in the crop rotation, along with the application (or lack thereof) of solid manure (Table 3.3). About 40% of fields had had corn and about 30% had had potatoes in their rotations in the last five years. Conversely, only five fields had received applications of solid manure, and are relative outliers in their RDA ordination (Figure S4B). When solid manure was removed from the model, potato and corn still explained significant amounts of variation in the volatile-mediated suppression of the three pathogens combined (Table 3.3).

Table 3.3 Multivariate regression models determined from soil properties, microbial community, and/or field history parameters measured from 50 soils; the dependent variable was overall *in vitro* suppression of *Rhizoctonia solani, Fusarium oxysporum,* and *Pythium intermedium* by soil volatiles, combined. Model parameters presented explain significant ($P<0.05$) amounts of variation in volatile suppression rates. Parameters are derived from a reduced regression model after a forward selection procedure, which was only run on significant ($P<0.05$) models, and not non-significant (ns) models. The first model listed for each dataset is the best model determined and, for comparison, significant variables were removed, the model rerun, and the alternative models listed below the best model.

Dataset	Removed variable	R^2	*P*	Significant model parameters with respective partial R^2 (in brackets)
Soil properties		0.24	<0.001	DOC[a] (0.08), CO_2[b] (0.08), C:S (0.05)
	DOC	0.21	0.029	CO_2 (0.08), S-total (0.09), OM[c] (0.07)
	C:S	0.19	0.016	DOC (0.08), CO_2 (0.08), C:N (0.02)
	CO_2	0.20	0.049	DOC (0.08), C:S (0.05), Na (0.03)
Microbial community		ns	ns	
Field History[d]		0.18	0.004	corn (0.09), potato (0.07), solid manure (0.07)
	corn	0.16	0.045	potato (0.07), solid manure (0.07), liquid manure (0.02)
	potato	0.14	0.070	corn (0.09), solid manure (0.07)
	solid manure	0.14	0.025	potato (0.07), corn (0.09)
All significant parameters from soil properties + microbial community + field history		0.27	<0.001	CO_2 (0.09), corn (0.05), DOC (0.07),
	CO_2	0.13	0.002	DOC (0.07), corn (0.05)
	corn	0.22	<0.001	CO_2 (0.09), DOC (0.07), C:S (0.04)
	DOC	0.25	<0.001	CO_2 (0.09), corn (0.05), S-total (0.07)

[a]DOC=dissolved organic carbon

[b]Cumulative microbial respiration after 14 weeks incubation

[c]Organic matter content (%)

[d]Indicated field crop or management practice was either present or absent in previous field seasons

By reducing the number of input parameters into the multivariate regression models (dataset "All significant parameters") for overall suppression, the highest coefficient of variation was achieved (R^2 = 0.27 (Table 3.3)). In this case, cumulative CO_2 production, DOC and corn were the significant model parameters. Removal of CO_2 resulted in the highest decrease of variation explained: 14% lower than when it is included in the model, and it was not replaced by any other variables (*i.e.* DOC and corn remained the only two significant parameters (Table 3.3)). The importance of this parameter further supports the notion that overall pathogen suppression by volatiles is driven by the consortium of soil microorganisms and that microbial activity is more relevant to volatile production than the absence or presence of particular microbial species.

Conclusions and future directions

We have presented here the first multi-soil survey of the effects of volatiles emitted from soils on *in vitro* biomass production of three different pathogens. The edaphic parameters we have identified as being significant in their effect on the combination of these pathogens are also properties that can be directly linked to microbial metabolic activity, either as a substrate source (DOC) or an activity indicator (CO_2). While this link has been previously postulated, our statistical confirmation of the relevance of these parameters should provide an impetus for future hypothesis-testing. The focus of this future experimental work should be on 1) more mechanistically exploring the role of microbial substrate, including DOC, and its influence on VOC production/quality, and then 2) determining management practices which effect substrate-driven microbial activity and thus, may enhance VOC-mediated pathogen suppression *in situ*. Previously it has been shown that VOCs from soil positively correlate to reduced disease incidence *in situ* (Chapter 2) which supports the potential for VOC-mediated suppression in agricultural fields. This implies VOC-mediated pathogen suppression could be an important component of general disease suppression in agricultural soil, and should be considered as a natural control mechanism for reducing crop-loss and moving towards sustainable agriculture.

Acknowledgements

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Table S3.1 Primers, adapters, PCR conditions and mastermix used for the 454 amplicon libraries of bacterial 16S, and fungal and oomycetal ITS rDNA fragment sequencing.

	Bacteria 16S	Fungal ITS	Oomycete ITS	References
Primer F	577F (5′-AYTGGGYDT AAAGNG-3′)	ITS86F (5′-GTGAATCAT CGAATCTTTGAA-3′)	OOMUP18Sc (5′-TGCGGAAGG ATCATTACCACAC-3′)	577F/926R: Rosenzweig, N., Tiedje, J.M., Quensen, J.F., Mang, Q., Hao, J.J., 2012. Microbial communities associated with potato
Primer R	926R (5′-CCGTCAATT CMTTTRAGT-3′)	ITS4 (5′-TCCTCCGCTTAT TGATATGC-3′)	ITS2-OOM (5′GCAGCGTTCTTC ATCGAATGT -3′)	common scab-suppressive soil determined by pyrosequencing analyses. Plant Dis. 96, 718e725.
sequencing adapter F	(5′-CCATCTCATCCCTGC GTGTCTCCGACTCAG-3′)	(5′-CCATCTCATCCCTGC GTGTCTCCGACTCAG-3′)	(5′-CATCTCATCCCTGCGTGTCT CCGACTCAG-3′)	ITS86F: White, T.J., Bruns, S., Lee, S., Taylor, J. (1990) Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. *PCR Protocols: A Guide to Methods and Applications.* pp. 315-322
sequencing adapter R	(5′-CCTATCCCCTGTGTG CCTTGGCAGTCTCAG-3′)	(5′-CCTATCCCCTGTGTG CCTTGGCAGTCTCAG-3′)	(5′-CTATCCCCTGTGTGCCTTGG CAGTCTCAG-3′)	ITS4: Turenne, C.Y., Sanche, S.E., Hoban, D.J., Karlowsky, J.A., Kabani A.M. (1999)Rapid Identification of Fungi by Using the ITS2 Genetic Region and an Automated Fluorescent Capillary Electrophoresis System. J Clin Microbiol 37: 1846-1851
PCR conditions	2 minutes 94°C, 30 cycles 94°C 45s, 59°C 45s, 72°C 60s, final annealing: 10 minutes 72°C	2 minutes 94°C, 30 cycles 94°C 45s, 59°C 45s, 72°C 60s, final annealing: 10 minutes 72°C	2 minutes 94°C, 30 cycles 94°C 45s, 57°C 45s, 72°C 60s, final annealing: 10 minutes 72°C	OOMUP18Sc, ITS2-OOM: Lievens, B., Hanssen, I.R.M., Vanachter, A.C.R.C., Cammue, B.P.A., Thomma, B.P.H.J. (2004) Root and Foot Rot on Tomato Caused by Phytophthora infestans Detected in Belgium. Plant Dis 88: 86-86. Lievens, B., Thomma, B.P. (2005b) Recent developments in pathogen detection arrays: implications for fungal plant pathogens and use in practice. Phytopathology 95: 1374-1380.
PCR mastermix	25 μl, 0.15 mM each DNTP, 0.5 μM of each primer, 1 unit Titanium taq DNA polymerase, 1x Titanum taq PCR buffer (Clontech Laboratories, Palo alto, CA, USA)	25 μl, 0.15 mM each DNTP, 0.5 μM of each primer, 1 unit Titanium taq DNA polymerase, 1x Titanum taq PCR buffer (Clontech Laboratories, Palo alto, CA, USA)	25 μl, 0.15 mM each DNTP, 0.5 μM of each primer, 1 unit Titanium taq DNA polymerase, 1x Titanum taq PCR buffer (Clontech Laboratories, Palo alto, CA, USA)	

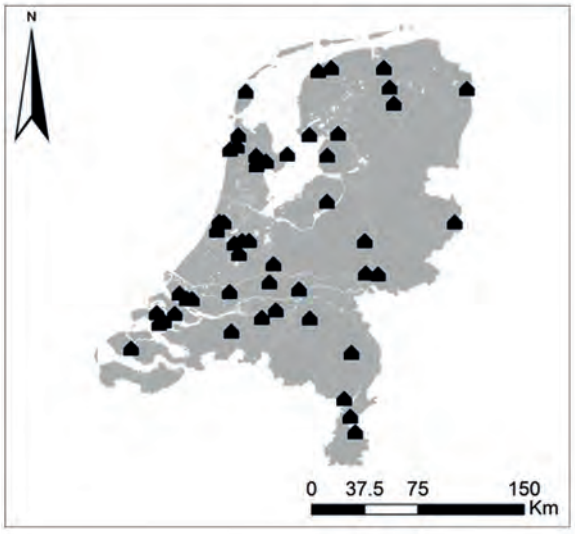


Figure S3.1
Locations in The Netherlands of 50 arable agricultural fields sampled.

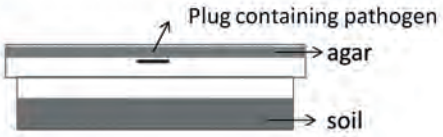


Figure S3.2
Illustration of the set-up used to determine the effect of soil-released volatiles on pathogen biomass. The bottom Petri-dish compartment contains fresh soil. The inner side of the lid compartment contains water yeast agar with a plug directly in the centre containing pathogen mycelium as inoculum. Para-film seals the lid to the bottom compartment.

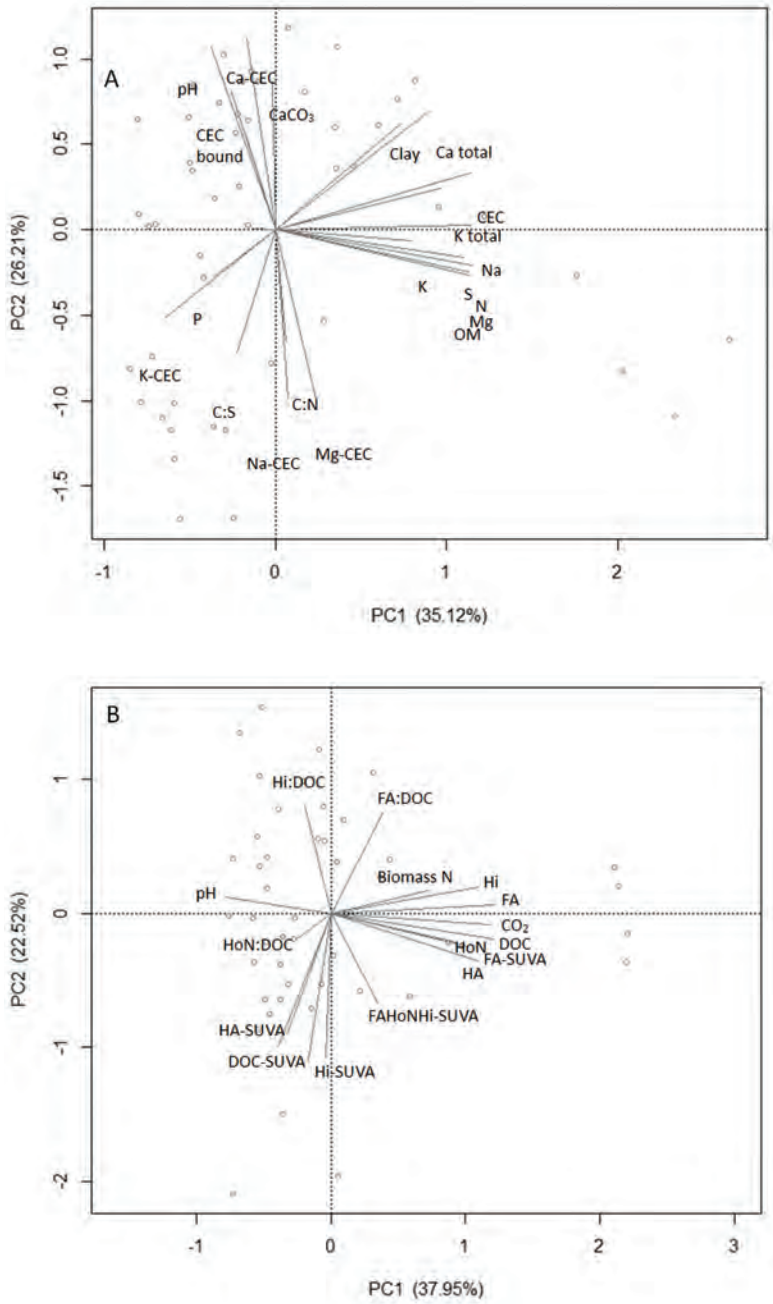


Figure S3.3 Principal component analysis of 50 soils' soil properties measured using A) routine near-infrared based or $CaCl_2$ extractions (OM=organic matter; CEC=cation exchange capacity). B) ultra-pure water extractions and dissolved organic carbon (DOC) fractionation (HA=humic acids; FA=fulvic acids; HoN=hydrophobic neutrals; Hi-hydrophilic compounds; SUVA=specific ultra violet absorption at 245 nm).

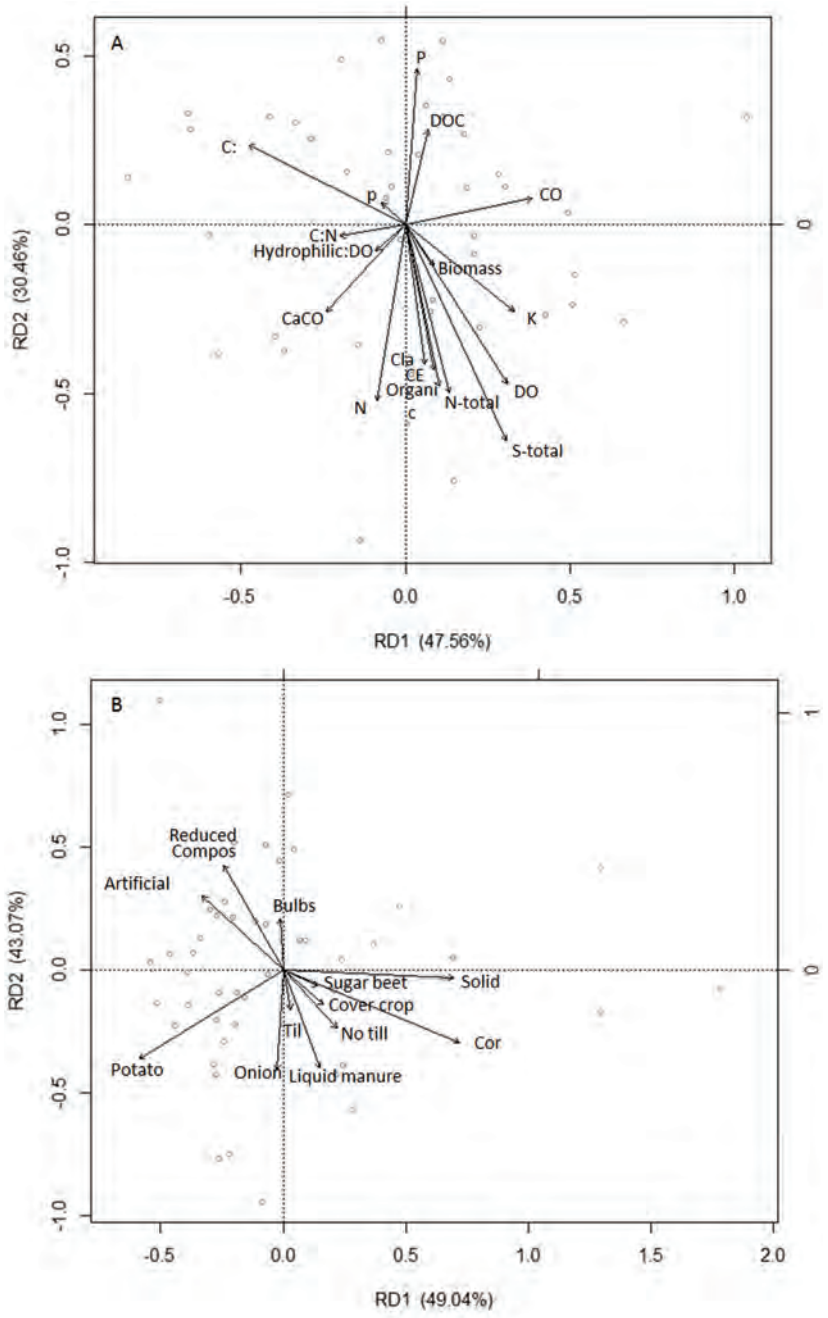


Figure S3.4 Redundancy analysis (RDA) plot of overall *in vitro* suppression of *Rhizoctonia solani, Fusarium oxysporum,* and *Pythium intermedium*(combined) by soil volatiles from 50 arable soils in relation to A) soil properties (DOC=dissolved organic carbon). B) field management practices.

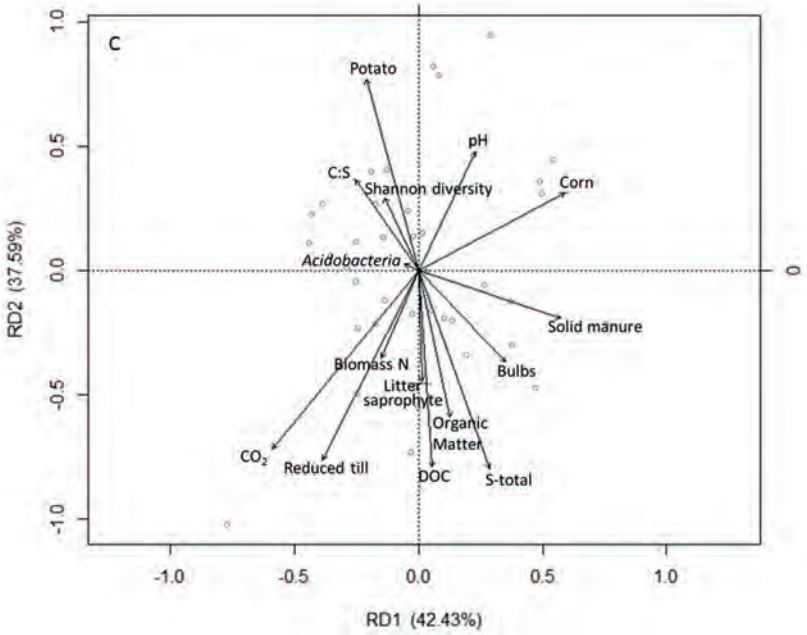
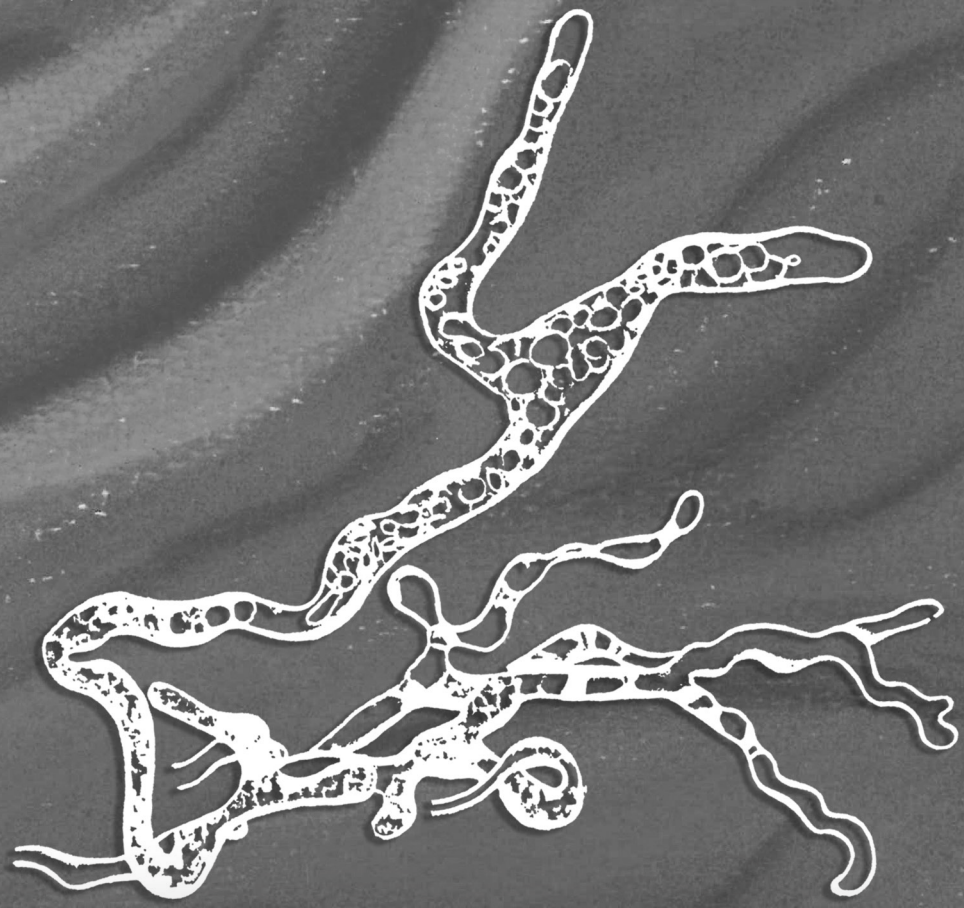


Figure S3.4 continued C) all significant ($P<0.05$) regression model parameters from soil properties, microbial community and field history datasets.

CHAPTER 4.

EXPLORING THE PHYTOPATHOGENIC SEEDBANK OF AGRICULTURAL SOILS



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Abstract

The pool of plant pathogenic propagules in soil, i.e. the so-called phytopathogenic seedbank, is a reservoir for future outbreaks of crop diseases. Next generation sequencing offers an unprecedented way to zoom in on the composition of this pathogenic reservoir. The aim of this study was to obtain insight in environmental factors that drive the composition of fungal and oomycetal plant pathogens present in soil. To this end, we assessed the alpha- and beta diversity of potential plant pathogenic fungi and oomycetes for 42 agricultural soils in the Netherlands and determined relationships with physico-chemical soil properties, crop and management history, spatial patterns and non-pathogenic microbial community composition. Our results indicate that the composition of pathogen propagules in soil is driven by pH, soil type, crop history, litter saprophytes and spatial patterns. The major driving factors differed for fungal and oomycetal pathogens as well for root- and shoot-infecting pathogens, suggesting interactions between environmental factors and pathogen traits like reproduction, survival and dispersal. This information appears to be of basic importance to identify risks for disease outbreaks and to suggest management strategies to prevent such outbreaks.

Introduction

As part of the total soil microbial community, soils harbor a reservoir of plant pathogenic fungi and oomycetes, forming the phytopathogenic seedbank (Gilbert, 2002; Oerke, 2006). In arable fields, with crop rotation and periods of fallow, survival and persistence of plant pathogens is crucial for their success. In the absence of living hosts, most pathogens can survive actively as saprotrophs or enter a dormant state in the form of resting propagules (Termorshuizen and Jeger, 2008; Lennon and Jones, 2011). This pool of surviving propagules, which we refer to as the pathogen seedbank, forms an important source of future disease outbreaks. Abiotic factors including pH, various nutrients, organic matter and clay content are not only major drivers of microbial community composition (Schutter *et al.*, 2001; Fierer and Jackson, 2006; Lauber *et al.*, 2009; Rousk *et al.*, 2010) but have also been shown to influence pathogen survival (Peng *et al.*, 1999; Mondal and Hyakumachi, 1998; Kühn *et al.*, 2009). However, little is known on the impact of environmental factors on abundance, diversity and species composition of the pathogenic seedbank. This lack of knowledge is hampering predictions on outbreaks of diseases.

The soil biotic community strongly influences the dynamics of pathogens (Garbeva *et al.*, 2006; Garbeva *et al.*, 2011; Pérez-Piqueres *et al.*, 2006). Competition for resources or even 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 (soil fungistasis), enhancing survival in unfavorable periods (Romine and Baker, 1972; Lockwood, 1977; Garbeva *et al.*, 2011; Lennon and Jones, 2011). Yet, propagule survival in soil is not unlimited as the propagules loose viability over time (Lockwood, 1977).

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 (Sturz *et al.*, 1997; Bockus and Shroyer, 1998). In addition, buildup of pathogens in agricultural soils is related to the availability of suitable host plants. Consequently, crop history can leave a pathogenic legacy in the soil (Bennett *et al.*, 2012). Root pathogens infect plants belowground and generally do not form airborne spores. Such pathogens have limited dispersal ability, whereas shoot-infecting pathogens often form airborne propagules, allowing 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 pathogenic seedbank.

For a long time, studies on distribution of pathogen propagules in agricultural soils had to rely on cultivation techniques, even though it was generally recognized that such methods had inherent biases, e.g. the restriction to only detect culturable microorganisms or the inability to detect propagules that remain in a dormant stage

(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 development of next generation sequencing technologies (Margulies *et al.*, 2005)2005 enables expanding the range of detection of potential pathogens in soils (Vettraino *et al.*, 2012)2012. The aim of our research was to get a comprehensive overview of the potential pathogens present in the fungal and oomycetal seedbank in agricultural soils using 454 amplicon pyrosequencing. Furthermore, we aimed to determine key environmental biotic and abiotic parameters that shape the seedbank.

Materials & Methods

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 (Figure 4.1). 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 (Table S4.1A,B). Sites were sampled in early spring 2013 (February-March), before the start of the growing season. Soil sampling (0-20 cm cores) was performed by BLGG AgroXpertus (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 about 3 kg soil sample per site. Upon arrival in the lab, samples were homogenized and split in equal subsamples that were used for (1) analysis of physico-chemical soil properties, (2) microbial community analysis. Regarding the first analysis, a broad set of soil physical and chemical parameters was determined by BLGG-AgroXpertus using standard procedures (Table S4.1). For the microbial analysis, two 1 g subsamples per site were taken for DNA extraction.

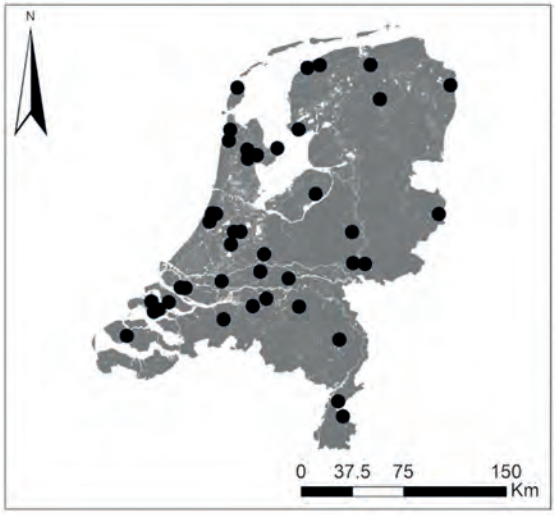

Figure 4.1 Map of the field locations in The Netherlands.

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

For each of the selected fields, genomic DNA was extracted in duplicate from 2x 0.5 g soil using the Mobio 96 well Powersoil® extraction kit (Mobio Laboratories Inc., Carlsbad, CA, USA). Subsequently, amplicon libraries were created using three PCR primer sets targeting part of the bacterial 16S ribosomal RNA (rRNA) genes, the fungal internal transcribed spacer (ITS) 2 region, and the oomycete ITS 1 region. Primer pairs used included 577F (5'-AYTGGGYDTAAAGNG-3') and 926R (5'-CCGTCAATTCMTTTRAGT-3') (Rosenzweig *et al*. 2012), ITS86F (5'-GTGAATCATCGAATCTTTGAA-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') (White *et al.*, 1990; Turenne *et al.*, 1999) and OOMUP18Sc (5'-TGCGGAAGGATCATTACCACAC-3') and ITS2-O (5'-CAGCGTTCTTCATCGATGT-3') (Lievens *et al.*, 2004; Lievens *et al.*, 2005b). All samples were assigned unique MID (Multiplex Identifier) barcode sequences according to the guidelines for 454 GS-FLX Titanium Lib-L sequencing (Table S4.2). 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 denaturing (45 s at 94 °C), annealing (45 s at 59 °C, except for OOMUP18Sc/ITS2-O for which the annealing temperature was 57°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 / purified 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 three 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).

Sequences obtained from the 454 pyrosequencing run were assigned to the appropriate sample (sequences from both replicates combined) based on barcode and primer sequences allowing zero discrepancies using Mothur (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 longer than 8 nucleotides were rejected, as were chimeric sequences detected by Uchime (Edgar *et al.*, 2011) implemented in Mothur. Sequences which passed all quality control procedures were used for

further analysis. In order to avoid bias from unequal sequencing depth when comparing different samples, all bacterial and fungal sequences were randomly rarefied to the minimum number of obtained high-quality sequences per sample (14789 for bacteria, 2745 for fungi). For the oomycetes, data were not rarefied due to the apparent absence or low number of obtained oomycete reads for several sites (average 720 reads, with a spreading from 2 to 2568 per field). Next, bacterial sequences were aligned to the Silva reference alignment (Pruesse *et al.*, 2007)2007, representing prealigned bacterial and archaeal 16S RNA gene sequences. Reads were also classified using a Bayesian classifier (Wang *et al.*, 2007)2007, and reads classified as mitochondrial or chloroplasts were removed. Fungal and oomycete sequences were clustered using the pairwise alignment algorithm CD-HIT-EST (Li and Godzik, 2006) v. 4.5.4 (parameters: word size 9, compare both strands, cluster sequences into most similar clusters instead of first cluster). Average neighbour clustering was done with a threshold of 97% similarity, a commonly used sequence similarity cut-off to define species-level OTUs. OTUs were assigned taxonomic identities, to the genus level where possible, based on BLAST (Altschul *et al.*, 1990) results of the OTU representative sequences using the NCBI nucleotidet database (Benson *et al.*, 2008), excluding uncultured/environmental entries. Taxonomic assignments were considered reliable when a ≥200 Blast score value was found (Lumini *et al.*, 2010)2010 and/or when more than 80% sequence similarity was found with sequences in GenBank. Sequences that did not meet these criteria were rejected from the dataset.

Data analysis

As the majority of soil phytopathogens are either fungi or oomycetes, fungal and oomycete datasets were screened manually to identify potential plant pathogens. The bacterial dataset was not included in this search. The identification was done based on extensive literature data available on fungal and oomycetal plant pathogens. When top hits revealed identical Blast scores for different species, among which at least one was a known pathogenic species, the OTU was considered potentially pathogenic. Furthermore, to assess the influence of life history strategies in the phytopathogenic seedbank, the OTUs representing potential fungal pathogens were classified on genus level in root- and shoot-infectors. OTUs from genera that can infect both roots and shoots were classified according to the Blast result on species level, which resulted in a few genera that are present in both the root- and shoot-infecting subset. An overview of the OTUs, representing potential plant pathogens that were found in this study, is presented in Table S4.4. Fungal dataset OTUs that could be accurately assigned to the genus level were classified into 8 potential functional groups (abuscular mycorrhiza, coprophiles, endophytes, fungal parasites, nematode parasites, wood saprophytes, litter saprophytes and other) based on literature screening. Similarly, the oomycete OTUs were classified into potential functional

groups (plant pathogens, animal/fish pathogens, hyperparasites, saphrophytes and other). Finally, all potential pathogenic OTUs (fungi and oomycetes) were combined in a so-called "pathogen subcommunity", whereas non-pathogenic OTUs (bacteria, fungi and oomycetes) were combined per site in a "non-pathogen subcommunity". Bacterial OTUs grouped at phylum level (phyla containing > 0.05% of the reads) were included in the non-pathogenic dataset in able to assess the relationship between bacterial community composition and pathogen communities. To correct for differences in the non-pathogenic community, the dataset was re-rarefied after splitting the data.

For each field, information on management and crop history was collected. An interview with the farmers was performed to document management practices, mainly focusing on tillage, fertilization, usage of a cover crop and crop rotation. To reduce the number of variables, results were grouped according to different management practices, i.e. different tillage practices were categorized into three groups, including deep ploughing, reduced tillage or no tillage. Similarly, fertilization was categorized as liquid manure, solid manure, compost application and/or artificial fertilizer.

All statistical analyses were performed in R (3.0.0) with the R packages vegan, spdep, PCNM, packfor, spacemakeR, ade4 and ape. Correlation between environmental variables was first examined using a principal component analysis (PCA). The results were used to make a sub selection of the most relevant variables and to reduce the number of input variables for redundancy analysis (RDA). To study pathogen and non-pathogen communities, both datasets were Hellinger-transformed to reduce effects of large abundances and zero values in the dataset (Legendre and Gallagher, 2001). The difference in pathogen community composition between sites (beta diversity) was studied by analyzing the pathogen community difference as a function of the environmental (abiotic), the field history data and the non-pathogenic community. Therefore, constrained-RDA analysis was performed, followed by permutation tests to test the contribution and significance of the selected abiotic variables on pathogen diversity. This was followed by a forward selection procedure with double stopping criterion, adjusted R^2 and $\alpha<0.05$ (Blanchet *et al.*, 2008) to create parsimonious models and identify the most relevant variables. Grouped field management data was dummy-coded and then used to explain pathogen community data using RDA and forward selection procedures. Next, these data were used in an RDA with forward selection to explain the variation in pathogen data by the non-pathogenic microbial community composition. Lastly, a Moran-Eigenvector Map (MEM) analysis was carried out to explore spatial patterns (and thereby dispersal limitation) of the pathogen community dissimilarity (Dray *et al.*, 2006). We modelled connectivity between sites using the geographical distances between sites. The spatial model used was based on the distance between the different sites: the larger the distance the less

connected two sites are assumed to be. Connectivity was determined by constructing different models ranging from a model in which each site was connected to at least one other and all sites being connected to all other sites. The model with the highest correct Akaike information criterion (AIC) value was selected and variables from this model were used for further analysis. Positive and significant MEM variables of each of the subsets of the pathogen community were used as the spatial model, describing spatial patterning of the data. These variables were subjected to forward selection and selected variables were used in a RDA model to explain pathogen beta diversity. Variation in the fungal, oomycete, shoot- and root-infecting pathogen communities was explained using the four parsimonious (forward selected) models (local abiotic conditions, field history, non-pathogen community and spatial patterning). The individual and combined effects of these four models was further elucidated by means of a variation partitioning. To study the alpha diversity and correlations with the significant factors in the RDA models, the Shannon index was calculated from the pathogen dataset and correlations were tested with Spearman's rank correlation coefficient. The latter was also used in combination with RDA/Anova to study the relationship of the environmental variables on all pathogens individually.

Results

Environmental parameters

The geographical spread (Figure 4.1) of the 42 arable agricultural fields sampled for this study ensured a wide variety of soil textures and management strategies. The selected sites included a variety of properties and crops, for example fields from organic-poor sandy dune areas, heavy river and marine clay fields, and former peatlands. This covered a broad range of soil textures with clay ranging from 1-45% clay and sand from 8-97%. The pH in the sites varied from 4.7 to 7.3, with a mean pH of 6.4. Organic matter content varied between 1 and 12% (mean value 4.1%) with the exception of 4 sites, representing former peat soils, having an organic matter content of 20-40% (Table S4.1).

Field and crop history

The, the sites did cover a broad range of crops and management practices. Corn (*Zea mays*, n =15) and wheat (*Triticum aestivum*, n =11) were the most grown crops in the year before sampling. Tillage regimes varied from intensive (deep ploughing) to reduced- or no-till. Liquid manure was the most common fertilizer, applied on 19 studied fields. Artificial fertilizers as single nutrient source were used in 13 fields. The remaining fields were fertilized by solid manure or compost, often in combination with artificial fertilizer (Table S4.1).

Fungal and oomycetal pathogens

From a total of 5,848 fungal OTUs, 169 OTUs from 65 genera harboring potential phytopathogenic species were identified (Table S4.3, S4.4). These included the well-known genera *Rhizoctonia*, *Fusarium* and *Verticillium*. OTUs representing potential fungal pathogens could be divided in two major groups: potential root pathogens or potential shoot pathogens. The total number of reads assigned to the pathogenic subcommunity, which combined the reads for all potential pathogenic OTU's , differed strongly among sites (Figure 4.2A). For most sites, potential shoot pathogens were more abundantly present than potential root pathogens. However, the average number of pathogenic genera per field was higher for root pathogens (Figure 4.2B), with an average of 8 genera encompassing root infecting species versus 5 shoot-infecting genera (Figure 4.2B). For oomycetes, in total 178 OTUs from 7 genera were detected (Table S4.3, S4.4) which represent potential pathogens. Genera harboring different potential oomycete pathogens contained mainly OTU's from the family Pythiales (i.e. the genera *Phytophthora* and *Pythium)* (Figure 4.2A, B).

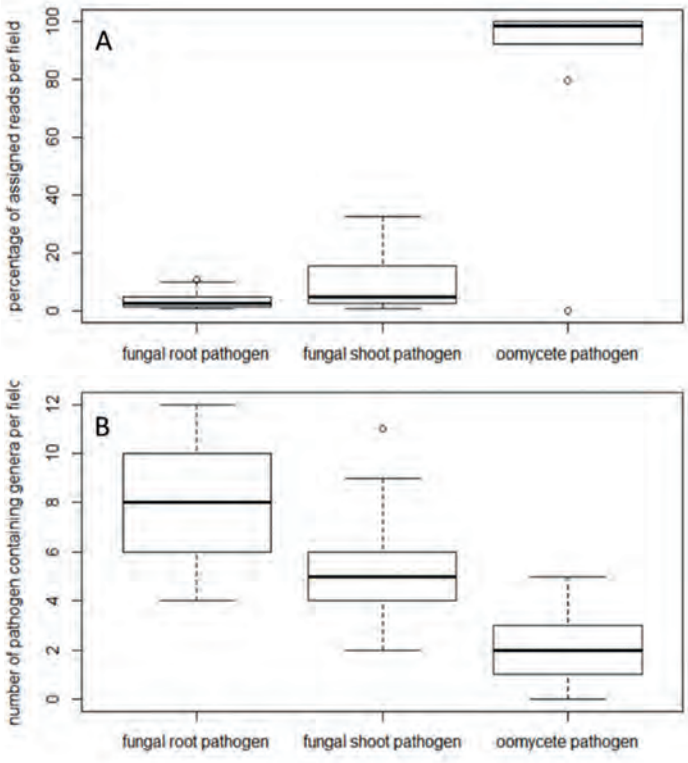

Figure 4.2 Boxplots of the relative abundance of potential pathogens and number of potential pathogenic genera **A** Percentage of total fungal or oomycetal reads assigned to the different groups of potential pathogens. **B** Average number of potential pathogenic genera per field.

Environmental variables influencing the potential fungal and oomycetal pathogenic communities

We were able to explain 37% of the beta diversity of the fungal potential pathogen community ($p<0.001$) using physico-chemical soil properties (PC), field history data (FH), non-pathogenic community (NP) and spatial descriptors. Physico-chemical variables alone were able to explain 19% ($p<0.001$); relevant variables were pH and the clay content of the soil (Figure 4.3A). Field history data were able to explain 14% ($p<0.002$); relevant variables were the presence of corn, bulbs and/or potato in the rotation and the presence of a cover crop (Figure 4.3A). Spatial patterning could explain 16% of the beta-diversity of the potential pathogen community ($p<0.001$). The non-pathogenic community explained 33% ($p<0.001$); relevant microbial groups were litter saprotrophic-, endophytic- and coprophilic fungi, and the bacterial phyla *Acidobacteria, Bacteroidetes, Spirochaetes, Chlorobi, Gemmatimonadetes* and *Planctomycetes* (Figure 4.3A, S4.5). Partitioning of the datasets revealed the individual contribution per dataset as well as the overlap between the sets. Plotting the significant variables in a RDA plot (Figure 4.4A) shows a strong separation between pH and the bacterial taxa *Acidobacteria, Spirochaetes, Chlorobi, Gemmatimonadetes*, *Bacteroidetes* on the one hand, and potential litter saprophytes and corn as previous crop pointing in the opposite direction (first RDA axis 45%).

In contrast to the observed relationship between potential fungal pathogen community composition and several biotic and abiotic variables, none of the RDAs performed on the potential pathogenic oomycete dataset revealed significant ordination (Figure 4.3B). Soil physical and chemical variables, crop history and management practices, the non-pathogenic community and spatial patterns could not explain the variance in oomycete pathogen diversity among sites.

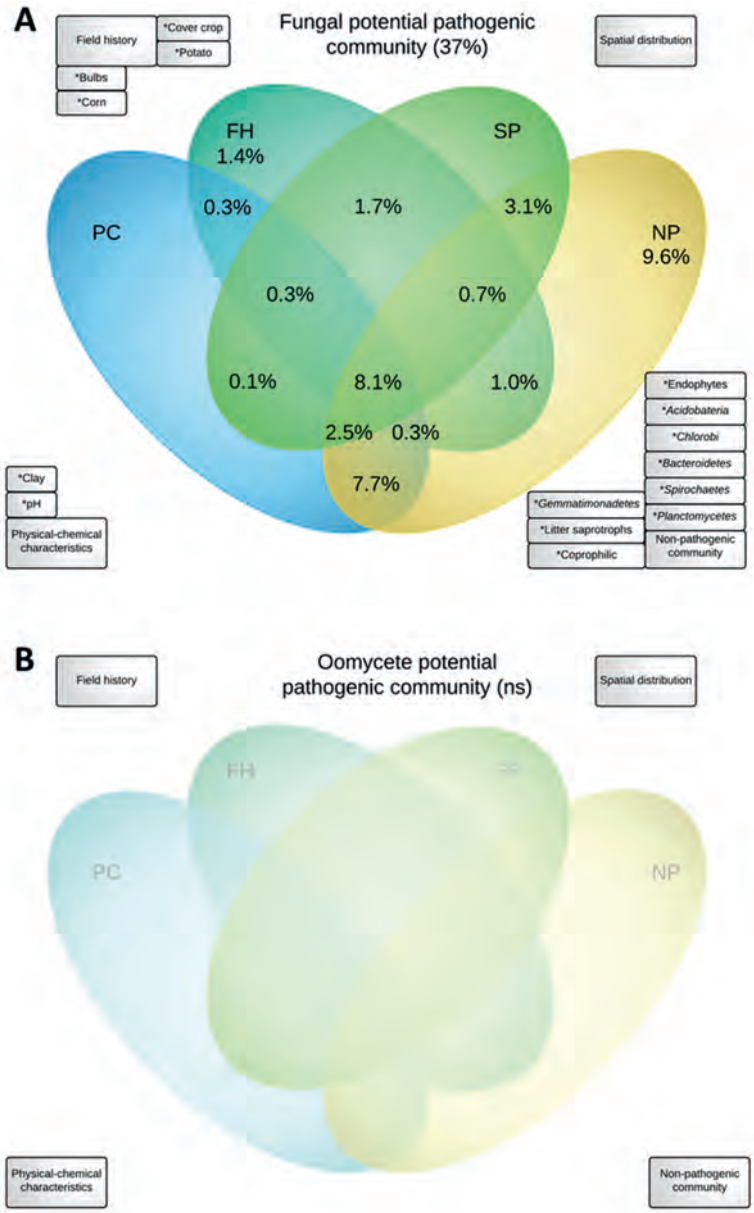

Figure 4.3 Venn diagram showing the contribution of each dataset to the beta diversity of the potential fungal and oomycetal plant pathogen community in agricultural soils. Explaining datasets are: Physico-chemical variables (PC), field history, including management practices and crop history (FH), spatial patterning (SP) and non-pathogenic microbial soil community (NP). Coloured ovals represent the contribution of each dataset, independent as well as overlapping contribution of datasets are given as percentages. Transparent ovals indicate no significant contribution of the indicated dataset. Significant contributing variables within each dataset are are indicated with (*). **A** Variation partitioning of the beta diversity of the total fungal potential pathogen community explaining 37% of the beta diversity. **B** Variation partitioning of the oomycetal potential pathogenic community (not significant).

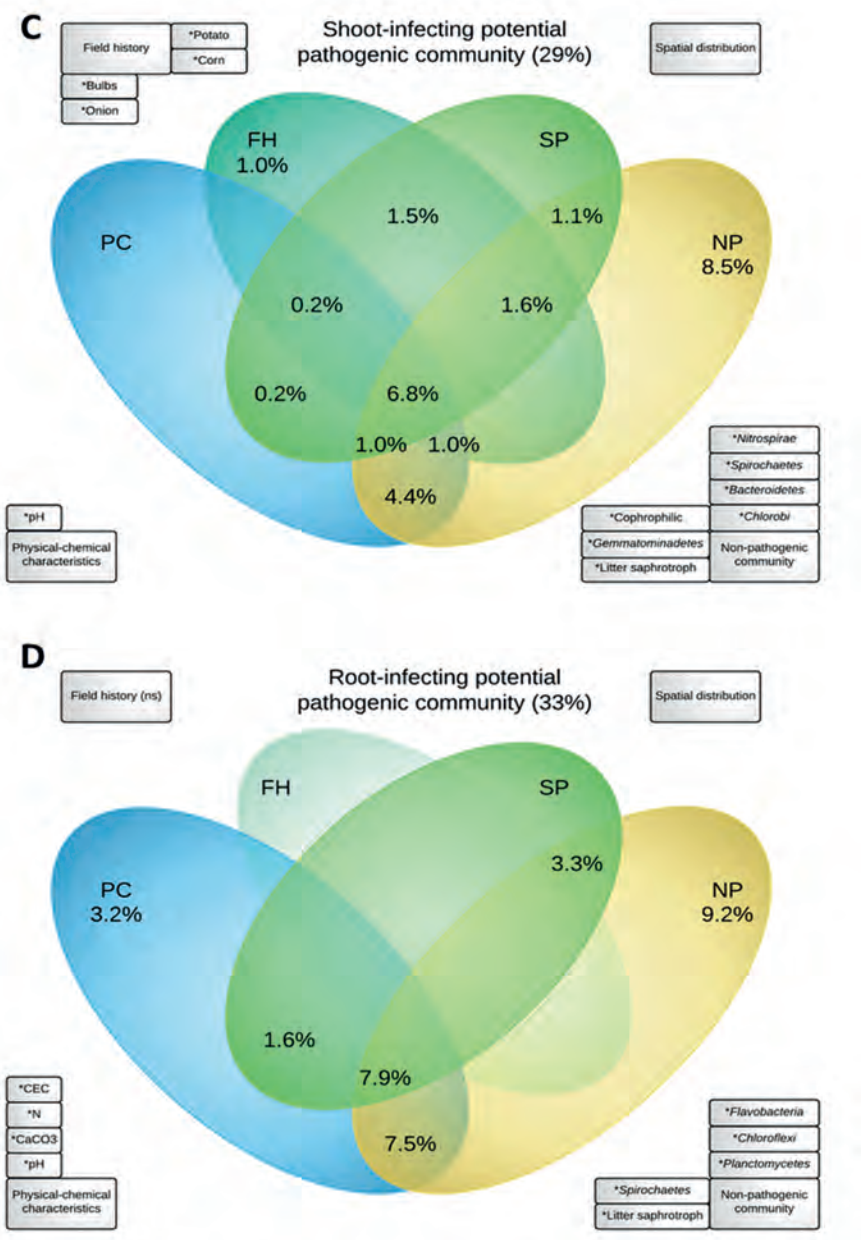

Figure 4.3 continued C Variation partitioning restricted to the potential shoot-infecting fungal pathogens explainingexplaining 29% of the beta diversity. **D** Variation partitioning if the beta diversity of the potential root-infecting fungalpathogens,fungal pathogensexplaining 33% of the beta diversity.

Environmental variables influencing the composition of potential root-and shoot infecting shoot-fungal pathogens

To take differences in life history strategies into account, the dataset of the potential pathogen community was split into potential root- and shoot-infecting pathogens. Due to lack of significant relations with any of the measured variables, oomycetes were not included in this analysis. We were able to explain 29% of the beta diversity of potential fungal shoot pathogens ($p<0.001$) using physico-chemical (PC), field history (FH), non-pathogenic community (NP) and spatial (SP) descriptors. Physico-chemical variables alone were able to explain 14% -($p<0.001$); the relevant descriptor was the pH of the soil (Figure 4.3C). Field history could explain 12% -($p<0.002$); significant variables were the presence of corn, bulbs, onions and/or potato in the crop rotation (Figure 4.3C). Spatial patterning could explain 12% ($p<0.001$). 24% of the beta-diversity of the potential shoot-infecting community could be explained by biotic variables ($p<0.001$); relevant microbial groups were *Nitrospira, Spirochaetes, Bacteroidetes, Chlorobi, Gemmatimonadetes* and coprophilic -and litter saphrophytic fungi (Figure 4.3C). A RDA plot including all variables from forward selection shows a strong separation on the first RDA axis (48%) between litter saprophytes and corn on the one hand and the other selected variables, including pH, onion and potato pointing in the opposite direction (Figure 4.4B).

We were able to explain 33% of the beta diversity of potential fungal root pathogens ($p<0.001$) using descriptors from physico-chemical properties, field history data, spatial patterns and biotic data. Physico-chemical properties were able to explain 20% ($p<0.001$); the relevant variables besides pH were cation exchange capacity (CEC), total nitrogen (N) and $CaCO_3$ (20%, $p<0.001$) (Figure 4.3D). Field history data could not explain the beta diversity of potential root-infecting pathogen community. Spatial patterning explained 13% ($p<0.001$). The non-pathogenic community could explain 28% -($p<0.001$); relevant microbial groups were litter saprophytes, *Spirochaetes, Flavobacteria Chloroflexi* and *Planctomycetes* (Figure 4.3D). A RDA plot including all variables from forward selection showed a strong separation on the first RDA axis (52%) between litter saprophytes, nitrogen and *Chloroflexi* on the one hand and the other selected variables, including pH and $CaCO_3$ in the opposite direction (Figure 4.4C).

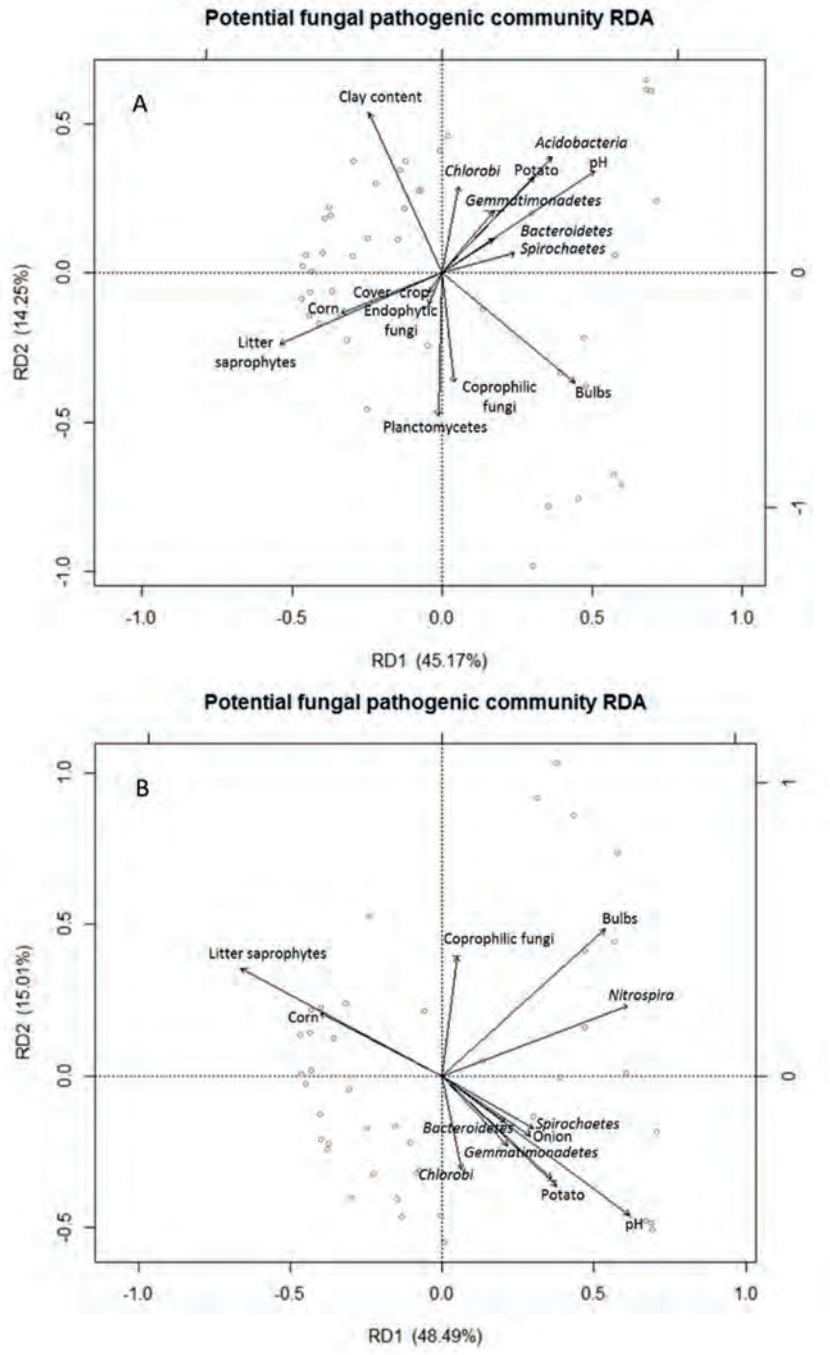

Figure 4.4 Redundancy analysis (RDA) ordination plot of -DNA sequences assigned to potential pathogens (Hellinger transformed) from different field sites as constrained factor. Arrows represent forward selected environmental variables significantly contributing to beta diversity of potential pathogens, dots represent the different field sites. CEC is the cation exchange capacity. **A** RDA analysis of the total potential fungal pathogen community. **B** RDA plot on potential shoot-infecting fungal pathogen community.

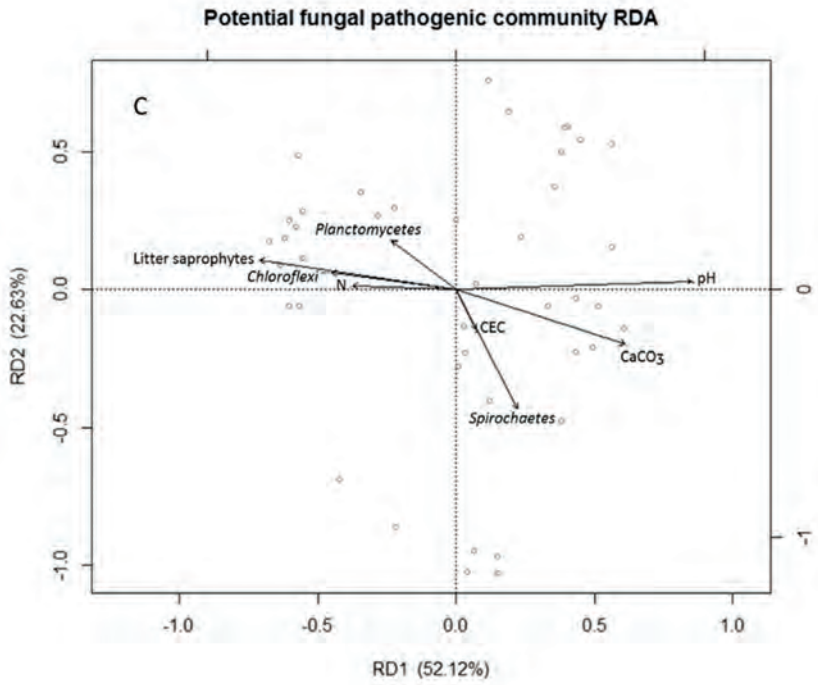

Figure 4.4 continued C RDA plot on potential root-infecting fungal pathogen community.

Alpha diversity and individual correlations of pathogens

In order to directly relate differences in pathogen diversity per site to measured soil-related properties the identified significant variables from the multivariate analysis were used in regression modelling. The correlations with these forward selected variables and the alpha-diversity (Shannon diversity index) of the potential pathogens from the different potential pathogen subcommunities (total fungal potential pathogens, shoot- and root-infecting potential pathogens) were tested (Figure 4.5, Table S4.6). Significant correlations were found with pH (the higher the pH the higher the Shannon index), the amount of litter saprophytes per field (the more litter saprophyte reads the lower the index) and the number of *Acidobacteria* reads (more reads, the higher the Shannon index, Figure 4.5A, B, C). Furthermore, the amount of nitrogen in the soil correlates with the Shannon index, the higher the amount of nitrogen per soil the lower the Shannon diversity of the potential root-infecting pathogens (Figure 4.5D, Table S4.6).

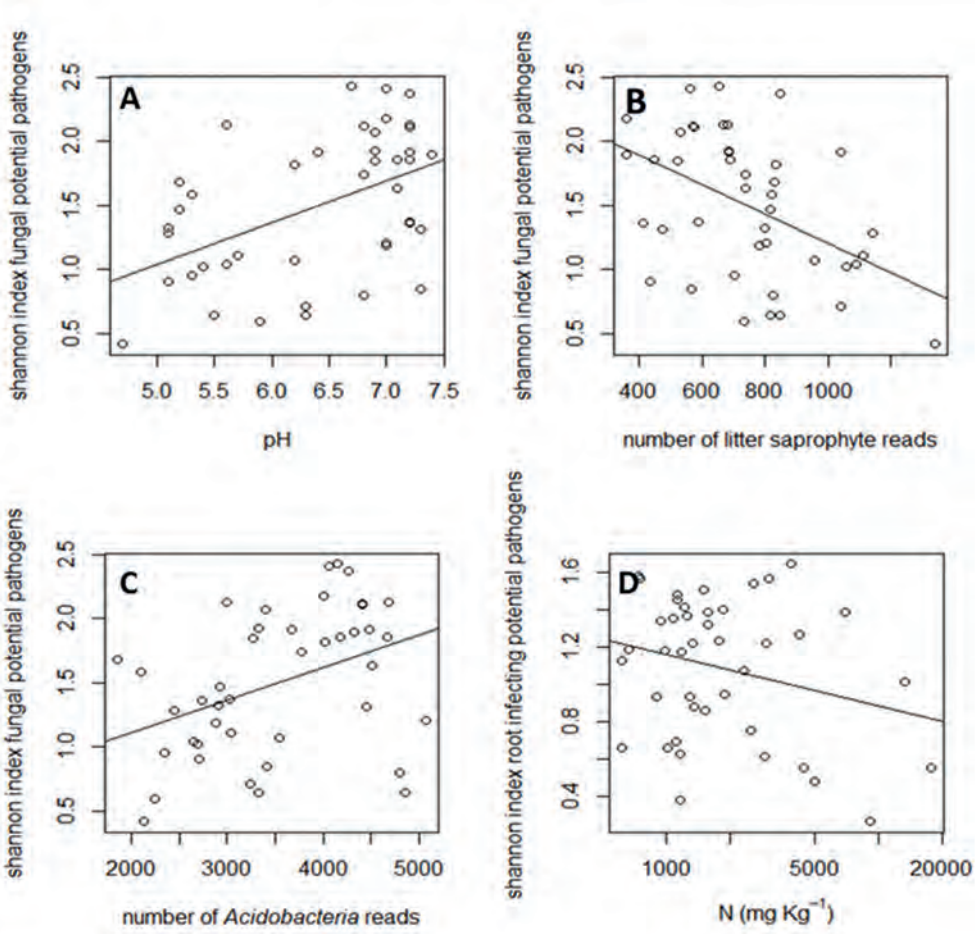

Figure 4.5 Significant correlations ($p < 0.01$) between Shannon diversity index of potential fungal pathogens and variables selected from the RDA analysis. **A** pH **B** Number of reads (after rarefication) assigned to saprophytic fungi. **C** Number of reads (after rarefication) assigned to *Acidobacteria.* **D**. Total nitrogen (N).

If, instead of the total community composition, individual genera harboring common pathogens are tested for their correlation with all environmental variables (including all datasets), significant relationships were found to be genus-dependent and show a diverse array of variables correlating with potential pathogen presence (Table 4.1). *Rhizoctonia, Spongospora* and *Ceratobasidium* show a significant correlation with the physico-chemical properties of the soil. Analysis of crop histories revealed mainly correlations with tuber and bulbous crops like potato, *Beta* species (beetroot and sugar beet) onion and flower bulbs. Bulbs and/or potato in the rotation correlated with *Olpidium* and *Pythium* (both crops, *Spongospora* and *Ceratobasidium* (flower bulbs), and *Verticillium* (potato).Significant correlations were also found for manure and compost application, correlating with *Mycophaerella* (liquid and solid

manure) *Albugo* (liquid manure and compost) and *Phytophthora* (liquid manure). Correlating non-pathogenic microbial groups are *Tenericutes (with Spongospora and Plectospaerella), Nitrospira (*with *Spongospora, Phytophtora* and *Pythium)*and *Gemmatimonadetes (*with *Mycospaerella),* and saprophytic and endophytic fungi (with *Plectospaerella* and *Phytophthora* respectively).

Discussion

Our study provides an overview of the pool of potential pathogens in a range of agricultural soils rpior to the growing season. With the aim to increase insights in the factors influencing the composition and diversity of this pool of plant pathogens a multiple field survey was performed including the collection of data for a wide range of agroecosystem characteristics and 454 pyrosequencing based microbial community analysis. From our inventory it becomes clear that it is hard to make generalizations of driving factors for the total (fungal and oomycetal) phytopathogenic seedbank. Strikingly, none of the variables tested in our study are significantly contributing to the variation in beta diversity of oomycetes. This could imply a reduced selective pressure of the environment on the distribution of oomycetes. In contrast to the observations for oomycetes, the among-site variation in potential fungal pathogen communities was significantly related to a range of field characteristics. Clay content and pH were indicated to be important abiotic factors explaining differences in composition of total potential fungal pathogens (including both root- and shoot-infecting pathogens), among sites. This is in accordance with earlier observations that pH and soil texture are important factors in structuring microbial communities (Garbeva *et al.*, 2004; Fierer and Jackson, 2006; Lauber *et al.*, 2009; Rousk *et al.*, 2010). However, when the fungi were grouped in potential shoot- and root- infecting pathogens variables explaining the differences in composition of pathogenic propagules between agricultural soils were not the same and only pH was maintained as common explaining abiotic factor. For the beta diversity of potential root infecting pathogens additional factors (total N, $CaCO_3$ and CEC) were contributing, which could point at a more important role of the physico-chemical environment for the composition of the seedbank of obligate soil-inhabiting pathogens than for that of shoot pathogens (Figure 4.3C,D).

Crop rotation, probably the oldest method used for controlling soil-borne diseases, is generally applied to reduce the build-up of the pathogenic seedbank. Indeed we found indications for a legacy of previous crops with respect to the composition and diversity of the pool of potential shoot-infecting plant pathogenic fungal propagules. The buildup of a pool of pathogens by crop monocultures and long term survival of highly resistant resting structures is a likely explanation for the observed importance of crop history (Bennett *et al.*, 2012; Termorshuizen, 2014). Although these legacies were not seen for potential root-infecting pathogens, some individual genera were

significantly correlated with crop history (Table 4.1). This may be ascribed to different host preferences of different pathogens (Agrios, 2005).

The importance of spatial variation for the distribution of soil biota has been shown by Ettema (2002) and spatial patterns are proposed to influence the evolution of both pathogen and host (Thrall and Burdon, 1997; Burdon and Thrall, 1999). We were expecting that dispersal limitation of pathogen propagules, and in particular of soil inhabiting root infecting pathogens, would result in spatial patterning of pathogen community composition independent of other variables.However, we did not see differences in the importance of spatial patterning between root- and shoot-infectors. In addition, spatial patterning of pathogen community composition was not independent of the other studied factors. This leads to the conclusion that spatial heterogeneity matters, but particularly in combination with the non-pathogenic community and the physico-chemical and field history conditions.

Several phyla of bacteria, as well different functional groups of non-pathogenic fungi, were indicated as independent explanatory factors for the composition of pathogenic fungal propagules in soils. This may indicate that interactions with indigenous microbes contribute to the among-site diversity of fungal phytopathogens. Interestingly, we observed that the amount of reads of fungal OTUs assigned to litter saprophytes is negatively correlated with the Shannon index of both potential root- and shoot-infecting pathogenic fungi. The dominant type of microbial interactions in soils is competition for organic nutrients and this is a major driver of fungistasis (Garbeva *et al.*, 2011; Lockwood, 1977). Fungistasis is one of the mechanisms of general suppression of diseases caused by plant pathogenic fungi and oomycetes (Garbeva *et al.*, 2011). Survival strategies of several root pathogens, (e.g. *Pythium* spp. and *Rhizoctonia* spp.) include a facultative saprophytic lifestyle on crop residues or other organic resources which can result in an increased inoculum density (Garrett, 1970; Chung *et al.*, 1988). The inoculum density can be reduced when a pathogen is outcompeted by other decomposer organisms (Chung *et al.*, 1988). The saprophytic fungi, which are major degraders of crop residues, show a significant explanatory value for fungal pathogen diversity. Competitive interaction during colonization of crop residues and organic matter by antagonistic saprotrophic fungi may reduce the survival of fungal pathogens. Hence, saprotrophic fungi may be involved in reducing the inoculum potential of plant pathogens of a given soil.

Microbial community composition has previously been shown to be related to the intensity of soil fungistasis (de Boer *et al.*, 2003). In the current study, the non-pathogenic community is significantly contributing to the variation in composition of potential pathogen communities, indicating that the presence and survival of potential pathogens in soil is affected by the composition of indigenous microbes (shown by the correlations of the Shannon index of the potential pathogens with litter saphrophytes and *Acidobacteria* in Figure 4.5). However, (potential) pathogen

presence does not automatically lead to crop disease, as inoculum potential and pathogen viability is affected by a range of factors, including disease suppression (Janvier *et al.*, 2007).

Bacterial phyla that contain known antagonists did not correlate significantly with the diversity indices of potential plant pathogens. For instance, *Proteobacteria,* including well-known antagonistic *Pseudomonas* species, did not correlate with the among-site diversity. However, this does not exclude the individual role of antagonistic bacteria in shaping the microbial community, as Mendes *et al.* (2011) showed that only by examining at species-level specific antagonistic strains were indicated to be related with disease suppression. The bacterial phyla that had a significant contribution to the among-site pathogen differences, e.g. *Nitrospira, Tenericutes* and *Gemmatimonadetes*, have not been examined so far in pathogen-microbe interactions. Obviously, the role of these phyla needs further investigation.

Despite the noise introduced by multivariate ordinations, due to biological variability and mechanistic uncertainties in techniques that lowers the total explained variation, our analysis could explain a large proportion (about 40%) of the beta-diversity of potential pathogens in agricultural soils. We also observed that the pathogenic seedbank harbored several unexpected taxa, such as pathogens of trees. Spores from fruiting bodies are known to be formed in massive amounts and may be deposited at a large distance, which may explain their occurrence in agricultural fields.

Our extensive inventory of the potential pathogenic community in agricultural soils highlights the relationships of the stock of propagules (seedbank) of potential pathogens with soil (microbial) properties. These relations may be life history-related, since we observed clear differences and even the absence of driving factors at taxonomical and functional level. Zooming in on the causal relationships of the variables related to pathogen community variability, for instance on the role of saprophytes on pathogen prevalence and survival, could lead to soil health indicators and evaluation of management practices. Further zooming into specific pathogens, crops or soil types may help setting up risk assessments and targeted to reduce the pathogenic seedbank in agricultural soils and ultimately reduce disease incidence in crops.

In conclusion, this DNA-based study provided an overview of the soils' phytopathogenic seedbank and identified environmental drivers of the composition and diversity of potential plant pathogenic pool in a range of agricultural soils. The major driving factors differed for fungal and oomycetal pathogens as well for root- and shoot-infecting pathogens, suggesting an importance contribution of pathogen traits like reproduction, survival and dispersal on the presence of pathogens. Knowledge on these driving factors of pathogen communities may improve risk assessment for disease outbreaks.

Table 4.1 Correlation between agroecosystem characteristics and relative abundance of selected genera of known fungal and oomycetal pathogens. Shoot/root indicates main infection zone, cor indicates the correlation coefficient, OM is organic matter content (%).

Mycospaerella (shoot)	***Rhizoctonia (root)***	***Spongospora (root)***	***Ceratobasidium (root)***	**Olpidium (root)**
liquid manure	beet	bulbs	bulbs	bulbs
($p<0,03$; R^2 0,19; cor 0,16)	($p<0,05$; R^2 0,12; cor -0,32)	($p<0,02$; R^2 0,22; cor 0,33)	($p<0,02$; R^2 0,24; cor 0,38)	($p<0,01$; R^2 0,32; cor 0,41)
solid manure	Total N	$CaCO_3$	pH	potato
($p<0,01$; R^2 0,26; cor 0,2)	(<p0,01; R^2 0,09; cor -0,43)	($p<0,01$; R^2 0,21; cor 0,36)	($p< 0,04$; R^2 0,11; cor 0,11)	($p<0,02$; R^2 0,12; cor 0,39)
Gemmatimonadetes	OM	*Nitrospira*		
($p<0,04$; R^2 0,18; cor -0,21)	($p<0,02$; R^2 0,09; cor -0,35)	($p<0,01$; R^2 0,50; cor 0,15)		
	C:N	*Tenericutes*		
	($p<0,04$; R^2 0,07; cor 0,18)	($p<0,03$; R^2 0,30; cor 0,16)		

Plectosphaerella (root)	***Verticillium (root)***	***Albugo***	***Phytophthora***	***Pythium***
litter saphrophytic fungi	potato	liquid manure	liquid manure	bulbs
($p<0,05$; R^2 0,21; $_{co}$r -0,21)	($p<0,05$; R^2 0,10; cor 0,48)	($p<0,02$; R^2 0,16; cor -0,24)	($p<0,05$; R^2 0,11; cor 0,17)	($p<0,01$; R^2 0,29; cor -0,39)
Tenericutes		compost	endophytic fungi	potato
($p<0,05$; R^2 0,23; cor 0,14)		($p<0,04$; R^2 0,16; cor-0,11)	($p<0,02$; R^2 0,21; cor 0,16)	($p<0,02$; R^2 0,17; cor -0,27)
		onion	*Nitrospira*	*Nitrospira*
		($p<0,03$; R^2 0,08; cor 0,10)	($p<0,01$; R^2 0,44; cor -0,27)	($p<0,03$; R^2 0,37; cor -0,43)

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Supplementary data

Table S4.1 Overview of physico-chemical properties, field history and site locations and the non-pathogenic microbial community of the sampled fields. **A** Physico-chemical properties per field.

	pH	Na	S-total	N total	C:N ratio	K total	CaCO3	Organic matter	Clay	CEC	C:S ratio	P total	Mg	P-available	K-available	Ca (CEC)	Mg (CEC)	K (CEC)	Na (CEC)	Ca avalaible	Ca stock	Silt	Sand
		mg kg^{-1} dry soil	mg kg^{-1} dry soil	mg kg^{-1} dry soil		mg kg^{-1} dry soil	% dry weight	% dry weight	% dry weight	mmol kg^{-1} dry soil		mg kg^{-1} dry soil	mg kg^{-1} dry soil	mg kg^{-1} dry soil	mg kg^{-1} dry soil							% dry weight	% dry weight
Site 1	6,9	18	370	1860	9	3,6	2	3,5	17	181	47	50	88	1,7	93	90	7,2	2	0,6	277	9410	34	44
Site 2	7,3	22	340	1160	10	5,7	6,5	2,4	7	102	35	161	91	7,1	127	85	8,9	5,6	0,8	171	5280	24	60
Site 3	7,2	22	500	3050	10	3,7	7,1	6	23	274	60	18	101	0,6	32	94	4,7	1,4	0,4	193	13745	52	12
Site 4	7,2	15	260	1110	11	6,6	2,1	2,3	9	123	45	61	60	2	137	86	8,3	5,4	0,8	25	6430	24	63
Site 5	5,6	17	680	2900	18	2,8	0,2	8,9	3	139	76	51	182	2,8	119	81	14	2	1	137	6440	13	75
Site 6	5,4	7	180	1170	10	3,5	0,2	2,2	11	76	62	30	103	1,6	61	60	18	4,6	0,7	25	2805	15	72
Site 7	6,2	40	1150	4250	13	6,3	0,3	10,9	42	422	47	42	143	1,5	79	91	6,6	1,5	0,4	377	19140	36	11
Site 8	5,9	17	170	990	11	5,9	0,2	2,2	11	88	64	36	100	3,1	128	81	12	6,7	0,6	220	4340	68	19
Site9	5,2	6	260	1350	14	2,1	0,2	3,3	1	46	74	51	69	5,6	66	71	15	4,6	0,9	27	2230	8	89
Site 10	5,6	38	2040	6910	15	8	1,7	20,4	17	398	50	38	289	1	216	84	8,9	2	0,6	268	14980	21	40
Site 11	6,2	19	1380	4980	9	12	0,9	9,4	39	314	34	56	189	1,9	324	77	14	3,8	0,7	40	12295	15	36
Site 12	5,3	6	240	1290	18	2,6	0,2	4	2	61	97	69	93	2,4	36	77	13	4,3	0,8	133	3110	17	77
Site 13	5,1	6	210	1130	14	2	0,2	2,8	1	41	77	52	81	4,6	85	67	13	4,9	1	221	1910	na	na
Site 14	6,3	37	350	2520	10	9,4	1,2	4,8	39	258	69	17	213	0,4	83	83	12	3,6	0,5	197	11740	47	8
Site 15	7,2	13	460	1570	9	8,6	3,5	2,9	20	165	31	59	77	2	139	87	7,6	5,2	0,7	1174	8730	31	43
Site 16	7,1	9	170	900	9	4,7	5,3	1,5	15	122	45	47	47	1,2	71	91	5,1	3,9	0,5	263	7250	26	52
Site 17	7,2	11	270	1250	10	5,6	4,9	2,4	16	149	44	43	56	1,1	80	89	6,5	3,8	0,5	122	8125	35	42
Site 18	7,2	14	870	3870	11	13,5	5,3	8,5	16	277	49	159	203	14,2	291	81	13	4,9	0,5	375	11725	35	35
Site 19	5,2	12	260	1880	12	3,6	0,2	3,9	7	84	87	17	122	0,5	71	69	22	4,3	1	27	3835	15	74
Site 20	5,1	78	3720	13270	11	12,7	1,4	30,2	24	419	41	45	560	1	207	67	19	3	1,4	16	11095	28	16
Site 21	4,7	65	6050	17700	12	10,1	2,3	41	15	554	34	29	395	0,8	189	72	12	1,8	0,8	71	14175	10	32
Site 22	5,7	28	370	2580	10	7,5	0,2	5,2	25	168	70	27	271	2,7	178	74	21	4,5	0,8	193	6670	18	52
Site 23	7	35	1110	2950	9	7,8	4,8	5,3	25	259	24	49	133	1,6	128	90	6,4	3	0,5	429	12505	28	37
Site 24	5,1	6	240	1210	17	2,1	0,2	3,5	2	42	85	53	29	1,7	36	72	16	5	0,7	162	2030	13	82
Site 25	6,3	18	220	1520	8	4,3	0,9	2,4	18	145	55	60	127	3,6	103	87	9,5	3	0,6	49	7710	39	40
Site 26	5,5	58	2380	9160	11	11,5	0,9	20,2	36	386	42	38	562	1	159	75	19	3	0,7	18	12515	21	22
Site 27	7	23	380	2320	9	6,9	2,7	4,1	32	260	54	66	146	2,3	95	87	10	2,7	0,4	328	12300	38	23
Site 28	6,4	8	180	670	10	3,6	0,2	1,3	2	41	36	45	39	5,4	83	68	22	8,8	1,2	146	2035	7	90
Site 29	7,4	16	200	1080	8	3,5	3,6	1,8	20	155	44	38	66	0,8	78	93	3,9	2,3	0,5	289	9505	37	38
Site 30	5,3	6	210	1020	15	2,1	0,2	2,7	2	33	75	42	101	1,2	91	61	26	6,4	1,2	28	1395	9	86
Site 31	7,3	12	210	750	15	3,4	6,1	2,3	8	111	55	64	76	2,6	98	93	4	3,1	0,5	220	6275	33	51
Site 32	7	14	350	1500	11	6,2	0,7	3,2	14	148	45	59	123	2,4	151	84	11	4,2	0,9	46	7145	29	53
Site 33	6,7	6	230	1560	11	3,9	0,2	3,5	10	96	76	68	93	3,6	157	83	12	4,1	0,6	115	4610	24	63
Site 34	6,8	44	1090	4410	11	5,4	1,7	10,1	20	303	46	22	237	0,4	105	89	8,8	1,8	0,6	377	13340	32	36
Site 35	7,2	15	890	1180	12	5,6	6,7	2,7	21	161	15	55	84	1,2	90	90	6	3,5	0,6	220	8855	35	35
Site 36	7,1	15	230	1330	11	6,5	6,2	2,8	18	161	61	71	82	1,5	98	87	7,9	4	0,6	25	8605	16	57
Site 37	7	13	180	1130	9	3,5	2,5	2,1	8	102	59	64	55	3,9	122	89	5,1	3,4	0,5	25	5560	12	75
Site 38	6,8	9	440	1770	9	4,8	2	3,1	45	358	35	15	126	0,6	42	78	11	1,3	0,6	207	16075	na	na
Site 39	6,8	6	240	940	10	2	0,2	1,9	1	56	39	56	55	4,2	72	87	8,9	3,6	0,7	29	3470	9	88
Site 40	6,9	6	220	740	11	2,4	0,3	1,6	1	54	36	41	45	3,2	48	86	8,3	4,4	0,7	87	3370	12	85
Site 41	6,9	6	190	620	11	1,9	2,2	1,4	1	44	37	30	18	1,7	42	85	9,3	4,3	0,9	436	2730	9	87
Site 42	7,2	6	320	620	13	2,5	6,4	1,6	1	57	25	58	32	4,7	69	86	4,9	4,4	0,7	1281	4240	4	88
method		CaCl2aCaCl2a	CaCl2a	NIRb	NIRb	NIRb	NIRb	NIRb	NIRb	NIRb	NIRb	NIRb	NIRb	CaCl2a	CaCl2	NIRb	NIRb	NIRb	NIRb	NIRb	NIRb	NIRb	NIRb
transformation for regression analysis		Log	log	log		log	sqrt	log	log	log		log											

a 0,01M CaCl2 extraction on soil dried at 40 C and ground (Houba *et al.*, 1999)b Near-infrared spectoscopy on soil dried at 40 C and ground (Malley *et al.*, 1999)

Table S4.1 B Overview of management practices, crop rotation and site locations.

	deep ploughing	no till	reduced till	artificial fertilizer	liquid manure	solid manure	compost	cover crop	onion in rotation	bulbs in rotation	potato in rotation	beet in rotation	corn in rotation	Latitude	Longitude
Site 1	1	0	0	1	0	0	0	0	1	0	1	1	0	53.26836	5.594041
Site 2	1	0	0	0	0	0	0	0	0	0	0	0	1	52.72872	4.941374
Site 3	0	1	0	0	1	0	0	0	0	0	0	0	1	51.69959	5.009675
Site 4	0	1	0	0	0	0	1	0	1	0	1	1	0	51.66924	4.010309
Site 5	1	0	0	0	1	0	0	1	0	0	1	1	1	53.05728	6.386659
Site 6	1	0	0	1	0	0	0	1	0	0	0	0	1	51.98090	6.074970
Site 7	1	0	0	1	0	0	0	0	0	0	0	1	0	53.14145	7.161042
Site 8	0	0	1	0	1	0	0	0	0	0	1	1	1	50.97235	5.955021
Site9	1	0	0	0	1	0	0	1	0	0	0	0	1	52.86227	5.497322
Site 10	0	1	0	1	0	0	0	0	0	0	0	0	0	52.30454	4.609889
Site 11	0	0	1	0	0	0	1	0	0	0	0	0	0	51.92551	5.085833
Site 12	0	1	0	0	1	0	0	0	0	0	1	0	1	51.47673	5.922771
Site 13	0	0	1	0	1	0	0	1	0	0	0	0	0	52.29536	7.001113
Site 14	0	1	0	1	0	0	0	0	0	0	0	1	1	51.74769	5.152791
Site 15	1	0	0	0	0	1	0	0	1	0	0	1	1	51.81364	4.292990
Site 16	0	1	0	0	0	1	0	0	0	0	1	1	0	51.71883	4.119227
Site 17	1	0	0	0	1	0	0	1	1	0	0	1	0	51.81497	4.240398
Site 18	0	0	1	1	0	0	0	0	0	0	0	0	0	52.04060	5.125986
Site 19	0	1	0	0	1	1	0	1	0	0	0	0	1	52.18534	6.067214
Site 20	0	0	1	0	0	0	1	0	0	0	0	0	1	52.18837	4.802480
Site 21	1	0	0	0	1	0	0	1	0	0	0	0	1	52.18785	4.874727
Site 22	1	0	0	0	0	1	0	0	0	0	0	1	1	51.97368	6.201275
Site 23	1	0	0	0	1	0	0	0	0	1	0	0	1	52.73832	5.266918
Site 24	1	0	0	0	1	0	0	1	0	0	0	0	1	51.61089	4.701128
Site 25	1	0	0	1	0	0	0	0	0	0	1	0	0	53.28662	5.729963
Site 26	0	0	1	0	1	0	0	1	0	0	0	0	1	52.10462	4.771814
Site 27	0	1	0	0	0	1	0	1	0	0	0	0	1	51.86183	4.677621
Site 28	0	1	0	1	0	0	0	1	0	1	0	1	0	53.13673	4.827415
Site 29	1	0	0	1	0	0	0	0	0	0	1	1	0	53.28507	6.287735
Site 30	1	0	0	0	1	0	0	0	0	0	0	0	1	51.69650	5.499270
Site 31	1	0	0	0	1	0	0	0	1	0	1	0	1	52.69329	5.044904
Site 32	0	0	1	1	0	0	0	1	0	0	1	0	0	51.65318	3.963712
Site 33	1	0	0	0	1	0	0	0	0	0	0	1	1	51.07073	5.906099
Site 34	0	1	0	0	1	0	0	0	0	1	0	0	1	52.66898	4.946282
Site 35	1	0	0	0	1	0	0	1	1	1	0	1	0	52.43758	5.680702
Site 36	1	0	0	1	0	0	0	0	0	0	1	1	0	51.49150	3.682580
Site 37	1	0	0	0	1	0	0	1	1	0	1	0	0	51.72202	3.935215
Site 38	1	0	0	1	0	0	0	1	0	0	1	0	1	51.88055	5.390049
Site 39	0	0	1	0	0	0	1	0	0	1	0	0	0	52.78763	4.739146
Site 40	1	0	0	0	0	0	1	1	1	1	0	0	1	52.85862	4.752689
Site 41	0	1	0	1	0	0	0	0	0	1	0	0	0	52.25156	4.540798
Site 42	0	0	1	0	0	0	1	0	0	0	0	0	0	52.30751	4.565290

Table S4.1 C Overview of the non-pathogenic bacterial and fungal communities per site.

	abuscular mycorrhizal		coprophile		endophyte		wood saphrophyte		fungal parasite		nematode parasite		litter saphrophyte		other fungi		*Acidobacteria*		*Bacteroidetes*		*Firmicutes*		*Actinobacteria*		*Chlorobi*		*Chloroflexi*		*Cyanobacteria*		*Flavobacteriales*		*Gemmatimonadetes*		*Ignavibacteriae*		*Nitrospira*		*Planctomycetes*		*Proteobacteria*		*Spirochaetes*		*Tenericutes*		*Verrucomicrobia*	
	no. of reads		no. of reads		no. of reads		no. of reads		no. of reads		no. of reads		no. of reads		no. of reads		no. of reads		no. of reads		no. of reads		no. of reads		no. of reads		no. of reads		no. of reads		no. of reads		no. of reads		no. of reads		no. of reads		no. of reads		no. of reads		no. of reads		no. of reads		no. of reads		no. of reads	
Site 1	115	88	8	32	1	9	682	47	3333	1012	807	7	928	7	150	35	8	1327	3	66	210	4179	4	6	185
Site 2	374	48	7	17	87	39	564	45	3421	760	382	4	374	4	164	9	17	1236	15	81	240	5341	31	2	204
Site 3	177	115	10	68	27	13	665	100	4679	587	683	14	496	14	230	18	11	1305	23	40	152	4002	1	0	167
Site 4	232	128	20	61	6	12	412	192	2732	1445	727	16	402	16	112	7	12	777	23	43	202	6113	0	1	203
Site 5	65	92	5	38	17	33	1088	37	2652	622	677	7	416	7	277	9	6	1116	6	58	230	3801	2	42	155
Site 6	46	106	5	26	3	28	1056	32	2691	453	678	14	496	14	251	15	12	1123	66	45	232	4175	0	11	99
Site 7	244	38	49	25	6	29	832	31	4016	751	306	5	1030	5	188	65	3	1369	37	39	179	3711	9	1	293
Site 8	67	85	28	36	2	36	734	121	2240	621	492	17	352	17	249	28	0	1339	14	45	271	5008	16	6	255
Site9	64	167	9	20	8	13	814	11	2921	606	839	0	234	0	262	6	0	1079	4	108	282	3997	7	12	256
Site 10	46	70	19	29	1	48	681	80	3001	428	387	5	807	5	273	18	14	675	9	74	241	5500	3	17	116
Site 11	35	125	7	48	1	15	957	54	3550	704	385	30	337	30	171	15	2	835	33	36	186	4245	2	12	92
Site 12	86	167	10	53	6	24	703	37	2350	588	707	6	928	6	215	27	2	1392	6	49	242	4936	1	6	132
Site 13	42	288	14	65	1	26	798	56	2905	255	735	0	509	0	231	18	0	1496	3	61	238	4987	2	27	230
Site 14	250	160	22	31	0	10	1039	16	3238	598	584	4	574	4	176	67	1	1579	16	66	174	3647	11	32	220
Site 15	274	110	14	35	28	23	684	126	4487	813	641	13	325	13	157	5	11	1064	21	72	195	4082	1	0	196
Site 16	326	139	9	61	9	34	690	39	4179	717	684	6	503	6	220	16	20	1383	70	71	211	3946	14	6	139
Site 17	280	79	5	41	6	14	573	23	4414	656	796	13	356	13	226	6	38	1790	64	40	223	3495	4	1	192
Site 18	101	138	25	45	1	33	845	60	4270	558	431	8	870	8	182	10	5	693	19	43	218	4542	3	7	149
Site 19	112	100	14	38	7	103	831	78	1856	447	1059	8	326	8	305	12	2	775	17	27	306	4174	0	12	158
Site 20	30	179	2	34	5	21	435	45	2707	846	191	6	719	6	219	20	1	1287	15	27	180	4201	0	2	312
Site 21	8	54	2	42	3	10	1341	25	2130	1085	217	0	192	0	493	9	5	1450	15	24	379	3686	2	9	62
Site 22	502	62	10	24	72	4	1110	22	3034	567	625	3	456	3	220	16	0	1312	23	43	182	4207	2	20	116
Site 23	27	112	47	82	3	7	780	99	2873	3420	433	29	662	29	141	7	3	875	14	32	222	4184	2	1	197

	abuscular mycorrhizal	coprophile	endophyte	wood saphrophyte	fungal parasite	nematode parasite	litter saphrophyte	other fungi	*Acidobacteria*	*Bacteroidetes*	*Firmicutes*	*Actinobacteria*	*Chlorobi*	*Chloroflexi*	*Cyanobacteria*	*Flavobacteriales*	*Gemmatimonadetes*	*Ignavibacteriae*	*Nitrospira*	*Planctomycetes*	*Proteobacteria*	*Spirochaetes*	*Tenericutes*	*Verrucomicrobia*
	no. of reads	no. of reads	no. of reads	no. of reads	no. of reads	no. of reads	no. of reads	no. of reads	no. of reads	no. of reads	no. of reads	no. of reads	no. of reads	no. of reads	no. of reads	no. of reads	no. of reads	no. of reads	no. of reads	no. of reads	no. of reads	no. of reads	no. of reads	no. of reads
Site 24	56	150	28	42	12	30	1144	30	2451	634	491	374	0	368	9	0	1252	4	67	250	3498	12	3	194
Site 25	104	22	0	36	28	3	816	42	4856	912	640	550	3	170	11	10	1178	20	55	177	3755	12	0	255
Site 26	127	198	10	36	12	27	847	34	3334	856	424	650	0	169	15	19	1073	12	17	218	3662	0	15	124
Site 27	90	312	18	88	17	51	802	50	5079	742	398	331	5	189	89	13	2212	14	34	144	2790	22	0	262
Site 28	34	62	43	38	6	19	1039	44	3681	678	550	542	3	254	8	7	1150	18	132	315	4383	11	1	164
Site 29	107	97	7	410	17	36	359	28	4320	1488	786	315	24	192	7	14	1428	13	58	164	3635	11	1	243
Site 30	22	342	15	43	15	44	820	54	2102	1182	627	355	1	235	3	0	1189	8	45	212	4888	8	2	133
Site 31	142	120	17	54	30	36	475	19	4448	721	674	252	6	243	7	12	1416	20	125	197	3889	5	5	183
Site 32	46	132	11	823	1	74	359	86	4003	2237	488	372	4	194	14	16	1010	40	46	158	3490	2	3	457
Site 33	149	130	5	30	7	8	655	59	4149	1297	319	283	3	130	37	11	1281	35	51	248	4177	16	12	131
Site 34	83	147	3	77	4	9	823	47	4800	555	483	344	20	145	27	0	1142	15	60	152	4084	0	19	121
Site 35	359	41	4	48	0	14	447	42	4675	679	442	225	6	228	7	26	1826	67	129	146	3808	7	5	179
Site 36	253	39	4	31	39	6	737	17	4518	1042	811	390	17	246	3	14	1281	46	41	154	3910	8	1	140
Site 37	167	223	9	37	5	5	563	58	4069	818	589	560	5	223	8	23	1274	54	64	232	4134	2	2	234
Site 38	94	104	6	252	8	10	569	38	4416	545	725	654	20	156	12	8	1147	22	58	183	4254	2	3	176
Site 39	76	128	3	63	11	23	735	42	3771	769	544	470	7	194	78	11	1658	16	78	276	4270	13	3	207
Site 40	90	257	46	61	5	38	528	31	3397	915	616	619	3	185	27	6	1343	13	93	278	4461	13	18	152
Site 41	235	189	12	81	0	79	522	293	3267	975	404	352	2	226	1	10	932	17	102	371	5220	15	6	146
Site 42	133	107	9	65	6	71	588	169	3031	761	574	525	1	229	10	5	1364	18	94	411	4715	21	11	122

Table S4.2 Used primer combinations, fusion primers and MIDs.

A) Primers combinations used in this study

Primer combination	Target region	Primer name (direction)	Reference
577F/926R	16S V4/V5	515F (Forward)	Rosenzweig et al (2012)
		926R (Reverse)	Rosenzweig et al (2012)
ITS86F/ITS4	ITS-2	ITS86F (Forward)	Turenne *et al.* (1999)
		ITS4 (Reverse)	White *et al.* (1990)
OOMUP18Sc/ITS2-O	ITS-1	OOMUP18Sc (Forward)	Lievens et al (2004)
		ITS2-O (Reverse)	Lievens et al (2005b)

B) Fusion primer component sequences and structure[a]

454 GS-FLX Lib-L Adaptor Sequences

Application	Adapter	Sequence (5′-3′)
Forward Fusion Primers	Adaptor "A"	CCATCTCATCCCTGCGTGTCTCCGACTCAG
Reverse Fusion Primers	Adapter "B"	CCTATCCCCTGTGTGCCTTGGCAGTCTCAG

Target Specific Primer Sequences

Combination	Target	Primer	Sequence (5′-3′)	Direction
ITS86F/ITS4	ITS-2	ITS86F	GTGAATCATCGAATCTTTGAA	Forward
		ITS4	TCCTCCGCTTATTGATATGC	Reverse
515F/926R	16S	515F	AYTGGGYDTAAAGNG	Forward
		926R	CCGTCAATTCMTTTRAGT	Reverse
OOMUP18Sc/ITS2-O	ITS-1	OOMUP18Sc	TGCGGAAGGATCATTACCACAC	Forward
		ITS2-O	CAGCGTTCTTCATCGATGT	Reverse

Multiplex Identifier Sequences (MIDs)[b]

MID-3	AGACGCACTC	MID-28	ACTACTATGT	MID-50	ACTAGCAGTA	MID-72	TGTCACACGA	MID-94	CGTATGCGAC
MID-4	AGCACTGTAG	MID-29	ACTGTACAGT	MID-51	AGCTCACGTA	MID-73	TGTCGTCGCA	MID-95	CGTCGATCTC
MID-5	ATCAGACACG	MID-30	AGACTATACT	MID-52	AGTATACATA	MID-74	ACACATACGC	MID-96	CTACGACTGC
MID-7	CGTGTCTCTA	MID-31	AGCGTCGTCT	MID-53	AGTCGAGAGA	MID-75	ACAGTCGTGC	MID-97	CTAGTCACTC
MID-8	CTCGCGTGTC	MID-32	AGTACGCTAT	MID-54	AGTGCTACGA	MID-76	ACATGACGAC	MID-98	CTCTACGCTC
MID-10	TCTCTATGCG	MID-33	ATAGAGTACT	MID-55	CGATCGTATA	MID-77	ACGACAGCTC	MID-99	CTGTACATAC
MID-11	TGATACGTCT	MID-34	CACGCTACGT	MID-56	CGCAGTACGA	MID-78	ACGTCTCATC	MID-100	TAGACTGCAC
MID-13	CATAGTAGTG	MID-35	CAGTAGACGT	MID-57	CGCGTATACA	MID-79	ACTCATCTAC	MID-101	TAGCGCGCGC
MID-14	CGAGAGATAC	MID-36	CGACGTGACT	MID-58	CGTACAGTCA	MID-80	ACTCGCGCAC	MID-102	TAGCTCTATC
MID-15	ATACGACGTA	MID-37	TACACACACT	MID-59	CGTACTCAGA	MID-81	AGAGCGTCAC	MID-103	TATAGACATC
MID-16	TCACGTACTA	MID-38	TACACGTGAT	MID-60	CTACGCTCTA	MID-82	AGCGACTAGC	MID-104	TATGATACGC
MID-17	CGTCTAGTAC	MID-39	TACAGATCGT	MID-61	CTATAGCGTA	MID-83	AGTAGTGATC	MID-105	TCACTCATAC
MID-18	TCTACGTAGC	MID-40	TACGCTGTCT	MID-62	TACGTCATCA	MID-84	AGTGACACAC	MID-106	TCATCGAGTC
MID-19	TGTACTACTC	MID-41	TAGTGTAGAT	MID-63	TAGTCGCATA	MID-85	AGTGTATGTC	MID-107	TCGAGCTCTC
MID-20	ACGACTACAG	MID-42	TCGATCACGT	MID-64	TATATATACA	MID-86	ATAGATAGAC	MID-108	TCGCAGACAC
MID-21	CGTAGACTAG	MID-43	TCGCACTAGT	MID-65	TATGCTAGTA	MID-87	ATATAGTCGC	MID-109	TCTGTCTCGC
MID-22	TACGAGTATG	MID-44	TCTAGCGACT	MID-66	TCACGCGAGA	MID-88	ATCTACTGAC	MID-110	TGAGTGACGC
MID-23	TACTCTCGTG	MID-45	TCTATACTAT	MID-67	TCGATAGTGA	MID-89	CACGTAGATC	MID-111	TGATGTGTAC
MID-24	TAGAGACGAG	MID-46	TGACGTATGT	MID-68	TCGCTGCGTA	MID-90	CACGTGTCGC	MID-112	TGCTATAGAC
MID-25	TCGTCGCTCG	MID-47	TGTGAGTAGT	MID-69	TCTGACGTCA	MID-91	CATACTCTAC		
MID-26	ACATACGCGT	MID-48	ACAGTATATA	MID-70	TGAGTCAGTA	MID-92	CGACACTATC		
MID-27	ACGCGAGTAT	MID-49	ACGCGATCGA	MID-71	TGTAGTGTGA	MID-93	CGAGACGCGC		

515F/926R – Soil	MIDs 3-5,7-8,10-11,13-23,25-26,29-38,41-43,45,47-51,54-65,67-76,79-80,83-90,93-95,97,99-103, &106-109
OOMUP18Sc/ITS2-O – Soil	MIDs 14-36,39-48,51-53,55,57-61,64-86,89-98,101-103,105,107-111
ITS86F/ITS4 – Soil	MIDs 14-36,39-48,51-53,55,57-61,64-86,89-98,101-103,105,107-111

Fusion Primer Concatemer Structure

Forward Fusion Primers	5′ – Adapter "A" – MID – Forward Target Specific Primer – 3′
Reverse Fusion Primers	5′ – Adapter "B" – Reverse Target Specific Primer – 3′

a Fusion Primers designed for Roche GS-FLX XLR70 instrument and Lib-L Titanium chemistry (Roche Applied Science, Mannheim, Germany).

b Multiplex identifier sequencers were selected from extended MID set TCB No: 005-2009 (Roche Applied Science, Mannheim, Germany).

Table S4.3 Potential plant pathogenic genera identified in the sampled field sites.

Potential shoot infecting fungi	Number of OTU's	Total reads	Number of sites	Potential shoot infecting fungi	Number of OTU's	Total reads	Number of sites	Potential root infecting fungi	Number of OTU's	Total reads	Number of sites	Potential oomycete pathogens	Number of OTU's	Total reads	Number of sites
Aciculosporium	1	40	8	*Mycosphaerella*	3	225	25	*Ceratobasidium*	1	16	3	*Albugo*	1	12	3
Alternaria	1	2	1	*Myrothecium*	2	13	5	*Cochliobolus*	3	5	3	*Hyaloperonospora*	8	10	7
Alveopora	2	6	2	*Neonectria*	1	2	1	*Colletotrichum*	2	7	6	*Peronospora*	1	4	3
Cadophora	1	1	1	*Oculimacula*	3	97	23	*Curvularia*	1	510	16	*Phytophthora*	20	178	24
Calyptella	1	38	14	*Omphalotus*	1	1	1	*Cylindrocladiella*	1	1	1	*Plasmopara*	1	14	1
Chrysomyxa	2	1	1	*Peyronellaea*	3	3	3	*Fusarium*	4	45	8	*Pythiogeton*	3	51	11
Cladophialophora	2	25	3	*Phaeoacremonium*	1	5	5	*Heterobasidion*	3	4	3	*Pythium*	127	24610	33
Coleosporium	1	50	11	*Pseudocercospora*	2	10	5	*Magnaporthe*	1	4	3				
Colletotrichum	1	2504	29	*Puccinia*	3	127	27	*Microdochium*	2	531	41				
Corynespora	8	410	13	*Pucciniastrum*	3	39	10	*Olpidium*	6	218	31				
Craterocolla	2	4	2	*Pseudotetraploa*	1	8	3	*Phoma*	3	29	9				
Cronartium	2	17	4	*Septoria*	3	15	4	*Plectosphaerella*	3	5	4				
Didymella	1	3	7	*Sphacelotheca*	1	1	1	*Pyrenochaeta*	2	66	14				
Drechslera	1	1	1	*Spongipellis*	1	3	3	*Rhizoctonia*	28	331	33				
Endocronartium	1	1	1	*Stenocarpella*	1	1	1	*Setophoma*	1	1	1				
Entyloma	2	30	3	*Sydowia*	1	69	15	*Spongospora*	9	11	8				
Fomitopsis	1	76	13	*Taphrina*	1	11	6	*Thielaviopsis*	3	5	3				
Fusarium	10	1552	27	*Tritirachium*	2	1	1	*Typhula*	1	2	1				
Ganoderma	2	2	2	*Urocystis*	1	9	6	*Verticillium*	2	92	25				
Hypohelion	2	9	6	*Ustilago*	1	1	1	*Waitea*	7	56	18				
Itersonilia	2	384	16	*Venturia*	1	5	4								
Leptosphaerulina	2	239	34	*Verticillium*	1	1	1								
Monilinia	1	1	1												

Table S4.4 Overview of the OTU's identified as potential pathogenic including Blast results, scores and sequence similarities. This data has been archived in the Data and Information Portal of the NIOO-KNAW http://data.nioo.knaw.nl/index.php under number XXX and is available on request.

Table S4.5 Overview of the results of the redundancy analysis (RDA) of the various datasets explaining variation in community composition of potential plant pathogenic fungi and oomycetes before and after (RDA reduced) forward selection. Explaining datasets are: Physico-chemical properties (PC), field history including management practices and crop history (FH), spatial patterning (SP), and the non-pathogenic microbial soil community (NP).

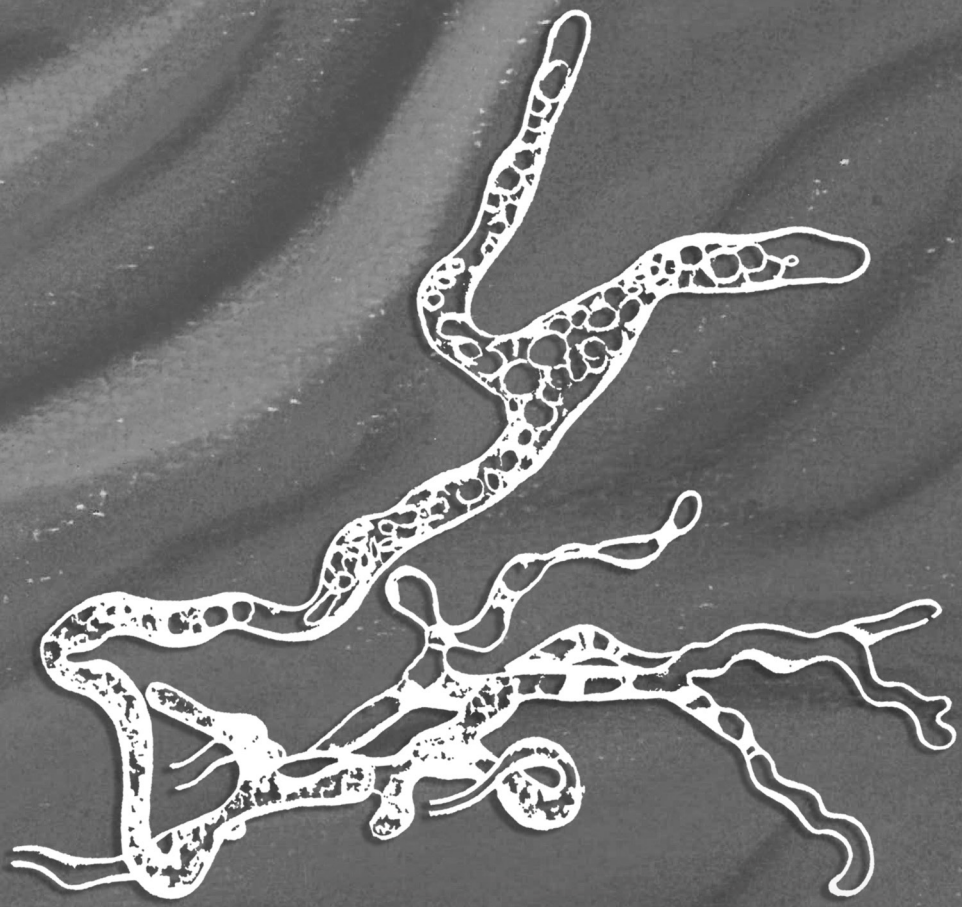
		RDA			RDA reduced			
		Df	R2adj	p value	Df	R2adj	p value	Permutation
Fungal	PC	10	0,21	0,001	1	0,18	0,001	1000
	FH	13	0,17	0,002	3	0,14	0,001	1000
	NP	23	0,35	0,001	8	0,33	0,001	1000
	SP	8	0,13	0,001	2	0,13	0,001	1000
Oomycete	PC	14	-	ns				
	FH	23	-	ns				
	NP	28	-	ns				
	SP	8	-	ns				
Shoot	PC	14	0,16	0,002	0	0,16	0,001	1000
	FH	23	0,18	0,002	3	0,15	0,001	1000
	NP	28	0,24	0,004	6	0,27	0,001	1000
	SP	8	0,11	0,005	2	0,12	0,001	1000
Root	PC	14	0,18	0,002	2	0,16	0,001	1000
	FH	23	-	ns				
	NP	28	0,43	0,001	8	0,43	0,001	1000
	SP	8	0,07	0,55	2	0,12	0,001	1000

Table S4.6 Significant correlation of the Shannon diversity index with forward selected variables of the RDA analysis.

x	y	cor	p
Acidobacteria	Shannon diversity index fungal potential pathogens	0,39	0,009
pH	Shannon diversity index fungal potential pathogens	0,48	0,001
litter saphrophytes	Shannon diversity index fungal potential pathogens	-0,46	0,002
pH	Shannon diversity index shoot infecting fungal potential pathogens	0,42	0,005
litter saphrophytes	Shannon diversity index shoot infecting fungal potential pathogens	-0,39	0,01
pH	Shannon diversity index root infecting fungal potential pathogens	0,32	0,041
litter saphrophytes	Shannon diversity index root infecting fungal potential pathogens	-0,31	0,049
N	Shannon diversity index root infecting fungal potential pathogens	-0,3	0,049

CHAPTER 5.

DEVELOPMENT OF A TWO-COMPARTMENT MODEL RHIZOSPHERE TO ASSESS GROWTH POTENTIAL OF PYTHIUM INTERMEDIUM IN AGRICULTURAL SOILS



Maaike van Agtmaal, Wietse de Boer

Abstract

Environmental triggers play key roles for germination and growth of soil-borne pathogens. This activation of survival structures of root pathogens in soils is the first step in the infection cycle. Host plant exudates can initiate the activation but other soil microbes may interfere by rapid consumption of the exudates or production of inhibitors. Therefore, it is difficult to predict if the presence of both host plant and pathogen will indeed result in infection and disease. The common approach to test if activation and infection will occur is to use plant bioassays which are often laborious and time-consuming. As an alternative for plant bio-assays we started developing a bilayer model system to study the response in soils of naturally occurring oomycete pathogen *Pythium* to influx of artificial root exudates. The model consisted of a top compartment containing a sterile organic-poor sandy soil enriched with artificial root exudates that could leak via 8 μm filter into the bottom compartment containing the field soil samples., This simple model was used to quantify the exudates-induced growth response of indigenous *Pythium intermedium* in a selection of agricultural soils by using qPCR. Results show that the *P. intermedium* DNA increase upon root exudate exposure over time is soil dependent, supporting the hypothesis that in situ microbial competition may successfully prevent *Pythium* outgrowth in the presence of root exudates. This effect was independent of the initial amounts of *Pythium* in the soil.

Introduction

Plant pathogens cause a considerable loss of crop yields worldwide (Oerke, 2006) and there is a need for development of methods that can estimate the sensitivity of a given soil to pathogen outbreaks. Soil-borne pathogens are difficult to control due to the complex environment they inhabit and their adaptation to survive periods without host. Propagules of plant pathogens can remain for long time in the soil in a quiescent state, waiting for favorable conditions to germinate and start the infection cycle. Outbreaks of disease are not only dependent on the amount of infective propagules in soil (inoculum potential) but are also influenced by physicochemical soil properties and activity and composition of microbial communities (Benson, 1994). These abiotic and biotic factors do also influence survival of pathogenic propagules, albeit that the mechanisms are largely unknown. This interferes with giving a reliable prediction of risks for outbreaks of soil-borne diseases.

Disease suppression, a naturally occurring phenomenon in soils is the sum of processes related to reduction of disease in the presence of pathogens. Competitive interactions play a major role in the biotic part of suppression. Natural suppression encompasses fungistasis, the restriction in germination and growth of pathogen propagules and thus infective ability of pathogens. This is either caused by nutrient deprivation (Lockwood, 1977) or by the release of suppressive compounds (Romine and Baker, 1972) keeping the infectious propagules in a dormant stage waiting for suitable conditions. Many studies have shown the inhibiting effect of indigenous soil microbes on germination and growth of fungal pathogens (reviewed by (Lockwood, 1977; Garbeva *et al.*, 2011). However, the relationship of these inhibiting effects with disease suppression is much less clear.

Environmental triggers play key roles at the start of a parasitic life cycle, a considerable part of the inoculum consists of resting forms and survival spores. To break the resting stage of the propagule, cues are needed to trigger germination (Curl and Truelove, 1986; Chet and Mitchell, 1976). Generally the presence of host plant cues can start the germination and growth of pathogens (Nelson, 2004). Root exudates consist of a mixture of different compounds such as amino acids, sugars and organic acids (Griffiths *et al.*, 1998) and have been proven to induce chemotaxis in zoospores of oomycetal pathogens (Deacon, 1996; Donaldson and Deacon, 1993; Chet and Mitchell, 1976). Besides, these general compounds also specific components exuded from the roots can trigger germination of oospores and sporangia (Nelson, 1991).

Pythium, an oomycetal genus containing many plant pathogens causes diseases such as damping off and root rot (Martin and Loper, 1999; Kamoun *et al.*, 2015) in a wide range of hosts including economically important crops. *Pythium* species are opportunistic pathogens and known to be sensitive to biostasis and disease

suppression (Chen *et al.*, 1988). The life cycle of *Pythium* has two stages an asexual and a sexual cycle. The first is characterized by the formation of zoospores of which the release is often triggered by the presence of water and specific exudate compounds such as amino acids and sugars (Donaldson and Deacon, 1993; Deacon and Donaldson, 1993). The sexual cycle is characterized by the formation of resistant thick walled oospores, a dormant survival stage that can remain viable during harsh conditions such as low temperatures and drought (Martin and Loper, 1999).

As *Pythium* is considered to be a poor in competitor for organic nutrients, the natural control of *Pythium* is related to the presence of indigenous exudate-consuming soil microbes outcompeting *Pythium* (Chen *et al.*, 1988; Van Os and Van Ginkel, 2001). Next to this, the natural control mechanisms in soil and rhizosphere include production of antagonistic secondary metabolites produced by competing microbes which may reduce growth and infection of *Pythium* in presence of a suitable host plant. However, currently fast methods lack to assess the activation of pathogens in soils to determine the soils' capacity of suppressing pathogen infection. With our method we aimed to mimic an exuding host plant to measure the responsive pathogen dynamics

Real time PCR, in comparison with conventional PCR amplification, includes a quantification step to allow for accurate quantification of the target pathogen. This enables to track pathogen dynamics, over time. This is the basis of our development of a two compartment bioassay to establish growth potential of the plant pathogen *Pythium intermedium* in agricultural soils induced by root exudates.

The aim of our research is to set up a method to estimate *in situ pathogen dynamics*. Our hypothesis is that in a suppressive soil no pathogen increase could be measured whereas in soils conducive to infection the soil microbial community cannot inhibit the pathogen to grow. The current common approach to test if activation and infection of soil-borne pathogens will occur is to use plant bioassays. There is a need for a fast and cheap method that can give an estimate of the risk for crop infection as an alternative for the costly, laborious and time consuming plant-bioassays. With our method we aimed to mimic an exuding host plant to measure the responsive pathogen dynamics. Estimating the suppressive potential in a soil is an essential step towards predicting crop diseases.

Material & Methods

Soil sampling and preparation

6 soils where *Pythium intermedium* was shown to be present were selected from a pathogen inventory of the 0-20 cm layer of 50 agricultural soils (Chapter 3). The 6 selected soils differed in properties such as pH, clay content and crop history (Table 5.1). Samples were homogenized by sieving with a 4 mm mesh sieve and were kept cold (4°C) until use. Moisture content was measured by drying the soil at 105°C for 24 hours and determining weight loss. Maximum water holding capacity (WHC) was determined by adding water until saturation.

Table 5.1 Physico-chemical soil properties of the selected agricultural fields. Total N, P, S, Mg, Na, K are in mg kg^{-1}. CEC is cation exchange capacity (mmol kg^{-1}), OM is organic matter (%).

	pH	Clay %	CEC	N	C:N	P	S	Mg	Na	CO_2	$CaCO_3$	OM	K	C:S
Soil A	4,7	15	554	17700	12	29	6050	395	65	2042	2,3	41	10	34
Soil B	5,2	1	46	1350	14	51	260	69	6	575	0,2	3,3	2,1	74
Soil C	5,6	17	398	6910	15	38	2040	289	38	955	1,7	20	8	50
Soil D	7,2	16	277	3870	11	159	870	203	14	534	5,3	8,5	14	49
Soil E	7,2	20	165	1570	9	59	460	77	13	290	3,5	2,9	8,6	31
Soil F	6,2	39	314	4980	9	56	1380	189	19	1372	0,9	9,4	12	34

Model system

A two compartment model rhizopshere was designed to ensure a physical separation between a nutrient rich layer (simulated rhizosphere) and a nutrient poor bulk soil. The two compartment microcosm (Figure 5.1) was constructed using ThinCert™ culture inserts consisting of a polystyrene housing and a polyethylene terephthalate (PET) capillary pore membrane (greiner bio-one®) in 6 well CELLSTAR®plates (Greiner Bio-one®). The pore membrane has 8 micron pores enabling the migration of microbes between the two compartments. Each well of the plates, representing the bulk soil compartment of the bioassay, was filled with field soil equivalent to 5 gram dry weight. Filter-sterilized root exudates solution (10 mg carbon/ml) was prepared according to Griffiths (1998) containing 0,2 M fructose; 0,2 M glucose; 0,2 M sucrose; 0,1 M succinic acid; 0,1 M malic acid; 0,05 M arginine; 0,05 M serine; 0,05 M cysteine. The culture inserts were filled with 5 gram dry weight sterile dune sand (Lisse 1% organic matter) in which root exudates were mixed to 1mg C per gram dry weight soil. The culture inserts were placed carefully in the soil containing wells ensuring optimal contact of the bottom filter membrane to the field soil. Control treatments received sterile water instead of the root exudate mixture. Moisture content in both compartments was adjusted to 60% WHC. Control and root exudate

treatment had tree replicates per time point. Plates were incubated at 10°C, the average annual temperature at the start of the growing season, for 0, 24, 48 and 72 hours after root exudate addition respectively.

At every time point 0.5g of soil was sampled destructively in both upper and bottom compartments in the middle of the well using a sterile 5mm stainless steel corer. DNA was extracted using the Powersoil 96 well extraction kit (Mobio) according the manual. After quality check on 1% agarose gels DNA was stored at -20°C. To determine the dynamics of *P. intermedium* quantitative PCR (qPCR) was performed using *P. intermedium* specific primers (Klemsdal *et al.*, 2008) namely ATGCAGAGGCTGAACGAA (forward) and CTGTATTCATAGCCGAAACGA (reverse) and a *P. intermedium* specific probe 5'-Cy5'- CAGCCACAGCGAGACACTTCACG -BHQ-3-3' including a black hole quencher and 5'-Cy5' dye (Jena biosciences). qPCR analysis was performed with the maxima probe qPCR mastermix (2x) (Thermo Scientific) using a concentration of 150nM (probe) and 80nM primer and 2 μl 10ng/μl of sample DNA in a final volume of 25 μl. Cycling conditions were: 10 min. at 95°C, followed by 40 cycles of 15 sec at 95°C, 45 s at 58°C, and 30 sec. at 72°C. The standard consisted of pure culture *Pythium intermedium* DNA, extracted with the ZR Fungal/Bacterial DNA MiniPrep™ Isolation kit.

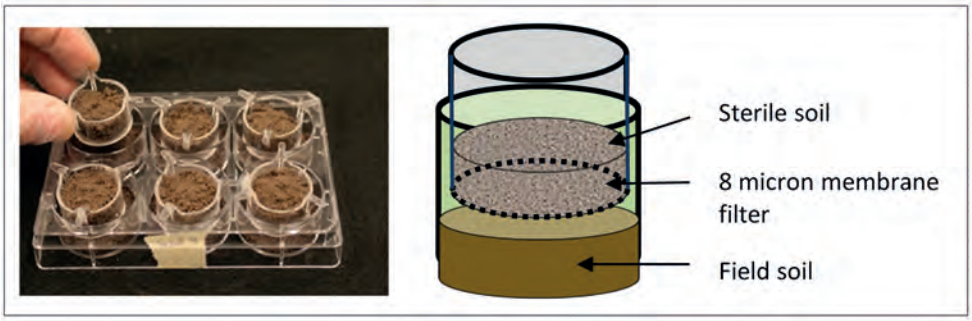


Figure 5.1 Two compartment model rhizosphere. The bottom compartment contains the sampled field soil, the upper compartment contains sterile organic-poor sandy soil amended with root exudates (control contains sterile water), the 8 micron filter acts as a physical barrier that divides the original soil from the enriched soil.

Data analysis

The DNA concentration of each soil sample was estimated using qPCR with a *P. intermedium* specific probe. A dilution series of *P. intermedium* (isolate P52, Applied Plant Research Flowerbulbs, Nursery Stock & Fruit, Lisse) was made to generate a standard curve ranging from 1 fg to 10 pg. DNA concentration of the 6 soils was calculated by plotting the measured cycle thresholds (Ct) values against the Ct values of the standard curve. Paired T-tests were performed to examine significant differences between control and root exudate amended samples per time

point. Similarly paired T-test were used to examine differences between indigenous *P. intermedium* concentrations (time 0) and the concentration at 24, 48 and 72 hours after root exudate addition.

Results

Pythium response to artificial root exudates in field soil compartment

In soils from 5 sites *Pythium* was detected at time 0, one soil, A was under the detection limit. The *P. intermedium* DNA concentration in soils B, C, D, E and F was in the range of 0.3-0.75 ng per gram soil. The presence of artificial root exudates in the sterile upper layer of the microcosm resulted in different effects on *P. intermedium* DNA concentration in the field soil compartment (bottom layer). For soils A, Band C no significant increase of the amount in *P. intermedium* DNA concentration was seen during the whole incubation period both for root exudate enriched treatments and controls (Figure 5.2). *P. intermedium* DNA concentration showed an increasing trend in soilD ($p<0.1$) in contact with the root-exudate containing upper layer. However, there was no significant difference between soil connected to the root exudate containing compartment and the control (sterile water addition) (Figure 5.2). A clear stimulating effect of artificial root exudates on *P. intermedium* DNA concentration was seen for soils E and F. In soil E, more than 30 fold increase ($p<0.01$) of *P. intermedium* DNA was measured after 48 hours. Soil F showed more than 20 fold increase after 48 hours and a 30 fold increase after 72 hours (both $p<0.01$) as compared to the control.

Pythium response to artificial root exudates in sterile soil compartment

None of the sterile soils in the polystyrene insert gave a positive *Pythium* signal at time 0 confirming that the soil in the insert was free of *Pythium* at the start of the experiment. During incubation *P. intermedium* was detected in the sterile top compartment for 3 soils (B,D, F) (Figure 5.3). The highest concentration of *P. intermedium* DNA in the sterile root-exudate compartment was found in soil F after 48 hours. For soil B and F *Pythium* invasion was also seen in the sterile compartment with water (control). There was no consistent pattern with respect to presence of *Pythium* DNA in the top compartment. For instance, soil B had significant amounts of *Pythium* DNA in the top compartments after 48h of incubation, but no DNA could be detected after 72 h.

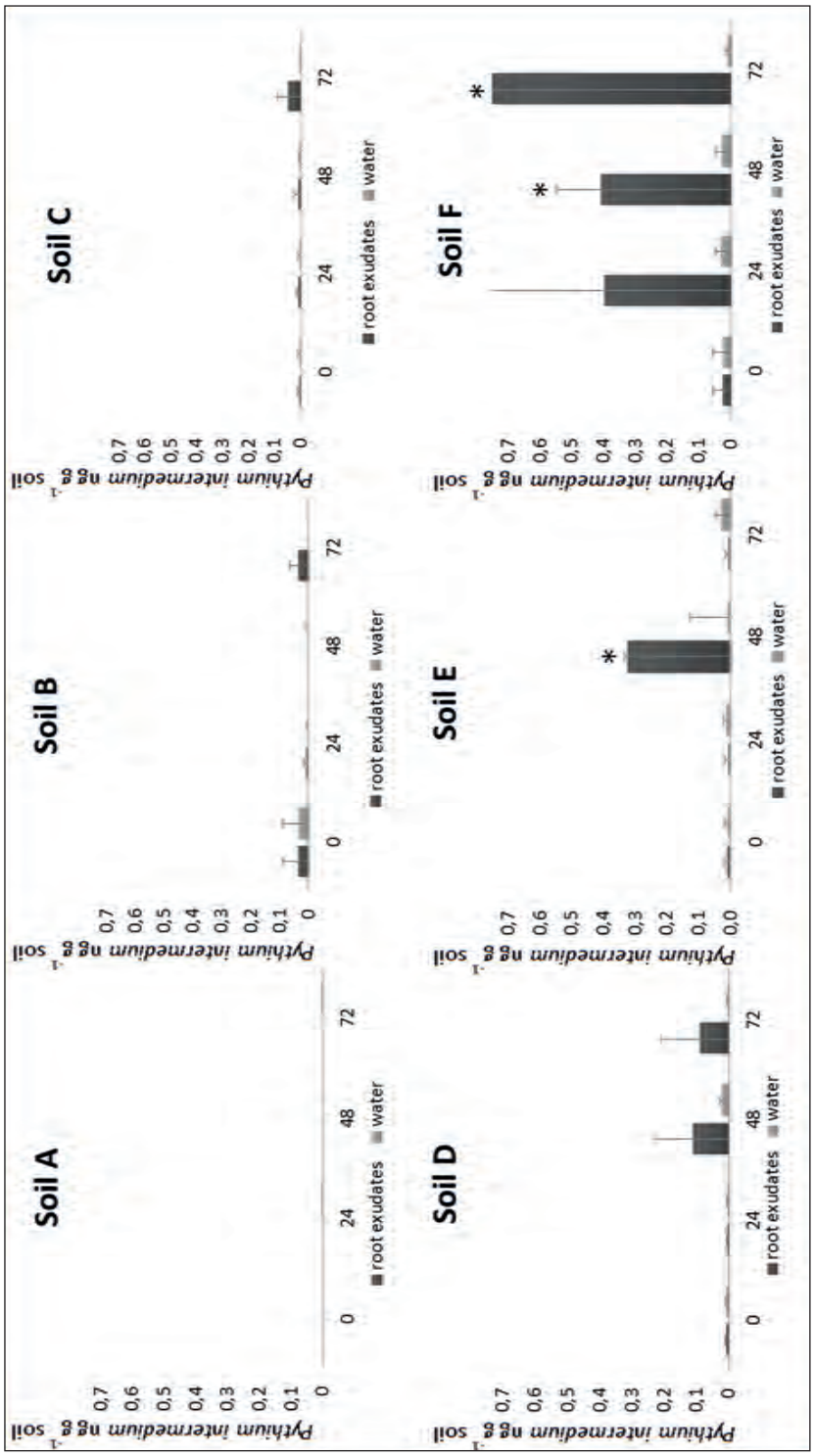


Figure 5.2 DNA concentration of *Pythium intermedium* at different timepoints in the bottom compartment, containing field soil exposed to an upper compartment with root-exudate containing sand or sterile water-containing sand (control). **A-F** DNA concentration at different timepoints in soil A-F. Significant differences between control and root exudate amended samples per timepoint are marked with *.

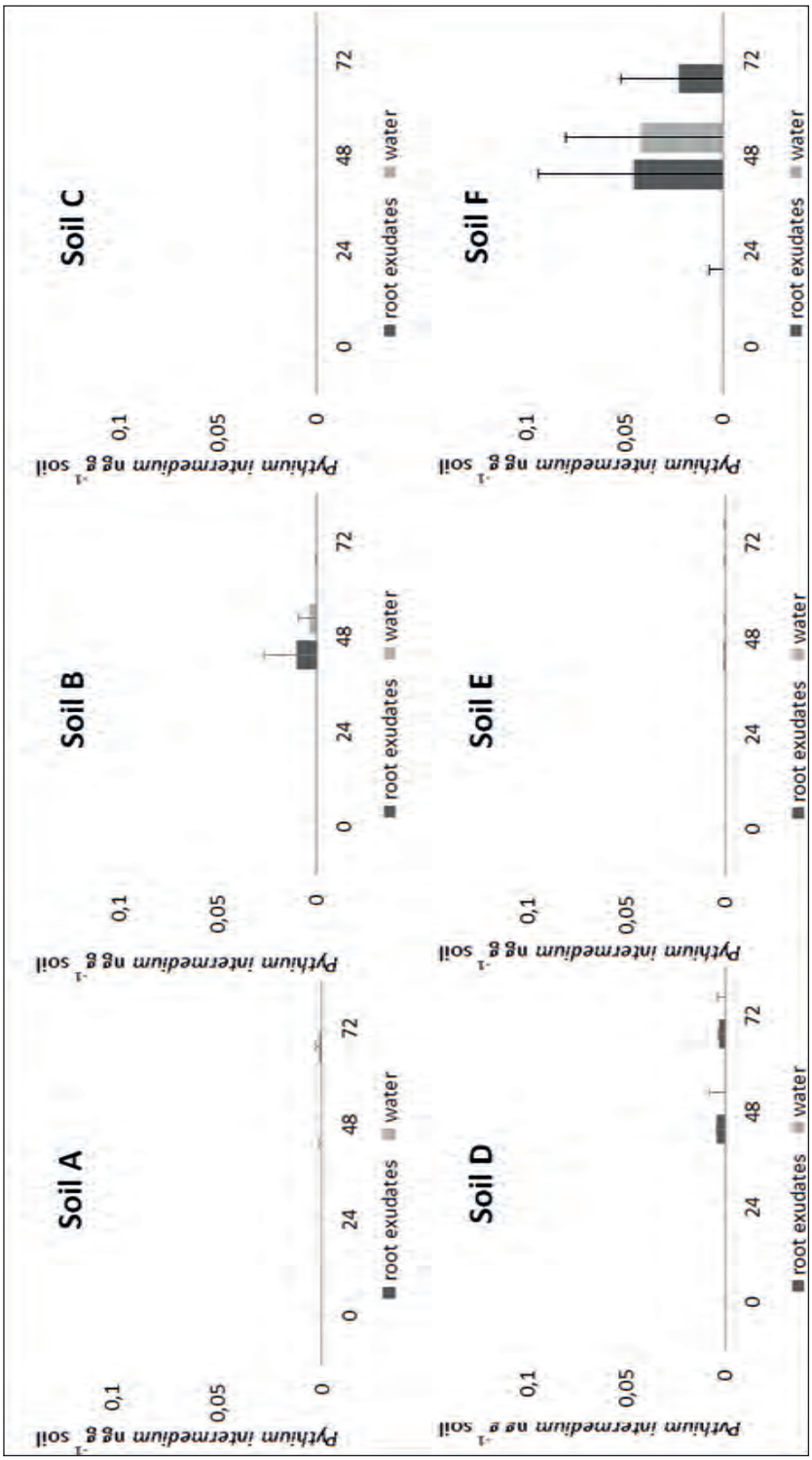


Figure 5.3 DNA concentration *of Pythium intermedium* at different timepoints in the upper layer of sterile organic-poor sand containing artificial root exudates or water (control). **A-F** *P. intermedium* DNA concentration at different timepoints in the upper layer above soil A-F.

Discussion

Chemical stimuli are often involved at the start of host parasite interactions (Nelson, 1991). Seed -or root exudates are important stimuli for germination and directed growth of propagules of a broad range of pathogenic fungal and oomycetal species (Nelson, 1991). Fungistastic suppression of pathogenic propagules can be overcome by the application of appropriate stimulatory signals (Lockwood, 1977; Lockwood, 1988). The results of the current study indicate that this relief from fungistasis by the addition of root exudates might be context dependent. The 6 soils that were used in this study were selected because of the presence of *P. intermedium* in the pathogen seedbank. Yet, the response of *Pythium* n these soils to contact with a sterile, root exudates containing compartment varied strongly. This may indicate that in situ (microbial) processes can successfully prevent *Pythium* development in some of the soils but not in others.

Low molecular weight organic compounds exuded by roots play a key role in microbial interactions by altering the structure and function of the community (Shi *et al.*, 2011). Root exudate compounds are easily accessible energy resources and are therefore thought to have a major regulating role in microbial community dynamics (Bais *et al.*, 2006). Competition for specific compounds that could activate dormant propagules may lie at the basis of prolonged suppression of germination despite the presence of a host plant (van Dijk and Nelson, 1998). This has been shown for *Pythium ultimum*, where the competition by soil microbes for plant derived fatty acids or seed exudates, which can elicit a germination response, suppressed the germination and subsequent plant infection (van Dijk and Nelson, 1998; van Dijk and Nelson, 2000).

Interestingly, the initial amount of *Pythium intermedium* DNA measured in the soils, was not related to the growth response after exposure to artificial root exudates. Lack of a dose response relationship between population densities of pathogens and disease incidence has been reported earlier for natural soil (Garber *et al.*, 1979). Whereas the study of Stasz and Harman (1980) (Stasz and Harman, 1980) showed a similar relation between disease severity versus proportion of diseased plants for two pea lines but a different threshold inoculum density for infection. They therefore suggested that biotic and abiotic soil properties mediate the pathogen response. This could be (partly) due to in situ microbial competition reducing the viability or preventing the germination of the pathogen propagules in the soil.

The addition of sterile water instead of artificial root exudates did not increase the *Pythium* DNA concentration significantly in any of the tested soils. This is in support of our expectation that root exudates form important triggers for the growth response of *Pythium.* However, the zone of the root where simple organic compounds compounds are exuded, the rhizosphere, is also a highly competitive environment

(Raaijmakers *et al.*, 2009). As pathogens are generally sensitive to suppression due to their limited saprophytic ability, outgrowth of *Pythium* hyphae may be suppressed by competition or antibiosis. The variation in *Pythium* response as observed for the different soils may be due to differences in intensity of competition for root exudates by the indigenous soil microbial communities.

The observation of *P. intermedium* growth into the sterile top layer might be due to the opportunistic nature of *Pythium* spp. Many *Pythium* species are facultative pathogens and as well fast growing pioneer species of new substrates. However they are poor competitors and have limited abilities to establish or infect priorly colonized substrates. Postma (2000) found that rockwool was suppressive to *Pythium* infection in cucumber when it was previously colonized by microbes, whereas it was conducive after sterilization. Sterile soils and substrates represent a biological vacuum which can be exploited by opportunistic microorganisms e.g. *P. ultimum* (Chen *et al.*, 1988) to escape the competition pressure. This may explain the increase of *Pythium* DNA in the top layer for different soils, including soil B that did not even show an increase in DNA concentration in the soil itself (bottom layer). The growth into the upper, root exudate containing layer may be stimulated by concentration dependent chemotaxis (Zentmyer, 1961; Deacon, 1996).

The variable results that were obtained with qPCR may be caused by biological variability e.g. the heterogeneity of *Pythium* distribution in natural samples. This may be the underlying cause of the observed variation between replicas per timepoint but also of the patterns of *Pythium* development during prolonged incubation which was not always showing a consistent incline

Our results confirm the potential of the two compartment model rhizosphere model to test plant-induced in situ pathogen dynamics and risk for infection. However, for a proper validation it has to be linked to bioassays to translate the observed differences in pathogen DNA concentration to actual values of plant infection and disease severity. If the disease incidence correlates to the observed response of soil Pythium DNA to presence of artificial root exudates than this relatively simple system can be used for prediction of disease. Bioassays are costly and time consuming. Therefore, our method may provide an alternative way to assess the susceptibility of a soil to certain soil-borne diseases. Q-PCR allows for detection of a wide range of pathogens and the quantification and differentiation between pathogens and non-pathogenic strains (Jiménez-Fernández *et al.*, 2010) which gives the potential to develop customized pathogen assays per field or crop. Furthermore, our method provides also the potential to monitor pathogens and specific antagonist populations side by side.

Acknowledgements

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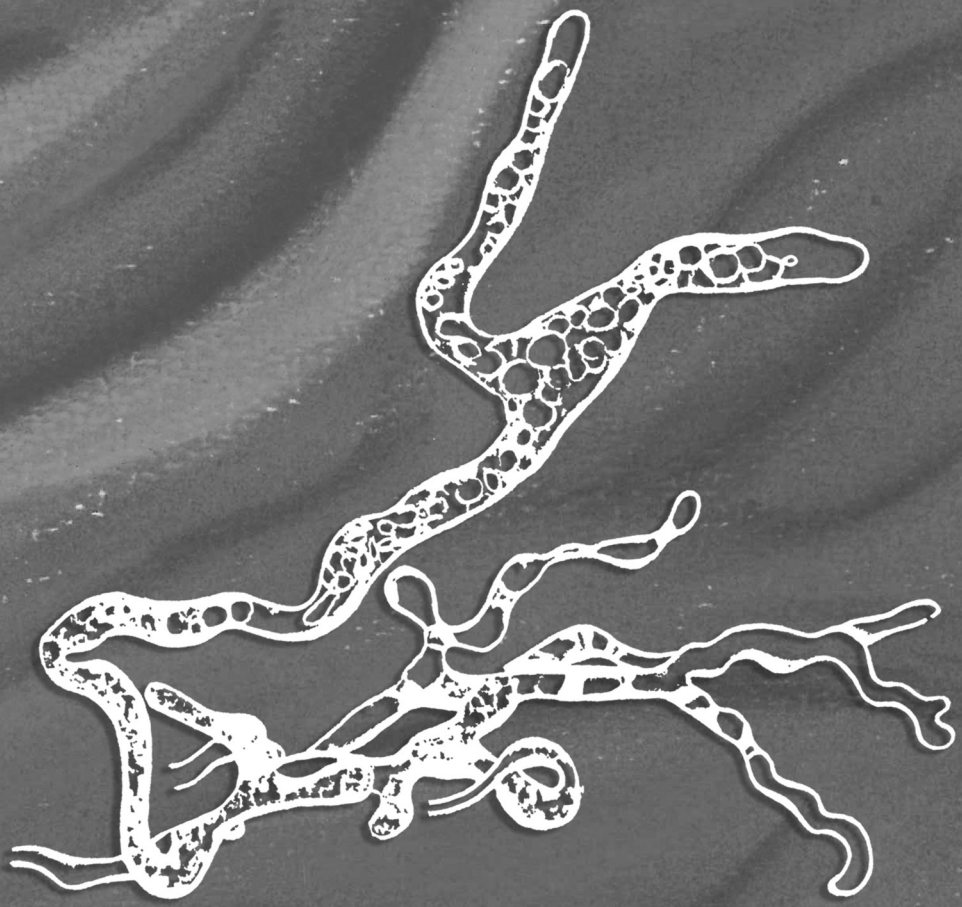
CHAPTER 6.
GENERAL DISCUSSION



Aim and outline

The aim of this thesis was to improve the insights into the interplay between microbial communities, soil-borne plant pathogens, edaphic properties and organic matter in Dutch agricultural soils. To accomplish this aim both field inventories and controlled experimental approaches were performed. In this synthesis I discuss the outcomes of these studies in the framework of general disease suppression.

In the first section of the synthesis I will discuss different aspects that added new insights to the current knowledge on general disease suppression. This section includes the relevance of volatile mediated inhibition of pathogen growth for disease suppression. In addition, the interplay between abiotic properties and microbial dynamics (composition, activity and functioning) will be discussed. Furthermore the role of pathogen life history characteristics in response to suppression will be addressed. Lastly, this section emphasizes some microbial groups that putatively play important roles in suppression.

The second section of this synthesis focuses on the implementation of the research presented in this thesis. It summarizes the main findings that are potentially important for agriculture and identifies which research needs to be conducted to translate this work to operational management practices for farmers. Furthermore this section, discusses how general 'general disease suppression' (GDS) is. This will be combined with a critical view on the value of an indicator of GDS.

The third section encompasses ideas and directions for further research. I will finish the synthesis with the major conclusions of this thesis

New insights in general disease suppression

Volatiles in disease suppression

Emission of volatiles by bacteria and fungi has been known for a long time (Stotzky *et al.,* 1976). Yet a limited number of studies have investigated their inhibitory effects on plant pathogens (reviewed by Campos *et al.,* 2010). Particularly, the link between effects of volatiles on pathogen germination and growth on the one hand and disease incidence on the other hand has not been made before. Therefore one aim of this thesis project was to investigate the relationship between production of soil volatiles and sensitivity of crops to disease, as has been described in chapter 2.

In Chapter 2 a strong correlation between volatile mediated pathogen suppression and root infection is reported, as indicated by root biomass development. Based on this I conclude that volatile mediated suppression may significantly reduce root infection of crops by soil-borne pathogens. This sheds new light on the role of volatiles in agriculture as it extends the role of volatiles from bulks soil processes

(fungistasis) to rhizosphere processes (suppression of root infection by pathogens).

Volatile mediated suppression of *in vitro* growth of pathogens has been and is being studied by several research groups. In most cases the studies report on effects of volatiles produced by soil bacterial or fungal isolates on fungi. My approach to measure the effects of volatiles that are produced in natural soil by indigenous soil microbial communities is only known from a few reports. The inhibition of *in vitro* pathogen growth by volatiles emitted from soil may be indicative for the effects of volatiles on pre-infective growth of pathogens. The results reported in Chapter 2 and 3 yielded different novel insights on the role of volatiles in the suppression of soil-borne pathogens. In summary I could show that: 1) volatiles emitted from soil can successfully inhibit the growth of different pathogens (Figure 2.1c; 3.1). 2) That this is a microbial effect (Figure2.1f; 2.2a, b). 3) That suppression by volatiles might be strongly related to microbial community composition (Figure 2.3). 4) That pathogens differ in sensitivity to volatile suppression (Figure 3.1). 5) That edaphic factors can have an indicative value for volatile mediated suppression (Table 3.2, Figure 3.2; 3.3; 3.4). 6) And lastly that the overall suppressive effect against three pathogens is related to microbial activity and organic substrate quality (Table 3.3).

Together these results point at the relevance of volatile mediated pathogen suppression as one of the mechanisms of disease suppression, implying a big role for small compounds.

Interplay between abiotic properties, microbial activity and pathogen suppression: the added value of an integrated approach

Whether a soil is conducive or suppressive to diseases is depending on a range of components (Alabouvette *et al.*, 1982), including soil factors that influence the inoculum density and pathogenic infection capacity. Inoculum density is related to soil characteristics, e.g. texture and pH can influence growth and spread of pathogens. Direct relations between abiotic soil properties, soil suppression and crop diseases have been found (Janvier *et al.*, 2007), for example lighter sandy loam soils with a low pH are often conducive to *Fusarium* (Stotzky *et al.*, 1961; Hoper and Alabouvette, 1996). Besides, the abiotic environment interacts with microbial community; physico-chemical properties shape the growth conditions for microbes and consequently the microbial community composition and activity. In chapter 4 we tested a range of soil related properties and found significant relations between soil characteristics and the pool of pathogens present in the soil. We found both pH and soil type to be the major drivers of the composition of community of pathogens surviving in the winter period. Zooming in on the root infecting fungi revealed more physico-chemical soil properties, namely $CaCO_3$, cation exchange capacity (CEC) and total N as significant drivers of the pool of potential root infecting pathogens. Altogether this would imply that soil abiotic properties are directly

related to the variability of the inoculum density of phytopathogenic propagules.

Next to the aforementioned direct abiotic effects on inoculum density, the results in chapter 3 do also suggest indirect effects of soil properties on pathogens suppression, namely via the effects of soil properties on the production of suppressing volatiles (influencing pathogenic infection capacity). Volatile mediated suppression of *Rhizoctonia* was related to pH and organic matter (OM), whereas volatile suppression of *Pythium* was related to S total. Multivariate analysis of the overall volatile mediated suppression of all three pathogens and soil properties indicated organic matter components, namely dissolved organic carbon (DOC), total organic matter (OM), C:S and C:N ratios, in different RDA models (Table 3.3). This may imply that a substantial part of volatile mediated suppression is resource driven. Carbon limitation is generally the driving force of competitive interactions in soil. Respiration, a measure for microbial activity, was also strongly correlated with suppression. Increased microbial activity may coincide with increased intensity of competitive interactions resulting in pathogens suppression.

Hoitink and Boehm (1999) reported that the concentration and availability of nutrients (easily degradable organic compounds) within the soil organic matter (OM) play a critical role in regulating soil-borne pathogens but that it is an challenge to characterize OM quality relative to disease suppression. The study of Straathof, van Agtmaal *et al.* (unpublished) took a first step in this by linking OM quality and respiration. Our aim in this research was to look in more detail to DOC characteristics in relation to microbial activity, hypothesizing that DOC fractions (differing in aromaticity and bioavailability) would be more indicative of the microbial activity than total DOC alone. The relation between variability of different dissolved organic carbon (DOC) components among agricultural soils and microbial respiration was complex but significant, conform our hypothesis. More detailed insight in the nature and complexity of organic substrates, microbial activity and interactions of these two components with soil pathogen occurrence and pre-infective growth suppression in agricultural soils could contribute to a more comprehensive understanding of GDS.

The importance of pathogen traits in disease suppression

The ability of soil-borne pathogens to survive unfavorable periods (outside the growing season and/or without presence of a suitable host crop) is determined by a multitude of factors among which competitive ability for different energy resources, sensitivity to fungistasis and survival abilities of resting structures. These factors have an important role in determining the inoculum potential (amount of in potential infective propagules in soil) and, consequently, in determining the risk for disease outbreaks.

Garrett (1970) grouped pathogens based on substrate utilization ability and saprophytic ability emphasizing trait-based differences in survival. The latter might be one of the underlying causes of the among site differentiation in composition of pathogen propagules (chapter 4), resulting in the selection of those pathogens that can successfully survive under particular environmental conditions.

Differences observed between the drivers of variation in community composition of root infecting (adapted to survive in soil) and shoot infecting pathogens were observed, implicating that they differ in sensitivity to environmental properties.

The variation in oomycete community composition showed no significant relations with any of the measured soil-related properties included in the analyses of chapter 4. This implies that among site variation in distribution of oomycete propagules has a stochastic basis or that the important steering factors were not included in the analysis. In contrast in vitro hyphal growth of *P. intermedium* appeared to be highly sensitive to one of the soil functions, namely emission of inhibiting volatile organic compounds. There was however, also no significant relation between the abundance of *Pythium* in the soil and volatile mediated suppression (Figure 6.1). To survive longer periods in the bulk soil, many plant-pathogenic fungi have dormant propagules (Mondal and Hyakumachi, 1998). Volatiles can thus be beneficial for some plant-pathogens, by preventing germination/growth under conditions without host. Though, prolonged fungistasis may lead to an irreversible loss of viability of fungal propagules (Lockwood, 1977) so in longer term volatile exposure may reduce the inoculum potential.

Results from this thesis, as reported in both chapter 3 and 4 support the importance of pathogen traits like survival and sensitivity to fungistasis and propose that differences in presence and dynamics of pathogens might be trait based.

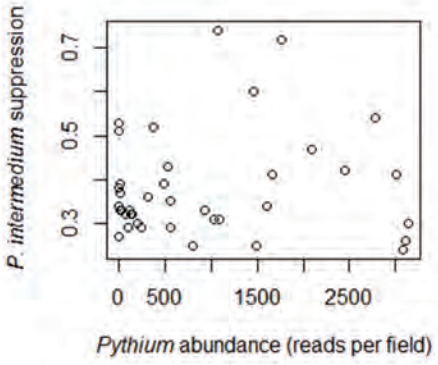


Figure 6.1 Relationship between *in vitro* suppression of *Pythium intermedium* by soil volatiles from 40 agricultural fields and the abundance of the genus *Pythium* in these fields (reads per field from 454 pyrosequencing). Suppression of *Pythium* by volatiles is expressed as the proportion of reduction of mycelial biomass in comparison to a soil-free control (control = 0 (no suppression), 1 = maximum suppression).

Unexplored contributors to suppression: litter saprophytes and *Acidobacteria*

Litter saprophytes

In concordance with the previous paragraph, the sensitivity of soil-borne pathogens to general disease suppression is often referred to as the result of their limited saprophytic ability compared to the vast majority of soil microbes. Therefore one of the main competitors for resources in the nutrient poor bulk soil would be the utilizers of the more recalcitrant soil organic matter, the litter saprophytes. The results of this thesis are indeed in agreement with this, as the relative abundance of saprophytes is a driving factor for the composition of the pool of plant pathogen propagules in the soil (chapter 4).

Our results indicate that litter saprophytes may have an important contribution in controlling pathogen survival and may, therefore, be involved in lowering risks for disease outbreaks. However their modes of action and potential to serve as indicators of soil health needs to be further examined.

The results from chapter 2 (Figure 2.3b) indicated *Acidobacteria* as promising candidates for an active role in volatile mediated pathogen suppression. However, despite the fact that it is one of the most abundant bacterial phyla in the soil, their ecological role in soil is largely unknown. The research on the role of *Acidobacteria* in suppression is largely hampered by the limited amount of isolates.

In contrast to the results of chapter 2, chapter 3 and 4 show a negative correlation between the relative amount of *Acidobacteria* in soil and the volatile suppression of *Rhizoctonia* (chapter 3) or to pathogen diversity (chapter 4). Hence, at this stage no conclusions can be drawn on the role of *Acidobacteria* in natural disease control.

In general, to unravel role of microbial groups and their impact in general disease suppression future research needs to take the step from explorative research (who is there) to more mechanistically approaches to understand their effects on pathogens.

Implementation for agriculture

How the results of this thesis could be brought into practice

The ultimate aim of this PhD was to provide an easily measurable variable (indicator) that could give an estimation of the soils' suppressive capacity. The value of such an indicator is to inform farmers on the he risk of yield losses due to soil-borne plant disease. The indicator could also be used to see if crop rotation or other agricultural management strategies increase GDS and lower the risk for disease outbreaks.

In this paragraph I will discuss how the results from this thesis can contribute to current and future farmers' practices, propose a few candidate variables with the

potential as GDS indicator and identify which research efforts needs to be done to bring this work into practice.

The major findings that have the potential to be implemented in agricultural practices are listed and discussed below:

1) Pathogen community composition is driven by environmental factors
2) Volatile organic compounds reduce pathogen growth and are positively correlated with root biomass/disease incidence
3) Volatile mediated suppression of multiple pathogens is a general phenomenon and is correlated with organic matter quality and microbial activity
4) Pathogen dynamics in the presence of root exudates is soil specific and can be determined by qPCR

1) The among field variation of pathogen communities is related to biotic and abiotic variables which implies that edaphic properties have an important role in pathogen establishment (Figure 4.3). A number of soil properties contributed significantly to variation in pathogen diversity, especially for potential root infection pathogens, namely pH, $CaCO_3$, N and cation exchange capacity (CEC). Those properties can be used to identify areas of risk. Thus all the identified properties significantly contributing to the variation in pathogen community composition may be at the basis for developing management strategies to reduce the occurrence of certain pathogens. For instance, the (relative) amount of litter saprophytes and the amount of total nitrogen in the soil were related to pathogen diversity. Fertilization and/or organic matter management can be used to increase both variables and consequently may decrease the pathogen diversity and the inoculum density.

2) Emission of inhibitory volatile organic compounds was identified as an important mechanism in in vitro pathogen growth suppression. Chapter 2 (Table 2.3) identified a subset of volatile organic compounds as candidate suppressive agents. Thus volatile organic compounds could be potential indicators of disease suppression. Measuring for example the emission of compounds that are known to be antimicrobial could give an indication of the soils suppressive capacity. Simple devices that monitor a few of these compounds, like an electrical nose (Bastos, 2007) could even been taken into the field. However before "sniffing on soil health" can become a method in soil assessment, fundamental research on volatile profiles of soils, and especially on key compounds that give an indicative value for soil suppression, needs to be performed. Specific suppressive volatiles need to be identified, as currently only a fraction of the emitted compounds is known (Effmert *et al.*, 2012). Besides the circumstances under which volatiles are produced and the role of substrate therein needs to be further unraveled.

3) Volatile mediated pathogens suppression is likely to be a substrate driven process. The edaphic parameters that were significant in the multi soil survey were

properties that can be directly linked to total microbial metabolic activity, either as a substrate source (DOC) or an activity indicator (CO_2). The role of substrate or growth medium on in vitro production of suppressive VOCs shows a strong signature of the growth medium composition on the volatile profiles (Garbeva *et al.*, 2014b). This could implicate that quality/chemical composition of the dissolved organic matter in the soil may determine the profile of emitted volatiles in combination with the microbial community composition. A clear relation between the community composition and the volatile producing function of the soil was found in chapter 2. Soil disturbance, by performing anaerobic disinfestation resulted in a shift in the microbial community coinciding with the loss of suppression. By influencing the interplay between organic matter and indigenous microbes, management of organic matter in agricultural fields could potentially improve volatile driven suppression. For example practices which effect substrate-driven microbial activity could enhance VOC-mediated pathogen suppression *in situ*.To accomplish this the role of microbial substrate, including DOC, and its influence on VOC production needs to be mechanistically explored.

4) The presence of a pathogen does not necessary relate to disease incidence. Pathogenic propagules often need a host trigger to germinate and start the infection cycle. Competition for these root-derived compounds or for other compounds during pre-infective growth can prevent successful pathogen infections. The development of a bilayer model system to measure pathogen dynamics in the presence of root exudates enabled quantifying (by qPCR) the responsive growth of a pathogen in a model rhizosphere (chapter 5). This model system was developed to link the pathogen presence to disease risks. Our observation that the response growth of the pathogen is soil specific opens the opportunity to further develop this model. The first step is to link the model to bioassays, to examine the relation between the dynamics measured in the model system and the disease incidence. If a series of bioassays match the risk estimation detracted from the responsive growth of the pathogen, this model has the potential to be a cheap and fast alternative method for bioassays.

Toward an indicator of general disease suppression

An indicator of general disease suppression could give an overall estimate of the soil health and the risk of pathogen infections, serving as a tool for crop selection and decisions on management practices. To develop cheap routine analysis that could give an estimation of GDS, the potential candidate variables need to be further investigated. Before implementation of for example, volatile-inhibition profiles of a soil (E-nose (Bastos, 2007)), the abundance of litter saphrophytes (ergosterol (de Ridder-Duine *et al.*, 2006)), and organic matter analysis (near infra-red spectrometry NIR (Malley *et al.*, 1999)) for each of these candidates standardized methods need to be developed, together with a reference databank. This reference dataset, accounting

for seasonal variation and validated with field data, could determine a critical range for low levels of natural suppression.

How general is 'general disease suppression'?

General disease suppression is described as 'caused by multiple organisms and acting against multiple organisms' (Weller *et al.*, 2002). Extrapolating the results of volatile suppression to general disease suppression casts doubts on the validity of the GDS concept. Different responses of pathogens to suppressing soil volatiles were observed as none of the soils included in the survey was highly suppressive against all 3 pathogens. Furthermore, no correlation was found between the volatile mediated suppression of pairs of 2 pathogens. In contrast, the sensitivity to volatiles and the variables correlating with the volatile mediated suppression were found to be pathogen specific. Similarly, the soil-related properties explaining the variation in pathogen community composition in soil differed for the root and shoot infecting fungi as well as for the oomycetes. Our results on different aspects of general disease suppression point to a pathogen specific response, which is seemingly a contradiction in terminis.

However, the overall volatile mediated suppression of the three pathogens was related to DOC and respiration. These results are in accordance with the commonly accepted view on general disease suppression, namely that "GDS is directly related to microbial metabolic activity and mediated by availability of nutrients and energy available for growth of the pathogen through the soil" (Hoitink and Boehm, 1999). Hence, it seems that part of volatile suppression for a particular pathogen is based on general microbial activities, but as well that the individual response is pathogen specific.

The observation that the sensitivity towards disease suppression differs between pathogens would question the value of an overall indicator of general disease suppression. From a farmers perspective it is most valuable to have an indication of the level of suppressiveness against the pathogens forming a threat for their cropping system instead of an overall indicator. Thus, zooming in on suppression per soil type, geographical area or related to specific crops could give a more detailed picture. An integrated and customized approach could therefore provide a more specified risk assessment.

The role of management in disease suppression: opportunities and risks

Ultimately, for fields exhibiting a low degree of suppression, management practices that can improve the suppressiveness of a soil need to be identified. Organic matter management is one of the promising candidates, as substrate availability and quality might be one of the key variables involved in disease suppression. The meta analysis of Bonanomi et. al (2010) on compost amendment showed a variety in effects, either

suppressiveness, neutral response or conduciveness to soil-borne pathogens. Thus the effects of organic matter addition, in interplay with the indigenous microbial community, can be variable. It would therefore be advisory to study such amendments in combination with microbial parameters. One of the potential new management strategies is to stimulate microbial groups, like litter saprophytes, involved in suppression.

Besides organic matter management other, more pathogen-specific relations with farm practices were found in this study. No till yielded a higher degree of volatile mediated suppression of *Fusarium* and the application of solid manure negatively correlated with the degree of *Pythium* suppression. However, before these correlation based results can be translated to farm practices they need to be studied more mechanistically.

'Managing soil suppression by managing soil' can introduce detrimental side effects. The biological soil disinfestation applied in chapter 2, commonly used to reduce the pathogen inoculum in soil, induced a strong disturbance in the soil and subsequently a shift in the microbial community. This coincided with the loss of *Pythium* suppression in the soil. Therefore the timing and impact of management practices must be carefully considered.

Future research

Many practical aspects of how to implement the results from this thesis have been discussed in previous paragraph. But besides the research on how management practices can improve soil health including the suppressive capacity of a soil more fundamental research could improve the insights in the mechanisms of general disease suppression.

One of the main findings of this thesis is the correlation between disease incidence (as indicated by root biomass) and inhibitory volatiles released from soil. This finding linked bulk soil processes (fungistasis) to actual disease suppression. However the role of crop plants was not including in our experiments, despite the knowledge that root exudate strongly influence the microbial community and, consequently, soil functions (Berg and Smalla, 2009).

The effect of changes in the microbial community on volatile mediated pathogen suppression is shown in chapter 2 (Figure 2.1). It is thus likely that exudation influences volatile mediated suppression indirectly, by shaping the microbial community performing this function. Plants are thought to select for specific microbes in the vicinity of their roots, and exudation is the driving force of this selection (Bais *et al.*, 2006; Berg and Smalla, 2009). The composition of root exudates, and potentially specific compounds mediate these plant-microbe interactions (Dennis *et al.*, 2010).

Plants may, besides shaping the rhizosphere, influence volatile production directly by

providing the substrate (exudates) for microbial metabolism. Garbeva *et al.* (2014b) showed that root exudate composition affected the production of fungus-inhibiting volatiles by the bacteria *Collimonas*, a higher amount of amino acids resulted in stronger inhibition of fungi.

Do plants call for help? From studies on insect herbivory it is known that plants can actively attract natural enemies of the herbivorous insect by releasing volatile compounds from the damaged leaves. For example (Kessler and Baldwin, 2001) showed that a plant could strongly reduce the number of herbivores (>90% reduction) by releasing volatiles, confirming that indirect (volatile) defenses can operate in aboveground plant defenses.

An observation made in one of our experiments (Figure 6.2) indicates that also belowground indirect defenses could be mediated by plants. The *Rhizoctonia* growth reduction upon exposure to soil emitted volatiles from two different soils (bulk soil and bulk soil after three weeks of crop growth in a pot experiment) showed stronger soil suppression after plant growth. One of the possible explanations of this effect is that plants selected for suppressive microbes via exudation.

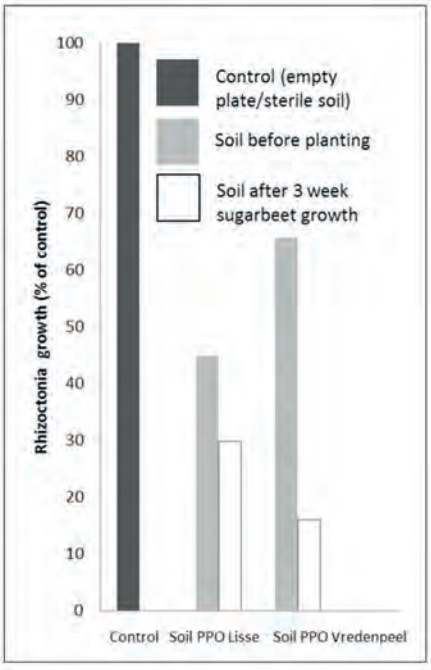


Figure 6.2
Average hyphal weight of *R. solani* hyphae that had been exposed to volatiles produced by 2 different soils before and after plant growth in the soil. *Rhizoctonia* biomass is presented as percentage of the empty plate control.

Future research on the role of host plant induced volatile mediated suppression should focus on the role of root exudates and single compounds therein. Besides identification of compounds, mutant lines, differencing in there exudation profile, could provide a mechanistic understanding of the fluxes of specific compounds and their effects on volatile mediated suppression in soil.

Conclusions

My thesis addressed different aspects of general disease suppression; research on these different aspects refined the existing knowledge on general disease suppression in agricultural soils. To summarize I could conclude that volatiles emitted from agricultural soils could reduce the growth of pathogens and that this coincided with reduced disease incidence as well as with a shift in the microbial community of the indigenous soil bacteria (related to the legacy effects of management practices). Not only the microbial composition could influence volatile mediated pathogen suppression, also a wide range of soil-related variables corresponded. The overall suppression of growth of different pathogens was linked to microbial activity and organic substrate. However different pathogens showed different sensitivity towards volatiles, and soil-related factors corresponding to volatile mediated suppression were pathogen specific.

All soils harbor a pool of pathogens. The composition of this pathogen seedbank is influenced by the environment. Pathogens differing in phylogeny or infection site respond differentially to soil-related variables. For example oomycete presence seemed unrelated to their environmental context, whereas variation in root and shoot pathogen community compositions were linked to soil physico-chemical properties and microbial community composition, with potentially a significant role of litter saphrophytes therein. The presence of pathogens in soil is not necessarily related to their growth in the presence of root exudates, as exposure to root exudates showed soil specific pathogen dynamics.

The results of this thesis gave insight into different aspects of disease suppression in agricultural soils which could serve as a fundament to develop environmentally-friendly control methods based on natural occurring ecological processes.

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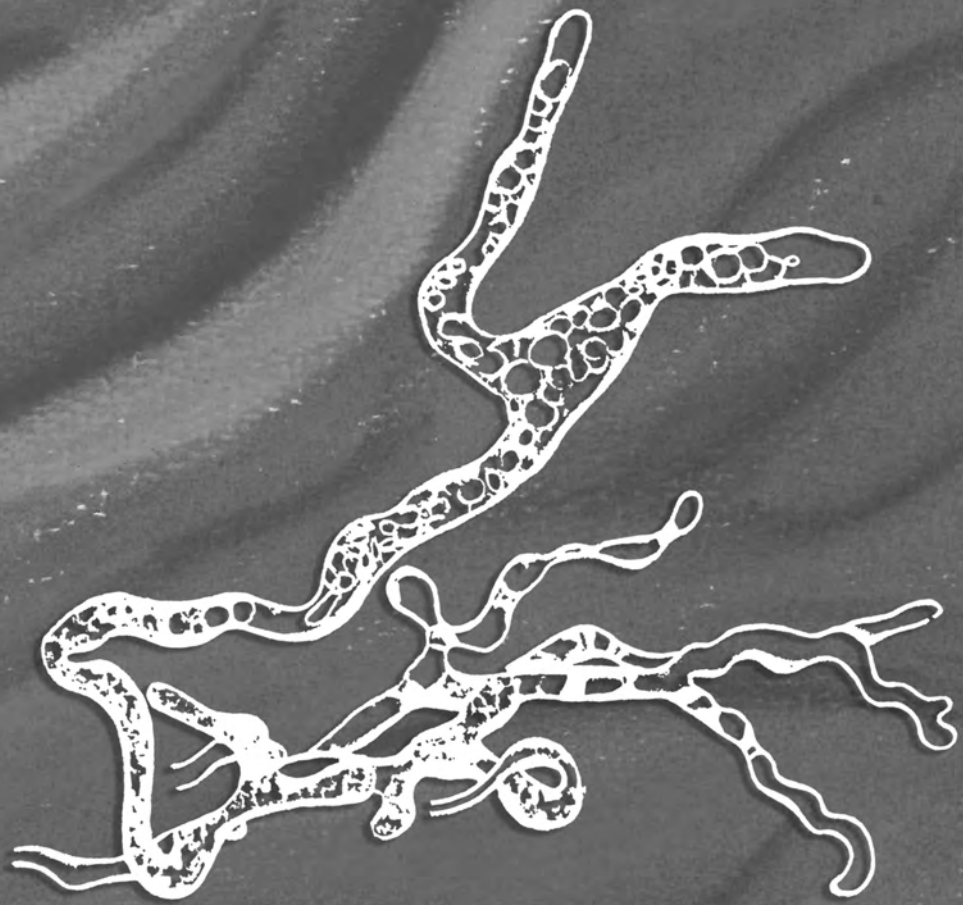

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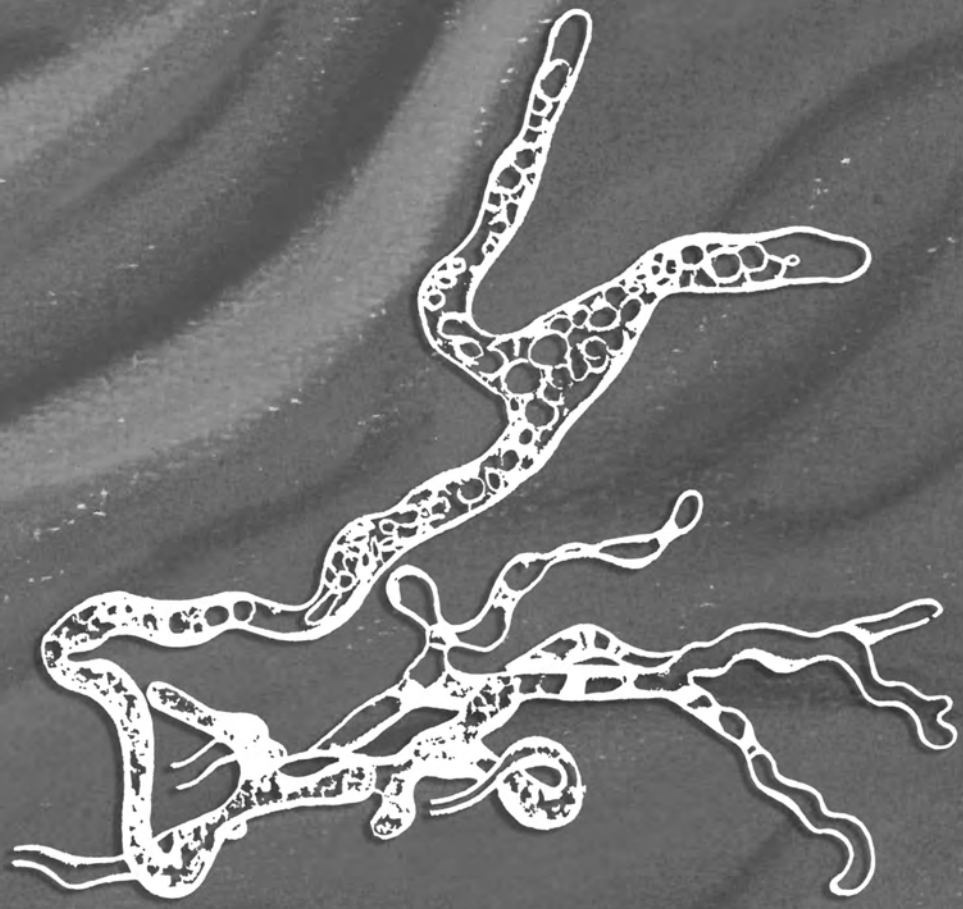


Samenvatting

Ziekteverwekkende organismen die in de bodem kunnen (over)leven en vanuit daar gewassen infecteren zijn moeilijk te bestrijden doordat ze een 'verborgen' leven leiden onder de grond. Dit beperkt de werkzaamheid van chemische en biologische middelen en zorgt wereldwijd voor grote opbrengst verliezen. Of er daadwerkelijk een gewasziekte uitbreekt in een akker is niet alleen afhankelijk van de aanwezigheid van pathogenen in de bodem, maar is ook gerelateerd aan bodemeigenschappen zoals fysisch-chemische bodemkaraketeristieken en de activiteit en samenstelling van de microbiële gemeenschap. Concurrentie tussen micro-organismen om het schaars aanwezige voedsel (afbreekbare organische stoffen) in de grond beperkt of voorkomt de kieming en groei van pathogenen en remt zo de uitbraak van een gewasziekte. Deze competitie kan op twee manieren plaatsvinden: direct door middel van snelle opname van de aanwezige voedingstoffen, substraat competitie genoemd, of indirect doormiddel van het uitscheiden van remmende stoffen, interferentie competitie genoemd.

Het totale ziekte onderdrukkende effect van alle op competitie gebaseerde mechanismen wordt 'algemene ziektewerendheid' genoemd. Het doel van mijn onderzoek was om verschillende aspecten van deze ziekteonderdrukking in de bodem te bestuderen om inzicht te krijgen in de wisselwerking tussen de microbiële gemeenschap, de dynamiek van pathogenen en de samenstelling van organische stof.

Als eerste werd de rol van vluchtige organische stoffen, geproduceerd door de microbiële gemeenschap, in de natuurlijke onderdrukking van pathogenen onderzocht. In hoofdstuk 2 wordt een serie van experimenten beschreven die nauw verbonden zijn met elkaar en gelijktijdig werden uitgevoerd. In dit hoofdstuk is gekeken naar een landbouwperceel dat, onderverdeeld in kleine proefvlakken, verschillende bewerkingen heeft gekregen. Hierin konden we aantonen dat er een sterke correlatie bestaat tussen wortelgewicht en onderdrukkende effecten van vluchtige stoffenop *in vitro* groei van de plantpathogeen *Pythium intermedium*. Er werd geen of slechts beperkte groeiremming van de pathogeen door deze vluchtige stoffen gevonden in de grond waarin veel wortels geinfecteerd waren, terwijl de gronden met een sterke pathogeen remming door vluchtige stoffen weinig infectie en een hoge wortelbiomassa lieten zien. Daarnaast was er ook een duidelijke verschuiving te zien in de samenstelling en diversiteit van de bodembacteriën die samenviel met de afwezigheid van pathogeen-onderdrukking, een nalatenschap van de grondbewerkingen die waren uitgevoerd. Als laatste is er ook bepaald welke vluchtige stoffen vrijkwamen uit de verschillend bewerkte gronden. Op basis hiervan is een lijst gemaakt met kandidaat remmende stoffen en van bacterie-groepen die mogelijk verantwoordelijk zijn voor de productie van deze vluchtige stoffen. Alles samengenomen geeft deze studie een sterke aanwijzing dat vluchtige stoffen die worden geproduceerd door bodem microben een belangrijke rol kunnen hebben in algemene ziektewering.

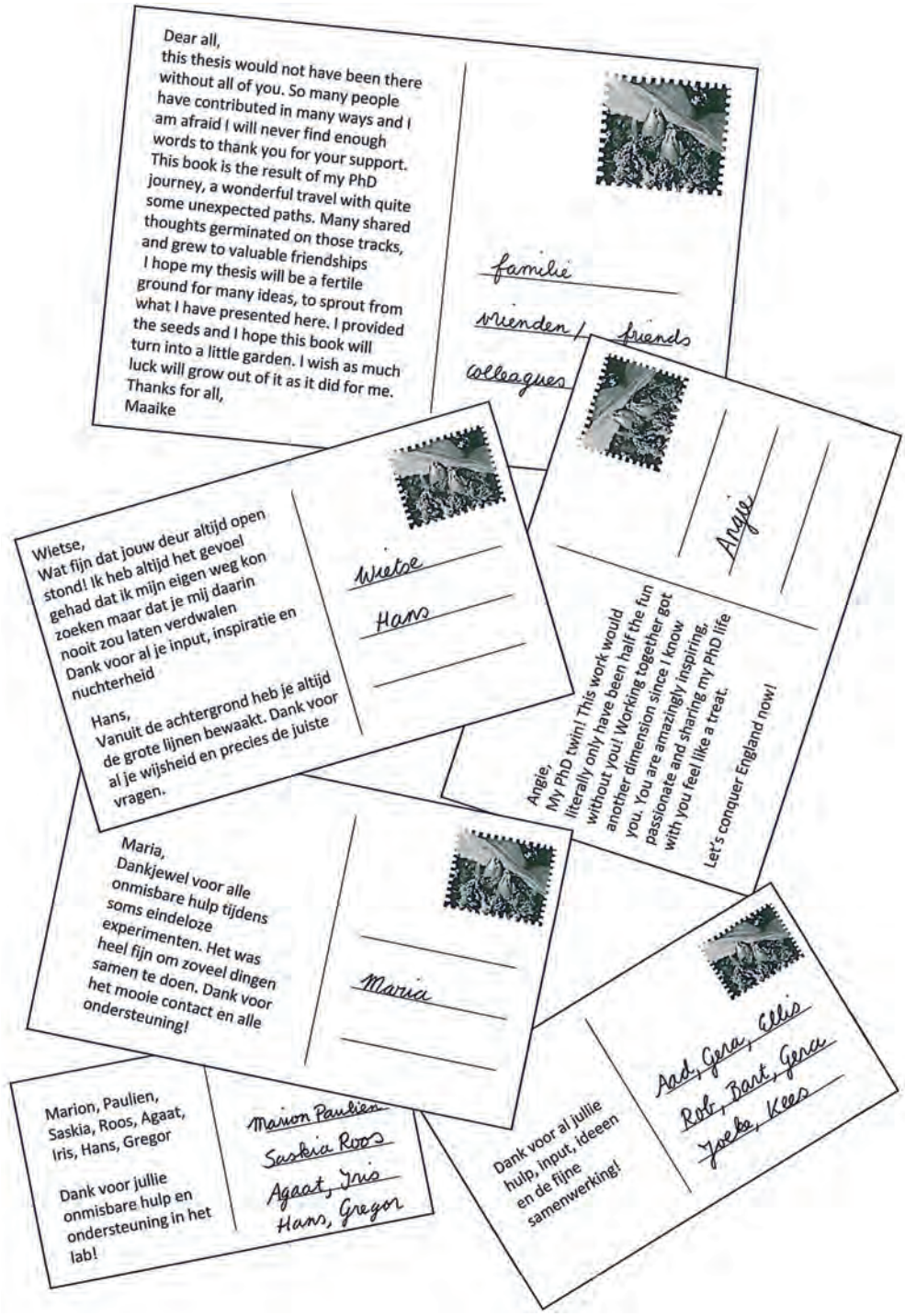
Als vervolg op dit onderzoek werd de invloed van bodemeigenschappen (biotische als abiotische eigenschappen) op de onderdrukking van de *in vitro* groei van verschillende plantpathogenen onderzocht (Hoofdstuk 3). Hiervoor werd een breed onderzoek

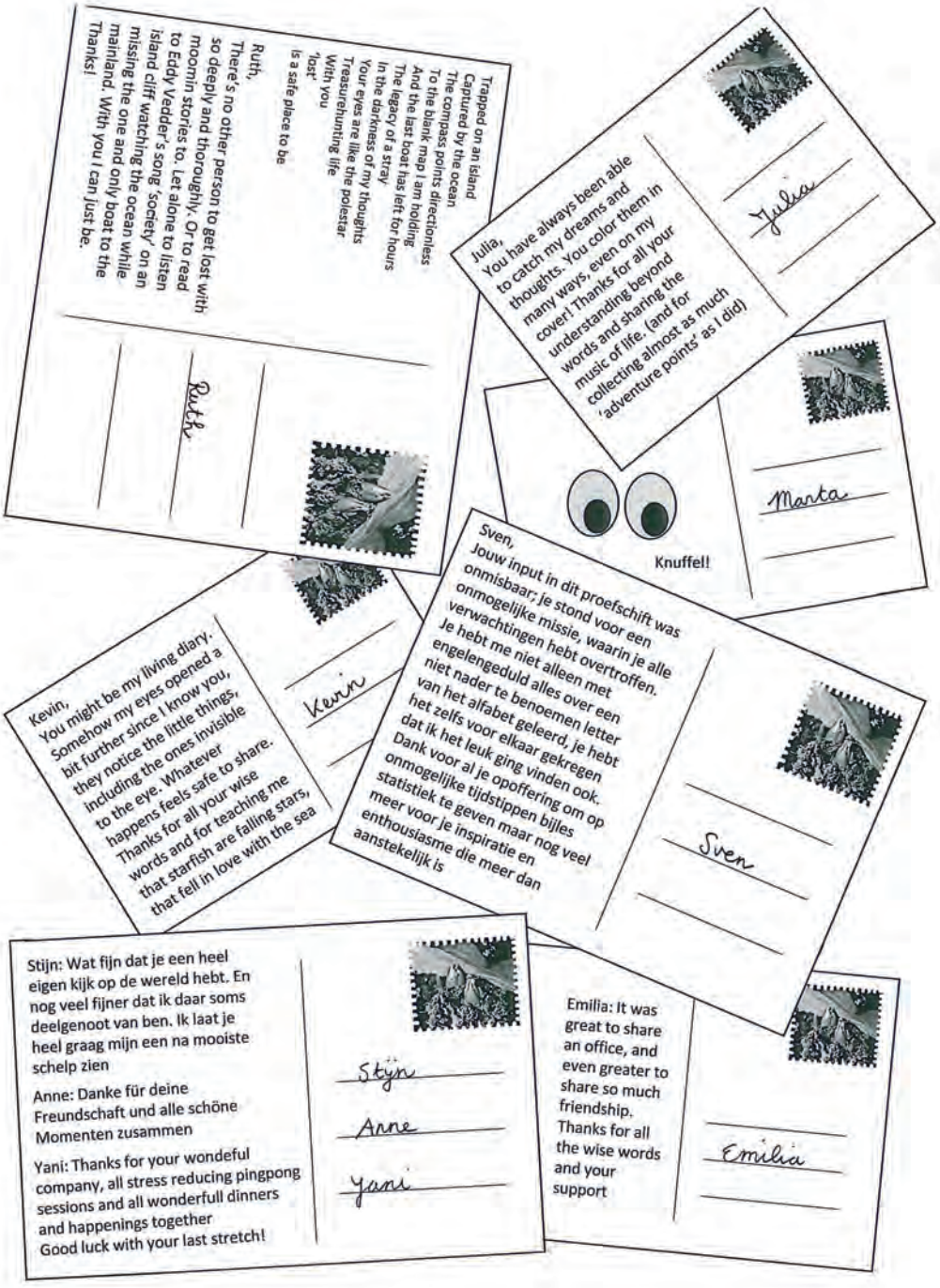
opgezet waarin grond uit 50 akkers verspreid over Nederland werd onderzocht. In dit onderzoek werd de onderdrukkende werking van vluchtige stoffen uit deze 50 bodems op drie verschillende plant pathogenen (*Rhizoctonia solani, Fusarium oxysporum* en *Pythium intermedium*) gerelateerd aan een reeks van bodemeigenschappen met de hulp van univariate en multivariate statistische modellen. De analyses gaven aan dat de onderdrukking van de drie pathogenen voornamelijk wordt bepaald door de activiteit van het bodemleven en de aanwezigheid van afbreekbare organische substraten. Echter, als de verschillende pathogenen individueel werden geanalyseerd waren er duidelijke verschillen te zien in gevoeligheid voor de vluchtige stoffen. Daarnaast verschilden ook de bodemeigenschappen die relateerden aan de onderdrukking van de groei per pathogeen.

In hoofdstuk 4 werd de aanwezige voorraad van (mogelijke) ziekteverwekkende schimmels en oomyceten in de 50 landbouwbodems onderzocht, aan het begin van het groeiseizoen. De aanwezigheid van deze overlevende ziekteverwekkende schimmels en oomyceten werd ook gerelateerd aan de eigenschappen van de akker waarin ze werden gevonden. Dit werd gedaan om de aanwezigheid van ziekteverwekkers te kunnen relateren aan omgevingseigenschappen; het doel was te bestuderen of deze verschillen in aanwezigheid van pathogen tussen verschillende akkers veroorzaakt wordt door bodem gerelateerde eigenschappen zoals grondsoort, gewasrotatie en grondbewerking. Uit het onderzoek blijkt dat het sterk aan het type pathogeen ligt welke bodemeigenschappen corresponderen. De aanwezigheid van oomyceten kon bijvoorbeeld niet worden verklaard met de gemeten grond-gerelateerde variabelen. Aanwezigheid van wortel- en bladinfecterende schimmels was daarentegen wel gerelateerd aan de bodem en omgevingseigenschappen zoals pH, klei, grondbewerking, gewasrotatie en de microbiele gemeenschap met mogelijk daarbinnen een belangrijke rol voor saprofytische schimmels.

Vaak begint de pathogeen alleen te groeien als er daadwerkelijk een waardplant aanwezig is. Echter, in een ziekteonderdrukkende bodem wordt dit voorkomen. Omdat de aanwezigheid van plant pathogenen in de bodem niet direct hoeft te betekenen dat er ook een ziekteuitbraak zal komen isde reactie van de pathogeen in de aanwezigheid van stimulerende stoffen uit de wortel van de plant onderzocht. Hoofdstuk 5 beschrijft de ontwikkeling van een test systeem dat de aanwezigheid van een wortel nabootst en met hulp van qPCR de groei van de pathogeen over de tijd volgt. De aanwezigheid van wortelexudaten liet bodem specifieke reacties op de groei van *Pythium intermedium* zien: in sommige bodems is er geen respons maar andere bodems laten een sterke toename van de plantpathogeen zien. Dit lijkt er op te wijzen dat inderdaad sommige bodems de aanwezige pathogen kunnen onderdrukken. Deze methode heeft daarom de potentie om de gevoeligheid van een grond voor ziektes door een bepaalde ziekteverwekker te voorspellen zonder dat bewerkelijke bioassays nodig zijn. De resultaten in dit proefschrift geven nieuwe inzichten in de verschillende aspecten van algemene ziekteweredheid in landbouwbodems. Dit zou als een opstap kunnen dienen tot de ontwikkeling van omgevingsvriendelijke bestrijdingsmethoden die gebaseerd worden op bestaande, natuurlijke ecologische processen. De ideeën hoe in de praktijk kan worden gebracht en voor verdere onderzoeksrichtingen worden bediscussieerd in hoofdstuk 6.

Acknowledgements




Trapped on an island
Captured by the ocean
The compass points directionless
To the blank map I am holding
And the last boat has left for hours
The legacy of a stray
In the darkness of my thoughts
Your eyes are like the polestar
Treasurehunting life
With you
'lost'
Is a safe place to be
Ruth,
There's no other person to get lost with so deeply and thoroughly. Or to read moomin stories to. Let alone to listen to Eddy Vedder's song 'society' on an island cliff watching the ocean while missing the one and only boat to the mainland. With you I can just be.
Thanks!
Ruth
Julia,
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Julia
Marta
Knuffel!
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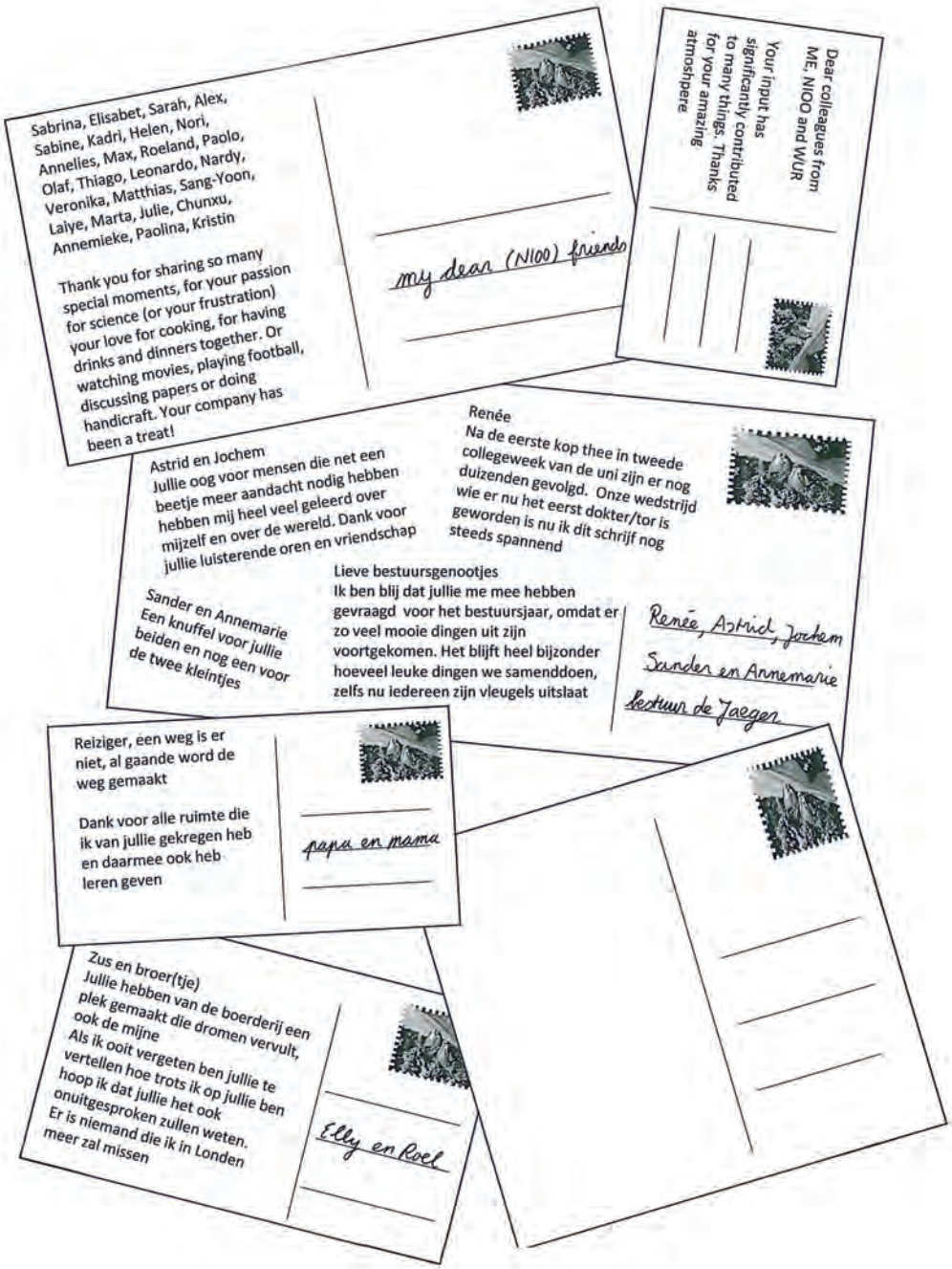
Sven
Stijn: Wat fijn dat je een heel eigen kijk op de wereld hebt. En nog veel fijner dat ik daar soms deelgenoot van ben. Ik laat je heel graag mijn een na mooiste schelp zien
Anne: Danke für deine Freundschaft und alle schöne Momenten zusammen
Yani: Thanks for your wondeful company, all stress reducing pingpong sessions and all wonderfull dinners and happenings together
Good luck with your last stretch!
Stijn
Anne
Yani
Emilia: It was great to share an office, and even greater to share so much friendship. Thanks for all the wise words and your support
Emilia


Sabrina, Elisabet, Sarah, Alex, Sabine, Kadri, Helen, Nori, Annelies, Max, Roeland, Paolo, Olaf, Thiago, Leonardo, Nardy, Veronika, Matthias, Sang-Yoon, Laiye, Marta, Julie, Chunxu, Annemieke, Paolina, Kristin
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my dear (NIOO) friends
Dear colleagues from ME, NIOO and WUR
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Lieve bestuursgenootjes
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Renée, Astrid, Jochem
Sander en Annemarie
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papa en mama
Zus en broer(tje)
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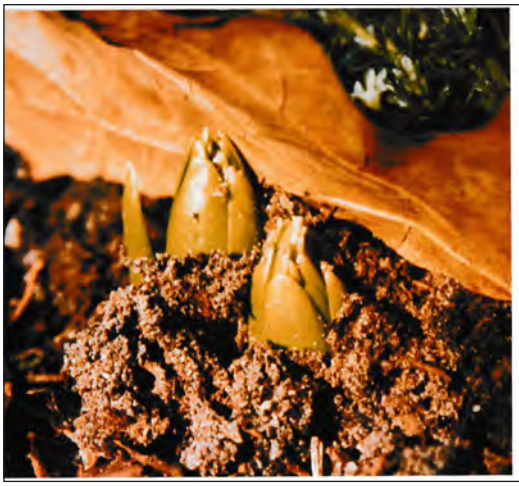
Elly en Roel

Curriculum vitae



O jij, zaad van mijn levensplant.
Je was zo lang verborgen in mijn ontbottende ziel.
J. Khan

Wij zijn dankbaar en blij met de geboorte van
onze dochter en zusje

MAAIKE

geboren op 20 mei 1984

Rolf, Mieke en Elly van Agtmaal

Piepenbeltweg 27, 6971 JH Brummen

Maaike van Agtmaal was born May 20, 1984. Her interest in soil and plant interactions could not have started earlier…..

Being raised at the family farm near the river IJssel and with a keen eye for the surrounding world the choice to study Biology at Wageningen University after finishing highschool in 2002 was very suiting to her amazement about the growing organisms around her. Her main interest during her bachelor was host-parasite interactions in both animals and plants. This topic was continued during her Masters' degree with a thesis project in Wageningen at the laboratory of Nematology and a stay in Edinburgh at the Institute for Infection and Immunity Research of Edinburgh University looking at the role of a group of nematode/hookworm secreted proteins in plant and animal infections. After finishing her Master Biology and a short stay as guest researcher at the laboratory of Phytopathology at Wageningen University she continued her studies in 2010 at the Netherlands Institute of Ecology (NIOO) in a PhD project focusing on disease suppression in agricultural soils, presented in this thesis. She will continue her research at Imperial College as postdoctoral fellow in Microbial Ecology.

PE&RC Training and Education Statement

With the training and education activities listed below the PhD candidate has complied with the requirements set by the C.T. de Wit Graduate School for Production Ecology and Resource Conservation (PE&RC) which comprises of a minimum total of 32 ECTS (= 22 weeks of activities).

Review of literature (6 ECTS)

- Prediction of disease suppressiveness

Post-graduate courses (5.6 ECTS)

- Autumnschool host microbe interactions; EPS (2011)
- qPCR Experience real-time PCR in plant pathology: diagnostics and research; COST (2011)
- Soil, biodiversity and life; PE&RC (2012)
- Dynamics of organic matter in soil; University of Copenhagen (2013)

Laboratory training and working visits (6.9 ECTS)

- DNA Array; Scientia Terrae, Sint Katelijne Waver, Belgium (2011)
- 454 Pyrosequencing; Scientia Terrae, Sint Katelijne Waver, Belgium (2013)

Deficiency, refresh, brush-up courses (1.5 ECTS)

- Basic statistics (2012)

Competence strengthening / skills courses (2 ECTS)

- Effective behaviour in your professional surroundings; WGS (2012)
- How to write a world class paper; WUR (2013)
- Selling science to society; NIOO (2013)

PE&RC Annual meetings, seminars and the PE&RC weekend (3.3 ECTS)

- PE&RC Weekend (2011, 2014)
- NERN (2011, 2013, 2014)

Discussion groups / local seminars / other scientific meetings (9.6 ECTS)

- Microbial ecology group meeting (2010-2014)
- KNPV Symposia (2010-2014)
- STW Meeting (2010-2014)
- KNPV Working group soil-borne pathogens meeting (2010-2015)

International symposia, workshops and conferences (17.3 ECTS)

- Ecology of soil microorganisms; Prague (2011)
- International student conference om Microbial Communication MiCom; Jena (2011)
- Functions and ecology of the plant microbiome; Rhodos (2012)
- ISME; Copenhagen (2012)
- IOBC Biocontrol of plant pathogens; Reims (2012)
- CNRS-Jacques Monod conference: bacterial-fungal interactions: a federative field for fundamental and applied microbiology; Roscoff (2013)
- ISME; Seoul (2014)
- GSBI Conference; Dijon (2014)

Lecturing / supervision of practical's / tutorials (3 ECTS)

- Internship student (2011, 2013)
- Ecological aspects of biological interactions; WUR (2012)

Supervision of 2 MSc students

- Volatiles produced by bacterial community in the bulk soil suppress Rhizoctonia solani and Pythium intermedium
- Rhizosphere interactions and Pythium dynamics in Dutch agricultural soils

The research presented in this thesis was conducted at the department of Microbial Ecology of the Netherlands Institute of Ecology (NIOO-KNAW) in Wageningen and financially supported by the Dutch Technology Foundation STW, which is the applied science division of NWO and the Technology Programme of the Ministry of Economic Affairs.

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