

Oligotrophic Bacteria and Root Disease Suppression in Organically Managed Soils

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Oligotrophic Bacteria and Root Disease Suppression in Organically Managed Soils

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Abstract

The objective of this thesis was to obtain a better understanding of soil health in terms of microbial and chemical characteristics as well as suppression of soil borne plant pathogens. Organic soils were chosen as an appropriate model for studying soil health. Four different organic amendments were applied on two experimental fields with different crop history, providing a diverse range of soil quality levels. Many soil microbial variables were measured, including copiotrophic and oligotrophic bacterial populations, the abundance and diversity of *Eubacteria*, *Alphaproteobacteria*, *Pseudomonas* and fungal communities, as well as several microbial genes involved in nitrogen cycling. Plant disease suppressiveness was used as a quantitative integrative parameter reflecting the health status of soils. *Rhizoctonia solani* on beet and *Fusarium oxysporum* on flax were selected as pathosystems; areas under disease progress curves were measured in bioassays with differentially amended field soils and were related to soil parameters. Combined rather than single amendments enhanced *Fusarium* suppression, but *Rhizoctonia* suppression was more related to crop history than organic amendments. No universal correlations were found between disease suppression and microbial and chemical parameters, although pH and organic matter affected microbial communities and *Fusarium* wilt. A significant relation between ammonia oxidizing bacteria and disease suppression was observed for both pathogens; this relation was likely indirect via nitrogen availability and pH. No direct relationship was found between quantities of N cycling genes and disease suppression. A specific emphasis was put on the potential role of oligotrophic bacteria in soil health and disease suppression. Bacteria isolated on low carbon medium (10 mg C/L) were repeatedly transferred onto this medium to select true oligotrophic bacteria. Most isolates could grow on both low carbon and higher carbon (1000 mg C/L) media and belonged to *Streptomyces*, *Rhizobium*, *Bradyrhizobium* and *Mesorhizobium*. A new oligotrophic isolate was identified as *Collimonas* sp. IS343 and its interaction with *R. solani* was studied. This strain was better adapted to oligotrophic conditions than a copiotrophic *Collimonas* reference strain and was more effective in controlling *R. solani*. This thesis provided a better understanding of some aspects of soil health and emphasized the role of oligotrophic bacteria, a poorly understood but very important group of soil inhabitants.

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Chapter 1

General introduction

Background

Soil health

The concept of soil health refers to the biological, chemical, and physical features necessary for long-term, sustainable agricultural productivity with minimal environmental impact. Healthy soils maintain a diverse community of soil microorganisms that help to: (1) recycle plant nutrients and improve soil structure with positive repercussions for its water- and nutrient-holding capacity; (2) form beneficial symbiotic associations with plant roots (e.g. nitrogen fixing bacteria and mycorrhizal fungi); (3) control plant diseases as well as insect pests; (4) improve crop production (Arias et al., 2005). A healthy soil has been defined as a stable soil, resilient to disturbances, with high biological diversity and high levels of internal nutrient cycling (Elliott et al., 1994; Mendes et al., 2011; Van Bruggen and Semenov, 2000). In healthy soils, cycles of C, N and P are balanced, without major losses to the environment (Kibblewhite et al., 2008). The absence of large concentrations of easily available nutrients prevents the establishment of fast-growing saprotrophic microorganisms, including pathogens (Van Bruggen and Semenov, 1999). Therefore, oligotrophic microorganisms are generally in the majority in healthy soils (Van Bruggen et al., 2006). In addition, the ability of the biological community to decrease disease incidence and severity by suppressing plant pathogens is characteristic of soil health (Hiddink et al., 2005; Van Bruggen and Termorshuizen, 2003).

Oligotrophy

Oligotrophs are adapted to exploit ecological niches characterized by low substrate concentrations and low energy flows. They have highly effective systems for the uptake of inorganic and organic nutrients that occur in nano- and picomolar concentrations, due to their efficient uptake and utilization systems characterized by unique metabolic regulation (Semenov, 1991). The ecological significance of oligotrophs is that they not only grow at low nutrient concentrations, but their activities are also responsible for reducing concentrations of low-molecular weight carbon compounds below threshold levels for catabolite repression for hydrolysis, so that production of enzymes can take place that are repressed by high catabolite concentrations. Oligotrophic bacteria can also contribute to the regulation of copiotrophic activity by keeping the concentration of low molecular weight substances at such low levels that they become inaccessible for the majority of eutrophs, including plant pathogens. Oligotrophs preferably occupy virgin soils, and are present in smaller numbers in conventionally managed than in organically managed soils (Semenov, 1991; Van Bruggen and Semenov, 2000).

Organic soil management

Organic management practices do not allow high inputs of mineral fertilizers and pesticides, therefore

organic soils are less eutrophic than conventionally managed soils. Organic soils often have higher stabilized organic matter contents and lower concentrations of carbon sources easily available to microorganisms associated with very fast turnover rates (Brock et al., 2012; Van Diepeningen et al., 2005). They also have more complex food webs, higher biodiversity and biomass, and contain much less toxic substances which makes them closer to natural soils (Cong et al., 2006; Kremen et al., 2012). In organic soils, oligotrophic bacteria can usually be found in higher numbers. Oligotrophic soils are low in low-molecular weight carbon or nitrogen sources that serve as easily available substrate for primary consumers. In general, organic (oligotrophic) soils can be considered more healthy than conventional (eutrophic) soils as additionally evidenced by enhanced root disease development, nitrate leaching and emission of greenhouse gases in conventionally managed soils (He et al., 2012). Root disease suppression in organically managed soils has been demonstrated for many soilborne pathogens (Bonanomi et al., 2010; Baysal et al., 2008; Bulluck et al., 2002; Grünwald et al., 2000; Hiddink et al., 2005; Liu et al., 2007; Workneh and Van Bruggen, 1994).

Root disease suppression

Soil suppressiveness towards plant pathogens can be divided into two types, general suppressiveness and specific suppressiveness (Weller et al., 2002; Mazzola, 2002). General suppressiveness is characterized by activity of the total microbial biomass and is not transferable between soils, whereas specific suppressiveness is characterized by the activity of a single or few species of microorganisms and is transferable. The classical example of specific soil suppressiveness is take-all decline in soils under continuous wheat production, where the accumulation of a specific 2, 4-diacetylphloroglucinol-producing *Pseudomonas fluorescens* population is believed to play an important role (Weller et al., 2002). General suppressiveness is expected to be the most important type of pathogen suppression recorded in soils amended with organic materials (Pérez-Piqueres et al., 2006) or with different histories of crop coverage (Garbeva et al., 2006). Increase in pathogen suppression in the same soil upon organic amendments or differences in crop histories thus can often be attributed to changes in the whole community or at least within major groups of the soil microbial community. The exact mechanisms of general disease suppression (as opposed to specific disease suppression) are mostly unknown, although general competition for nutrients and production of antibiotics or cell-wall degrading enzymes have been implicated. Soilborne pathogens are commonly suppressed in healthy soils (Van Bruggen and Termorshuizen, 2003).

Plant pathosystems used in this research

Two plant pathosystems were selected to perform root disease suppression assays: *Rhizoctonia solani* Kühn on sugar beets and *Fusarium oxysporum* f.sp. *lini* Snyder & Hansen on flax. These systems were

selected because they represent different types of diseases, resulting in different symptoms: necrotic lesions or seedling damping-off and vascular wilt, respectively. The pathogens also differ in their survival structures, autecology and synecology, saprophytic ability and infection mechanisms. Suppression of such different pathogens in the same soil may indicate general rather than specific suppression.

Rhizoctonia solani is the imperfect state of a basidiomycetous fungus, *Thanatephorus cucumeris*, that does not produce conidia. The teleomorph rarely occurs in temperate climates, but can be seen on infected plant tissues in the humid tropics (Sneh et al., 1996). Vegetative hyphae of *R. solani* are relatively wide, multinucleate, and have clear septae close to side branches that are at right angles. The hyphae of one colony anastomose readily with compatible hyphae of another colony. Several anastomosis groups are distinguished that have some specificity towards their host plants. In the research for this thesis *R. solani* anastomosis group AG2-2IIIB was used, which is pathogenic on a wide range of herbaceous, dicotyledonous hosts, including sugar beets. The pathogen is capable of long-term survival in soil and plant tissues because it has a high competitive saprophytic ability, thus can colonize soil organic matter as mycelium, and forms durable sclerotia. In the presence of a host sclerotia will germinate and produce infectious hyphae (Agrios, 1997). Hyphae of *R. solani* can be chemically attracted by roots and sloughed off cells, and then grow on and decompose plant cells or residues. It produces extracellular enzymes which degrade plant cell walls (Cubeta, 1997). After attachment to a root or hypocotyl, the fungus continues to grow on the plant surface producing an infection cushion that penetrates the plant, which releases nutrients required for further fungus growth (Alexopoulos, 1996). *R. solani* causes a variety of symptoms like damping off, sunken lesions on the hypocotyl, root rot, stem rot, and blight, affecting a wide range of field and vegetable crops. *R. solani* has a relatively high optimum temperature (around 30°C, depending on the isolate) and is sensitive to low oxygen concentrations (Parmeter, 1970). There is no uniform strategy for *R. solani* control and management. However, minimum-tillage systems, surface residues, neutral pH, a relatively low soil moisture content, and high available nitrogen commonly favour disease development, while regular or deep tillage, low pH and available nitrogen, high ammonia and high soil water content may suppress diseases caused by *R. solani* (Stone et al., 2004). Also, *R. solani* may be suppressed by enriching soil with microbial antagonists (e.g. actinomycetes, *Bacillus* spp., or *Pseudomonas* spp.) or stimulating competitors and antagonists by organic amendments, for example glucosinolate-producing *Brassica* species (Garbeva et al., 2011; Sacristan et al., 2011; Weller et al., 2002; Yulianti et al., 2006).

Fusarium oxysporum is a genetically heterogeneous polytypic morphospecies (Gordon and Martyn, 1997). Several teleomorphs have been found associated with *F. oxysporum*, including *Gibberella* and *Nectria* species, confirming its heterogeneity. *F. oxysporum* is a widespread soil inhabitant and endophyte. Many strains are nonpathogenic, but able to colonize the root cortex of a wide variety of plants, including monocotyledonous and dicotyledonous genera (Leoni et al., 2013).

However, there are also many pathogenic strains that are widespread. Plant pathogenic *F. oxysporum* strains cause wilts and sometimes root rots on many plant species (Gordon and Martin, 1997). Although many plant species may be affected by *F. oxysporum*, there is distinct specialization among strains, so that certain strains can only cause wilt in a limited number of closely related plant species or even a single plant species. These specialized strains are called formae speciales or f. sp., for example *F. oxysporum* f. sp. *lini* that induces wilt in flax, *Linum usitatissimum* L. Optimal temperature and moisture conditions are very much dependent on the forma specialis and its host. *F. oxysporum* can form three different spore types, macroconidia, microconidia and chlamydospores. Long-term survival is mainly in the form of chlamydospores, but *F. oxysporum* can also survive as conidia and as mycelium in plant materials. Pathogenic *F. oxysporum* has only moderate competitive saprophytic ability compared to non-pathogenic strains or *R. solani* (Takehara et al., 2003). *F. oxysporum* invades plants roots with germ tubes emerging from conidia or chlamydospores. Hyphae grow along junctions of epidermal cells and then penetrate intercellularly especially behind the root tip and where lateral roots emerge. Mycelium then proliferates in the cortex. Intracellular penetration is also possible in susceptible hosts; invasion of the vascular region through the caspary strip results in wilting of the infected plant (Agrios, 1997; Hall, 2013). The mechanism of wilt induction is still controversial, but seems to be partially dependent on the pathogen's ability to produce a toxin and partially on the response of the host plant producing gel-like materials in an attempt to arrest the fungus (Hall, 2007). Some pathogenic *F. oxysporum* strains produce mycotoxins that are toxic to animals. Infection by *F. oxysporum* is generally enhanced in the presence of root knot nematodes. Infection can be negatively affected by crop rotation, tillage practices, cover cropping or sowing resistant cultivars (Fravel et al., 2003). Suppressiveness against pathogenic *F. oxysporum* and other *Fusarium* species is often associated with non-pathogenic *Fusarium* spp., *Pseudomonas* spp. (Alabouvette, 1999; Oyarzun et al., 1994) or actinomycetes (Castano et al., 2011; Mendes et al., 2011). In conducive soil, *F. oxysporum* f. sp. *lini* causes flax wilt - plant leaves turn yellow, top leaves thicken, lower leaves may wilt, plant growth stops, and ultimately, the infected plant becomes brown, necrotic and dies.

General hypothesis

The general hypothesis behind this thesis was that agricultural soil managed according to strict organic principles, by addition of complex organic amendments that are relatively high in lignin and phenolic compounds, are relatively low in carbon and nitrogen sources that would be readily available for micro-organisms. This will favour nutrient scavenging bacteria (so called 'oligotrophs') over more copiotrophic types to which most phytopathogens belong. Low carbon and nitrogen availability in soils will have the following three consequences on microbial community composition and soil functioning:

1. organically managed soils low in easily available carbon and nitrogen are diverse in microbial species, in particular oligotrophs,
2. resilient towards disturbances and invading pathogens,
3. efficient in chemical transformations of essential elements like carbon and nitrogen.

Aim of the thesis

The main goal of this thesis was to assess the soil health status of organically managed soils in terms of plant disease suppression and oligotrophy.

Experimental objectives were:

- to determine the relationship between carbon availability and population density of bacteria isolated on media low in carbon available for bacteria from soils treated with different organic amendments and partially characterize the isolated bacteria based on 16SrRNA sequences;
- to analyze the effects of different organic amendments on suppression of disease development of *R. solani* on beets and *F. oxysporum* f. sp. *lini* on flax, and relate this suppression to soil chemical characteristics and bacterial communities as determined by polymerase chain reaction (PCR) and denaturing gradient gel electrophoresis (DGGE) on DNA directly isolated from soil;
- to investigate in vitro and in vivo interactions between a selected oligotrophic bacterial isolate, *Collimonas* sp., and *R. solani*;
- to investigate the relationships between number of genes involved in nitrogen cycling, amount of nitrogen available for micro-organisms in soil and suppression of *R. solani* and *Fusarium oxysporum* f.sp. *lini*.

Thesis outline

The research started with isolation of oligotrophic bacteria from soil samples collected from organically managed fields with different crop and management histories. Using repeated plating techniques, a selection for 'true' oligotrophs was attempted. As a result, a subset of bacteria isolated on low C medium (about 10 micrograms per liter) was obtained and partially characterized (chapter 2).

In order to study the interrelationship between soil health status and other soil parameters, suppressiveness tests against *F. oxysporum* f. sp. *lini* and *R. solani* were performed on flax and beet seedlings, respectively, using the same soil samples as mentioned for chapter 2. Various chemical characteristics of the soils were determined as well as microbial characteristics using plating and

molecular (DGGE, qPCR) techniques. Specific emphasis was placed on the abundance and community structure of eubacteria, fungi, *Pseudomonas* and ammonia-oxidizing bacteria. Various effects of previous crop history and recent organic amendments on disease suppressiveness were detected and correlations between microbial communities and soils suppressiveness were established (chapters 3 and 4).

A newly isolated and characterized oligotrophic strain of *Collimonas* sp. (IS343) was studied in more detail, its growth properties and interaction with *R. solani* were determined in comparison with a well-characterized chitinolytic strain of *Collimonas*. Suppressive effects of *Collimonas* IS343 on *R. solani* development both in vitro and in soil were investigated (chapter 5).

An additional study was performed where different potential N sources and functional genes involved in nitrogen oxidation and reduction in soils were correlated. Specifically, the relation between ammonia-oxidizing bacterial communities and disease suppression in organically managed soils was explored (chapter 6).

Finally, chapters 2-6 were discussed and overall conclusions were drawn (chapter 7).

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Chapter 2

Isolation and partial characterization of bacterial strains on low organic carbon medium from soils fertilized with different organic amendments

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Senechkin, I.V., Speksnijder, A.G.C.L., Semenov, A.M., Van Bruggen, A.H.C., Van Overbeek, L.S. (2010) Isolation and partial characterization of bacterial strains on low organic carbon medium from soils fertilized with different organic amendments. *Microbial Ecology* 60:829-839.

Abstract

A total of 720 bacterial strains were isolated from soils with four different organic amendment regimes on a low organic carbon (low-C) agar medium ($10 \mu\text{g C ml}^{-1}$) traditionally used for isolation of oligotrophs. Organic amendments in combination with field history resulted in differences in dissolved organic carbon contents in these soils. There were negative correlations between total and dissolved organic carbon content and the number of isolates on low-C agar medium, whereas these correlations were absent for bacterial strains isolated from the same soil on high-C agar medium ($1000 \mu\text{g C ml}^{-1}$). Repeated transfers (up to ten times) of the isolates from low-C agar medium to fresh low- and high-C agar media were done to test for exclusive growth under oligotrophic conditions. The number of isolates exclusively growing under oligotrophic conditions dropped after each subsequent transfer from 241 after the first, to 98 after the third transfer step. Identification on the basis of partial 16S rRNA gene sequences revealed that most of the 241 isolates (as well as the subset of 98 isolates) belong to wide-spread genera such as *Streptomyces*, *Rhizobium*, *Bradyrhizobium* and *Mesorhizobium*, and the taxonomic composition of dominant genera changed from the first transfer step to the third. A selected subset of 17 isolates were further identified and characterized for exclusive growth on low-C agar medium. Two isolates continued to grow only on low-C agar medium up to the tenth transfer step and matched most closely with *Rhizobium alarii* and *Pedobacter roseus* on the basis of the almost full length 16S rRNA gene. It was concluded that the vast majority of strains which are isolated on low-C agar media belong to the trophic group of microorganisms adapted to a 'broad range' of carbon concentrations, including well-known and wide-spread bacterial genera. Oligotrophy is a physiological, not a taxonomic property, and can only be identified by cultural means so far. We showed that true oligotrophs that are unable to grow on high carbon media are rare and belong to genera that also contain 'broad range' and copiotrophic strains.

Introduction

The impact of soil management on trophic groups of bacteria in soil is poorly understood. Several studies have been conducted to understand the relationship between microbial community structure and soil characteristics after different soil treatments or cropping regimes (Garbeva et al., 2004; Widmer et al., 2006). Others focused on the impact of organic amendments on bacterial communities (Calbrix et al., 2007) or on the relationship between soil nutrients (especially carbon and nitrogen) and microbial populations (Cookson et al., 2005). However, only a limited number of studies is available on the effects of organic substrates or inorganic nutrients on oligotrophic populations in soils (Hu et al., 1999; Zelenev et al., 2005). We define ‘oligotrophs’ as bacteria able to grow at (extremely) low and not at high nutrient availabilities, in accordance with definitions proposed before (Poindexter, 1981; Semenov, 1991).

Oligotrophic bacteria are widely distributed and have been isolated from different environments (Deming, 1986; Hattori and Hattori, 1980; Lango, 1988; Semenov, 1991; Tada et al., 1995). This trophic group is taxonomically diverse and includes Gram-positive as well as Gram-negative species (Horowitz et al., 1983; Witzel et al., 1982). Although oligotrophic bacteria have not been studied as frequently as copiotrophic or eutrophic strains, experts in this field contend that oligotrophs constitute the majority of bacteria in natural environments (Ohta and Hattori, 1983). Oligotrophs are distinct from other bacterial groups because of their trophic properties, i.e. their ability to exploit ecological niches that are low in substrate concentrations and energy flows (Semenov, 1991). Oligotrophs are *K*-strategists (although not all *K*-strategists are oligotrophs), while copiotrophs are often *r*-strategists (Watve et al., 2000). *K*-strategists grow slowly and consistently, both at low and high nutrient availabilities, whereas *r*-strategists grow faster and respond more abruptly to (high) amounts of easily available nutrients and may die or become dormant when their surroundings are deprived of readily accessible nutrients. Oligotrophic bacteria are commonly called ‘true’ or ‘obligate’ oligotrophs when they possess narrow reaction ranges in terms of extremely low K_m values, energy maintenance coefficients, and low respiratory rates (Semenov, 1991). There is no clear consensus on the definition of oligotrophic bacteria, but they share their common preference for (extremely) low nutrient availabilities (Semenov, 1991).

These bacteria are of great interest because they may play an important role in the decomposition of organic matter and nutrient dynamics, as they can bring the glucose concentrations below the threshold level for catabolite repression of hydrolytic enzymes, thereby contributing to the activity of eutrophic bacteria (Semenov, 1991; Suwa and Hattori, 1984). The limited amount of knowledge about this group of bacteria restricts further exploration on their role in important soil processes, like mineralization and plant growth support. Main constraining factors are: (1) the lack of (known) functional commonalities among oligotrophs that distinguish them from copiotrophs, (2) the

intrinsic difficulties to cultivate them with respect to nutrient availability and composition of their growth media (Poindexter, 1981) and (3) their taxonomic heterogeneity.

Standardized techniques for detection of oligotrophs in natural environments are not available, irrespective whether they are based on culture-dependent or independent approaches. Only traditional carbon limited culture media can be used for isolation of oligotrophs from the environment. Common low-carbon media used for isolation of these bacteria are 10^4 fold-diluted broth (Hattori, 1980; Suwa and Hattori, 1984; Watve et al., 2000) or 100 fold-diluted S medium originally containing $1000 \mu\text{g C ml}^{-1}$ (Van Bruggen et al., 1988). The obtained isolates are sometimes tested for the absence of growth on high-C agar medium, to distinguish (true) oligotrophs from copiotrophs (Hashimoto et al., 2006; Ohta and Hattori, 1983; Saito et al., 1998; Whang and Hattori, 1988). Molecular markers in oligotrophic bacteria suitable for detection have been proposed (Lauro et al., 2009), but not yet developed. Oligotrophic bacteria commonly belong to particular bacterial groups, like the *Alpha*- and *Gammaproteobacteria* (Cho and Giovannoni, 2004; Hashimoto et al., 2006; Saito et al., 1998; Zavarzin et al., 1991). Knowledge about characteristics of oligotrophic bacteria is important for development of detection tools, based on phylogenetic (e.g. 16S rRNA-gene-based) or functional (physiological) markers that can quantify oligotrophic populations in natural environments like soils.

Based on our previous research (Hu et al., 1999) we hypothesized that the oligotrophic bacterial fraction will be lower in soils with relatively higher amounts of available carbon and that repetitive growth on low-C agar medium will result in the selection of true oligotrophs.

The aims of this study were to:

- isolate bacteria on low organic carbon media from soils differing in organic matter management,
- investigate the relation between the population densities of these bacteria and the carbon contents in the soils,
- test the growth rates of selected isolates on high- and low-C agar media and determine the proportion of true oligotrophs among the isolates growing on low C agar media,
- tentatively identify the isolates on the basis of the nearest matches of their 16S rRNA sequences with 16S rRNA genes available in databases.

Materials and methods

Site description and sample collection

Two fields (denoted as field 1 and 6) located at the organic experimental farm Droevendaal, The Netherlands (coordinates, W 5.66 and N 51.99) were selected for sampling. These fields differed in

the history of agricultural management practices and crop rotation during at least three years before sampling (in 2005). Field 1 was previously used for conventional arable crop production (potatoes in 2001), whereas field 6 was a pasture with organic management (i.e. without chemical fertilizers or pesticides). In the last three years before sampling, both fields were managed organically and planted with field crops rotated with a grass-clover ley. In both fields small plots were established in 2002 with different organic amendments plowed under to a depth of 20cm. There were four treatments: 1) 72 m³ ha⁻¹ slurry (liquid cattle manure) (S), 2) 12 ton ha⁻¹ plant-derived carbon amendments (green waste compost) (C), 3) 43 m³ ha⁻¹ liquid and 27 ton ha⁻¹ solid cattle manure (dung) (SD), and 4) 37 m³ ha⁻¹ slurry, 27 ton ha⁻¹ dung and 11 ton ha⁻¹ plant-derived amendments (CSD). The treatments were applied in duplicate plots in each field. Fields were 36x28m in size including paths, and plot sizes were 8x9m. From each plot, duplicate soil samples (10 kg, including roots) were collected in September 2005 from the top layer (0-20 cm). The samples were stored for not longer than one week at 15°C for chemical and microbiological analyses

Soil chemical analyses

Subsamples (12 g) of the large soil samples were dried at 40°C for 24 h and ground. Total carbon was measured by the Dumas method (Nieuwenhuize et al., 1994) followed by detection by a CHN1110 element analyzer (CE Instruments, Milan, Italy). Total nitrogen was determined by the Kjeldal method (Bremner and Mulvaney, 1982). Nitrate content was determined with an Autoanalyzer II (Technicon Instrument Corporation, Tarrytown, NY) after addition of 0.01 M CaCl₂. Phosphorus and potassium contents were measured according to Novozamsky protocols (Novozamsky et al., 1983). The pH and dissolved carbon were determined according to the protocol of Houba (Houba et al., 1998).

Dilution plating on organic low-C and high-C agar media

For bacterial isolation, 0.5 g subsamples were suspended in 5 ml sterile tap water, sonicated (47 kHz) for 1 min, vortexed for 10 s and the suspensions were 10-fold serially diluted. Fifty microliters of each suspension was pipetted onto high-C agar medium (0.5g MgSO₄.7H₂O, 0.5g KNO₃, 1.3g KH₂PO₄.3H₂O, 0.06g Ca(NO₃)₂.4H₂O, 2.5g glucose, 0.2g enzymatic casein hydrolysate, 15g technical agar [Oxoid nr.3], 1 l demineralized water; and 100 mg l⁻¹ filter [0.22 µm pore size]-sterilized cycloheximide (100 mg l⁻¹) was added to the medium after autoclaving at 121°C for 20 min). The low-C agar medium (Semenov et al., 1999) had the same composition as high-C agar medium, except that enzymatic casein hydrolysate and glucose were 100-fold lower (2 and 25 mg, respectively) and that Noble Agar [Difco Labs, Detroit] was used. All plates were incubated at 25°C for 2 d (high-C agar medium) or for 15 d (low-C agar medium) before colony counting. Log-transformed CFUs were calculated g⁻¹ of dry soil.

Selection for oligotrophic isolates

A total of 45 colonies per sample (16 samples) from the highest diluted suspensions (10^{-4} dilution) on low-C agar media were aseptically transferred to fresh low- and high-C agar media (first transfer). Upon incubation, those colonies from low-C agar medium that did not form visible colonies on high-C agar medium, but did on low-C medium were selected and subsequently transferred to fresh low-C and high-C agar media (second transfer). Upon incubation, again, those colonies that exclusively grew on low-C agar medium were further selected (third transfer). All isolates from the first and third transfer steps were streaked onto fresh low-C agar medium to be used for taxonomic identification. A selected subset (17) of the colonies obtained after the third transfer were further tested for growth on low- and high-C agar media up to the 10th transfer step.

Colony growth measurements

Colony growth of the selected subset of 17 isolates was followed on low- and high-C agar media during 14 d at the same incubation temperature. Colonies were inspected at 50 x magnification using a StemiSV11 binocular (ZEISS, Germany) attached to an AxioCam MRc camera (ZEISS, Germany) and images of individual colonies were daily digitized. Diameters from 10 digitized images per isolate were measured using Axio software (ZEISS, Germany) for calculation of colony growth rate.

Molecular identification of oligotrophic isolates

Bacterial cells from pure colonies were suspended in liquid low carbon medium (same composition as low-C agar medium, except that agar was omitted). DNA extracts were made from these cell suspensions using the PUREGENE Genomic DNA Isolation Kit (Gentra systems, USA) according to the protocol for cultured cells provided by the manufacturer. Partial 16S rRNA gene fragments were amplified from these extracts by PCR using bacterial primers 27F (Lane et al., 1985) and 1492R (Rochelle et al., 1992). Fifty microliter PCR reaction mixtures were prepared containing 1 μ l of DNA extract (5 – 50 ng), 200 μ M of each deoxyribonucleoside triphosphate, 0.2 μ M of each primer, 1 \times SuperTaq buffer (HT Biotechnology LTD, Cambridge, UK), and 5 U SuperTaq Polymerase (HT Biotechnology LTD). Amplification was performed in a PTC-100 thermal cycler (MJ Research, Inc., Tilburg, The Netherlands) using a program of 94°C for 4 min followed by 35 cycles of 94°C, 1 min; 55°C, 1 min; 72°C, 1 min, followed by a final extension step at 72°C for 5 min.

PCR fragments of approximately 1400 bp were purified using the Wizard DNA Clean-Up kit (Promega, USA) for sequencing. For that purpose, purified PCR products were added to reaction mixtures containing 5 μ l of sequencing mixture, 1 μ l of DETT Dye (DYEnamic ET Terminator Cycle Sequencing Kit, Healthcare, GE), 3 μ l of dilution buffer and 1 μ l (0.5 μ M) of primer 1492R. Linear

amplifications were performed for 25 cycles at 94°C, 20s; 50 °C, 15s; 60 °C, 60 s. The amplified products, approximating 600 bp in size, were sequenced in an ABI prism automatic sequencer by making use of the services of Greenomics (Plant Research International, Wageningen, The Netherlands). For sequencing of larger fragments of 16S rRNA genes, PCR amplicons were first PCR amplified with the following primers: 27F, R530 (Muyzer et al., 1993) and 968R (Heuer et al., 1997) and 1492R. Then contiguous fragments of approximately 1400 bp in size were assembled. Sequences from all fragments were compared with RDP database sequences using the RDP analysis tool Sequence Match (<http://rdp.cme.msu.edu/>) and Basic Local Alignment Search Tool (BLAST) at default settings (Altschul et al., 1997; Cole et al., 2005). All 16S rRNA gene sequences were deposited in the EMBL database and are available under accession numbers AM709973 through AM710213.

Statistical analysis

The experiment was designed according to a randomized split-plot scheme, consisting of two fields, divided into two blocks having four plots each with one treatment per plot including two sampling points per plot. Duplicate values of the logarithm of the original CFUs on low- or high-C agar medium g⁻¹ of dry soil from the same plot were averaged and so were the duplicate values of each chemical variable. The Shannon diversity values were calculated on the basis of phylogenetic differences in low-C for each treatment. For calculation, the following equation was used: $H = -P_i \log P_i$, where $P_i = n_i/N$ and n_i is the number of isolates per genus on low-C agar medium and N is the total number of isolates on this medium. Pair wise comparisons of treatments and fields with respect to Log CFU, chemical and Shannon diversity values were made by two-tailed *t*-tests using SAS statistical analysis software (SAS Institute, Cary, NC). Quantitative relationships between the fractions of total and dissolvable organic carbon and Log transformed CFUs were determined using linear regression analysis in SAS statistical analysis software. Differences were considered to be significant at levels of $P \leq 0.05$.

Results

Soil chemical and microbiological measurements

Chemical parameters measured in soils from the 16 plots differed per treatment and field (Table 1). The pH values among the eight soil samples ranged between 4.48 – 4.95 and they were significantly lower in field 1 than in field 6. Per treatment, the pH was lowest in C-treated soils of field 1 and in SD-treated soils of field 6.

Total organic carbon in the differently treated soils ranged between 10.03 – 18.16 g kg⁻¹ soil

Table 1. Mean values of soil chemical and microbiological parameters measured in differently treated plots from fields 1 and 6 at the Droevendaal experimental farm of Wageningen UR, the Netherlands.

Soil parameter	Field 1#				Field 6			
	S	C	SD	CSD	S	C	SD	CSD
pH	4.88 ^a	4.74 ^b	4.95 ^a	4.89 ^a	4.57	4.5	4.48	4.58
Total C (g kg ⁻¹)	10.03	11.12	11.27	10.06	15.25	18.16	16.84	17.94
DOC (mg kg ⁻¹)	65.47 ^b	68.16 ^b	74.13 ^a	75.03 ^a	91.40	98.66	101.67	89.68
Total N (g kg ⁻¹)	1.15 ^b	1.24 ^{ab}	1.42 ^a	1.32 ^a	1.68 ^a	1.58 ^b	1.51 ^b	1.53 ^{ab}
N-NO ₃ (g kg ⁻¹)	0.18	0.23	0.24	0.26	0.22	0.19	0.17	0.16
C/N ratio	8.76 ^a	8.98 ^a	7.96 ^b	7.65 ^b	9.11 ^b	11.52 ^a	11.19 ^a	11.98 ^a
LogCFU (low-C media)*	6.73	6.61	6.69	6.50	6.55	6.50	6.45	6.47
LogCFU (high-C media)*	5.00	5.09	5.11	4.99	4.80	4.78	4.75	4.62

S, C, SD and CSD denote soils treated with, respectively, slurry, compost, slurry and dung, and compost, slurry plus dung. Values with different letters indicate a significant difference ($P \leq 0.05$), where $a > b$.

* Expressed in log CFU g⁻¹ dry soil.

and the dissolvable organic carbon content ranged between 65.47 – 101.67 mg kg⁻¹. Per treatment, values for total carbon and dissolved organic carbon were significantly lower in field 1 than in field 6 and the lowest values in total C were for S-treated soils in both fields.

Values for total nitrogen ranged between 1.15 – 1.68 g kg⁻¹ and those of nitrate between 0.16 – 0.26 g kg⁻¹. Values for total nitrogen were highest in SD- and CSD-treated soils of field 1 and in S-treated soils of field 6, whereas nitrate was approximately the same for all treatments.

Values for total C/N ratio ranged between 7.65 and 11.98 and the C/N ratio was lower in field 1 than in field 6 soils. Values were lowest in CSD-treated soils of field 1, and in S-treated soils of field 6.

Log transformed CFUs on low-C agar medium were between 6.45 and 6.73 g⁻¹ dry soil and these numbers were 1-2 orders higher than log CFUs on high-C agar medium, which ranged from 4.62 to 5.11 g⁻¹ dry soil. The average log CFUs on low-C agar medium were about the same in both fields (6.63 in field 1 and 6.61 in field 6, both g⁻¹ of dry soil) and so were the average log CFUs on high-C agar medium (5.05 in field 1 and 4.97 in field 6). Per treatment, no significant differences were found between CFUs of both trophic groups.

Negative linear correlations were present between log CFUs on low-C agar medium and total carbon (C_{tot}) and dissolvable organic carbon (DOC) fractions in the 16 soil samples (Table 2), indicating that the carbon contents in these soils have a negative effect on the number of CFUs recovered on low-C agar medium. No correlations were found between the number of CFUs on high-C agar medium and the concentrations of total or dissolvable carbon in the soils.

Table 2. Equations describing the linear relationships between the number of CFU on low-C agar media and total (C_{tot}) and dissolved organic carbon (DOC) in the differently treated soils.

Dependent variable	model	p-value	R ²
LogCFU (low-C media)	$6.851 (\pm 0.118) - 0.021 (\pm 0.008) \times C_{tot}$	0.04	0.51
LogCFU (low-C media)	$7.049 (\pm 0.156) - 0.006 (\pm 0.002) \times DOC$	0.02	0.62

Bacteria isolated on low-C agar medium and selection of oligotrophic isolates

In total 32 Petri plates with 10^{-4} diluted suspensions were obtained for the low-C agar medium and the same number of plates for the high-C agar medium. On average, 22 colonies grew on each low-C plate after 15 days of incubation (longer incubation till 21 days did not lead to significantly more CFU, but led to overlapping of colonies (Semenov et al., 1999)). A total of 717 colonies (three colonies did not grow further after transfer) were obtained on low-C agar plates with 10^{-4} diluted suspensions from all soil samples. These isolates must be considered as the culturable fraction that could grow at a broad range of carbon concentrations, including oligotrophic conditions. All isolates were tested for exclusive growth on low-C agar medium in the absence of growth on high-C agar medium. After the first transfer, 241 isolates were found to grow exclusively on low-C agar medium and this number decreased after the second (124) and third (98) transfer steps (Fig. 1). There was thus a tendency for leveling off towards a number of oligotrophic isolates that exclusively grew on low-C agar medium (Fig. 3).

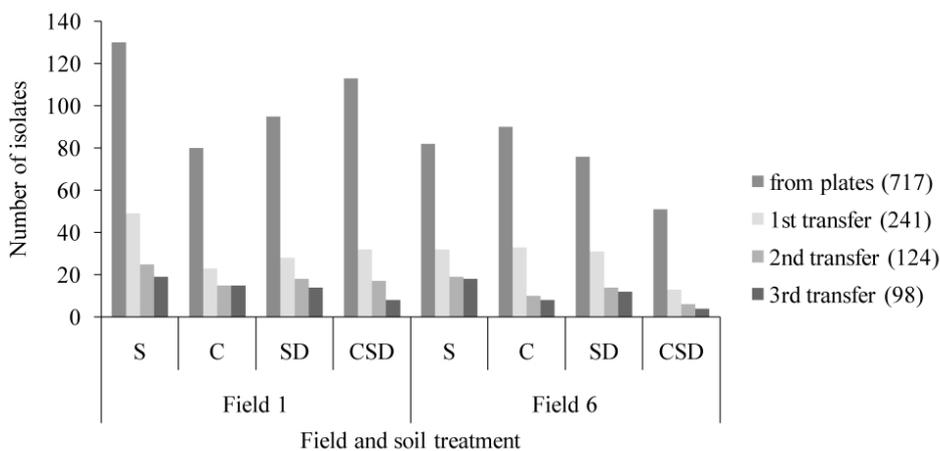


Figure 1. Distribution of bacterial isolates on low-C agar medium over the differently treated soils after subsequent transfer steps to fresh low- and high-C agar media. Only those isolates that exclusively grew on low-C agar medium were used in the succeeding transfer step. S, C, SD and CSD stand for soils treated with, respectively, slurry, compost, slurry and dung, and compost, slurry plus dung.

Preliminary identification of oligotrophic isolates

All 241 isolates obtained on low-C agar medium after the first, and the subset of 98 isolates obtained on the same medium after the third transfer step were identified by comparison of their 16S rRNA gene sequences with those present in the RDP database. Oligotrophs selected after the first transfer showed best matches with bacterial sequences from 32 different genera (Fig. 2). The genera occurring at the highest frequencies among this pool of isolates were: *Streptomyces* (30.7%), *Mesorhizobium* (11.2%), *Bradyrhizobium* (10.4%), *Rhizobium* (7.1%) and *Nocardia* (7.1%). The isolates selected after the third transfer step belonged to 11 genera and the most frequent occurring ones were: *Mesorhizobium* (27.6%), *Bradyrhizobium* (25.5%) and *Rhizobium* (17.3%). The clearest difference between both sets of isolates was the number of isolates affiliated with *Streptomyces* species, which was absent among the isolates after the third transfer step. Most remarkable is the high abundance of isolates affiliated with *Alphaproteobacteria* (73.5 % after the third transfer step), indicating that most of the oligotrophs in these soils belonged to this bacterial class.

The effect of soil treatment on CFUs on low-C agar medium

After the first transfer step, the total number of isolates on low-C agar medium were highest in S-treated soil from field 1 (42) and lowest in the SD-treated soil from field 6 (19) (Table 3). Taking the data for both fields together, the highest number of isolates was found in S treated soils (80) and the lowest in the CSD-treated soils (48) and so were the isolates in the *Alphaproteobacteria* (respectively, 31 and 8), *Bradyrhizobium* (respectively, 11 and 2), *Mesorhizobium* (respectively, 10 and 2) and *Rhizobium* (respectively, 8 and 1). However, no clear effect of soil treatment was found for the Shannon diversity values for low-C isolates, which were highest in SD-treated soils from field 1 and lowest in CSD-treated soils from field 6. After the third transfer step, the numbers of oligotrophs per treatment in fields 1 and 6 were, respectively: 19 and 18 for S-, 14 and 12 for SD-, 15 and 8 for C-, and 8 and 4 for CSD-treated soils; the highest numbers were again found in S-treated soils and the lowest in the CSD-treated ones. This indicates that soil treatment has an effect on the abundance of the dominant groups of oligotrophs in the differently treated soils, but not on the oligotroph species diversity (only after the first transfer step). S-treated soils were the ones lowest in Ctot and DOC, whereas in CSD-treated soils both variables had higher values (Table 1), indicating that the amount of carbon present in soils affect the most dominant oligotrophic groups in the different soils.

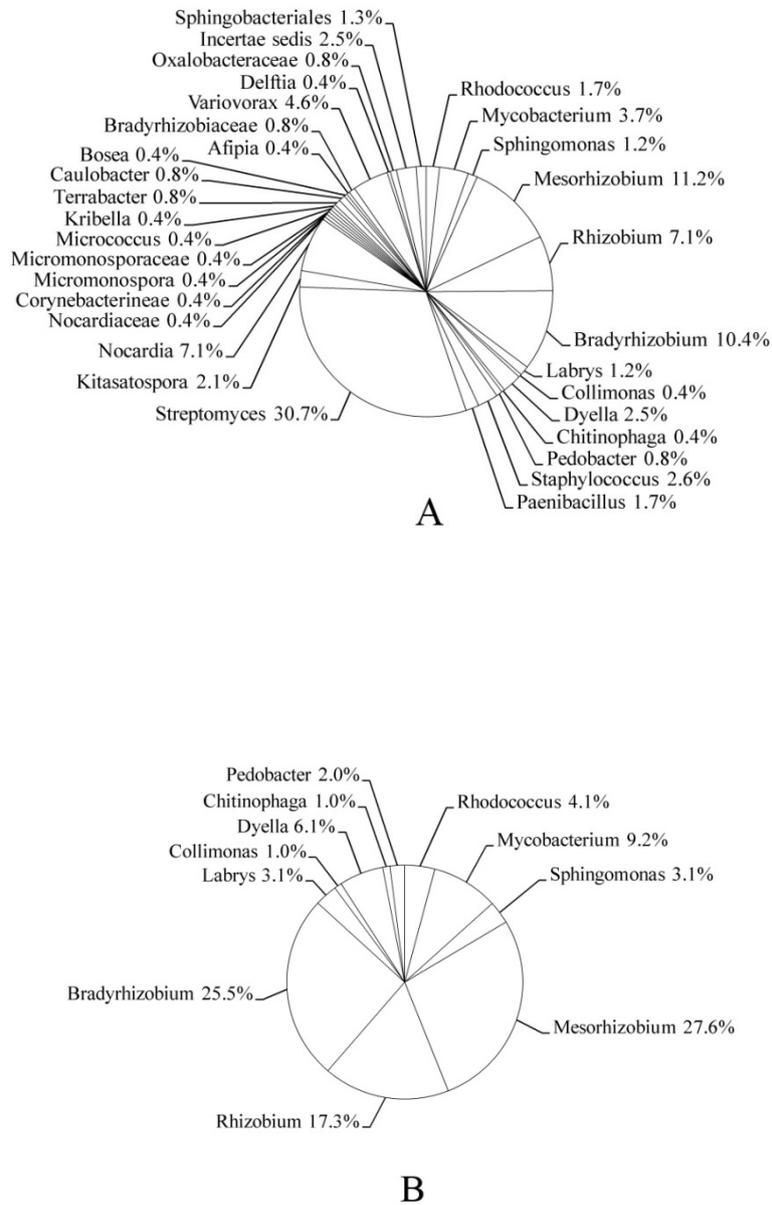


Figure 2. Taxonomic identity of isolates from all treated soils that exclusively grew on low-C agar medium after the first (A) and third (B) transfer step.

Table 3. Distribution of bacteria isolated on low-C agar medium after the first transfer step over different taxonomic groups and Shannon diversity values for the differently treated soils from fields 1 and 6 at the Droevendaal Experimental Farm of Wageningen UR, the Netherlands.

Number of oligotrophs and diversity	S [#]		C		SD		CSD	
	1	6	1	6	1	6	1	6
Total number of isolates	42	38	35	25	34	19	21	27
<i>Alphaproteobacteria</i>	15	16	12	8	16	6	6	2
<i>Bradyrhizobium</i>	8	3	3	2	5	2	1	1
<i>Mesorhizobium</i>	4	6	3	6	2	4	2	0
<i>Rhizobium</i>	3	5	3	0	5	0	1	0
Shannon diversity (H [†])	0.724	0.788	0.786	0.578	0.836	0.767	0.655	0.558

[#] S, C, SD and CSD denote soils treated with, respectively, slurry, compost, slurry and dung, and compost, slurry plus dung in field 1 and field 6.

[†] Calculated on the basis of all genera present among the low-C isolates from the different soils

Identification and characterization of selected true oligotrophic isolates

For better identification, larger stretches (approximately 1400 bp in size) of the 16S rRNA genes of 17 selected isolates (at least one from each genus) obtained on low-C agar medium after the third transfer step were compared and their growth rates were determined. One isolate had probably died in the freezer and could not be re-activated on low-C medium. From the remaining 16 isolates, 15 showed nearest matches at similarity levels of 96% or higher with culturable type strains in the RDP database, whereas one (denoted as IS204) matched at a similarity level of 91.6% with *Pedobacter roseus*. Nine of the selected oligotrophs were identifiable within the class of *Alphaproteobacteria*, two within the *Betaproteobacteria*, one within the *Gammaproteobacteria*, three within the *Actinobacteria* and one within the *Sphingobacteria*. One isolate showed a nearest match with an uncultured bacterium at a lower level (91.4 %), indicating that this isolate may represent a hitherto uncultured bacterial group. Therefore, it can be concluded that the majority of low-C isolates closely resembled already described bacteria from soils.

Growth rates of these 16 isolates on low- and high-C agar media were measured. Because not all isolates were able to grow in a uniform suspension in liquid media, it was decided to estimate growth rates from colony diameters on agar surfaces.

Estimated growth rates of the isolates ranged between 0.1 and 0.6 mm d⁻¹ on low-C agar medium and between 0 and 1.0 mm d⁻¹ on high-C agar medium, demonstrating that there were still ‘broad range’ bacteria among these isolates (Table 4). Growth rates on the high-C agar medium were

Table 4. Taxonomic identity and colony growth of a selected subset of bacterial isolates that grew exclusively on low-C agar medium at the third transfer, but not necessarily after additional transfers on low- and high-C agar media.

Isolate	Nearest match with non-type strains	Similarity (%)	Nearest match with type strains	Similarity (%)	Colony growth*	
					Growth rate (o/c) mm d ⁻¹	App (d)
Alphaproteobacteria						
IS6	Rhizobium sp. CCBAU 85046	99.6	Rhizobium huatdense	97.2	0.1/0.3	1/3
IS63	Rhizobium sp. CIAM1414	99.8	Mesorhizobium ciceri	98.9	0.1/0.2	1/2
IS19	Mesorhizobium loti LMG 6123	98.5	Mesorhizobium septentrionale	98.3	0.3/0.3	1/1
IS119	Mesorhizobium sp. USDA 4322	99.9	Mesorhizobium septentrionale	98.9	0.2/0.2	1/1
IS183	Rhizobium sullae	97.8	Rhizobium alarii	97.4	0.1/0	1/-
IS252	Mesorhizobium amorphae CCBAU 45139	99.8	Mesorhizobium amorphae	98.9	0.1/0.3	1/1
IS354	Bradyrhizobium japonicum	99.6	Bradyrhizobium japonicum	99.0	0.6/1	1/1
IS152	Labrys methylaminiphilus DSM 16812	97.6	Labrys monachus	96.9	0.2/0.2	2/1
IS42	Sphingomonas sp. kmd_118	99.5	Sphingomonas asaccharolytica	97.8	0.2/0.7	1/1
Betaproteobacteria						
IS184	Beta proteobacterium EC4	98.4	Duganella zoogloeoides	97.6	0.1/0.3	1/1
IS343	Collimonas sp. wged41	99.4	Collimonas fungivorans	98.4	0.3/1	1/1
Gammaproteobacteria						
IS173	Dyella marenensis CS5-B2	98.9	Dyella koreensis	98.0	0.5/0.5	2/1
Actinobacteria						
IS39	Mycobacterium sp. IMER-B1-12	98.5	Mycobacterium septicum	96.7	0.5/0.4	1/1
IS100	Rhodococcus tukisamuensis	99.4	Rhodococcus tukisamuensis	99.4	0.5/0.7	1/1
IS316	Rhodococcus maanshanensis	98.0	Rhodococcus maanshanensis	98.0	0.4/0.8	1/1
Sphingobacteria						
IS204	Uncultured Sphingoterrabacterium sp.	97.0	Pedobacter roseus	91.6	0.1/0	2/-

* measured after 3 transfer steps: o/c indicates oligotrophic/copiotrophic agar medium, App – first appearance of visible colonies

mostly similar or higher than on the low-C agar medium. In three cases the growth rates on the low-C agar medium was higher than on the high-C agar medium, while two of these isolates did not grow at all on the latter medium. These two isolates (IS183 and IS204) were only able to grow on low-C agar medium up to the 10th transfer step. Apparently, these two isolates, showing nearest matches with *Rhizobium alarii* and *Pedobacter roseus* type strains, can be regarded as ‘true’ oligotrophs as they most likely do not possess the capacity to grow at higher nutrient levels in the agar medium. One of these two true oligotrophic isolates matched even more closely to a hitherto uncultured bacterium.

Discussion

Many 'broad range' and a few true oligotrophic bacteria were recovered from soils that were amended with different organic substrates. These soil treatments are realistic in organic farming where organic amendments like compost, manure and slurry are commonly applied for crop growth and disease control (Van Diepeningen et al., 2006). Agronomic cultural practices were shown to affect the microbial community structure in soils most likely as a result of changes in chemical composition of the soils (Van Diepeningen et al., 2006; Van Elsas et al., 2002). Here, we demonstrated that the carbon status in soils treated with different organic amendments affected the numbers of 'broad range' and oligotrophic bacteria isolated from these soils. Soils that have been managed organically for several years, and received solid composted farm yard manure, are expected to be relatively low in available nutrients (Van Bruggen and Termorshuizen, 2003). Therefore, it was hypothesized that oligotrophs would be dominant in such soils. Indeed, the initial number of isolates grown on low-C agar medium was higher than that on high-C agar medium in our soils and the number of low-C isolates was reduced at higher carbon concentrations in these soils, whereas the culturable copiotrophs were not. Based on these culturable cell counts, we could accept our hypothesis. Most likely, it is the reduction in the amount of available carbon that favors growth of broad range and oligotrophic bacteria in these soils rather than the increased amount of total carbon that favors growth of hydrolytic copiotrophic bacteria and possibly suppression of oligotrophs. Our observations were in line with observations

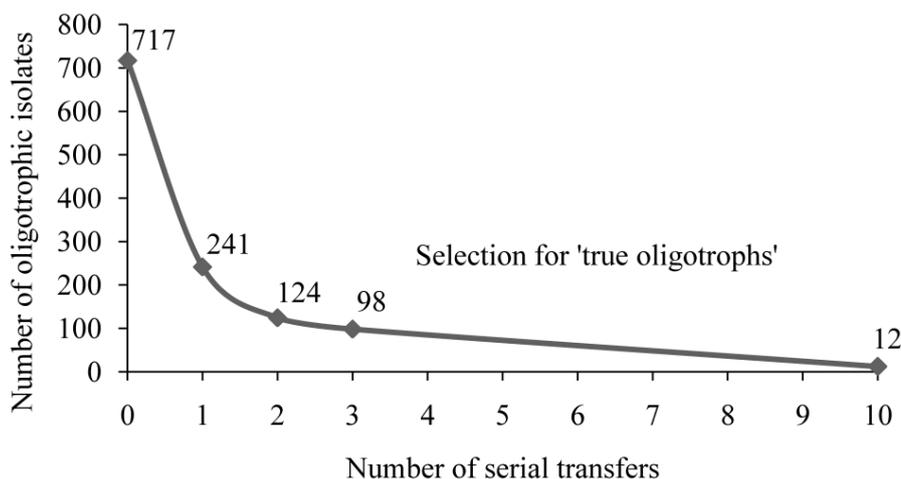


Figure 3. Number of oligotrophic bacterial isolates from soil selected for exclusive growth on low-C agar medium after repeated transfers to low- and high-C agar media. The number of 'true' oligotrophs in the graph is an estimate derived from the number of 'true' oligotrophs (2) obtained from the selected subset of 16 isolates after the third transfer step that were further tested for growth on low-C and high-C agar media up to the 10th transfer step.

made by Hu and coworkers (Hu et al., 1999), who demonstrated that putative oligotrophic bacteria peaked in their growth after the copiotrophs. Copiotrophic bacteria were more numerous at high carbon availability levels, while populations of putative oligotrophic bacteria declined at high carbon levels (Hu et al., 1999). Under these circumstances this latter group of bacteria would likely be outcompeted by copiotrophs. However, as soon as carbon availability declines, the copiotrophs become arrested in growth and activity whereas oligotrophs remain active at lower carbon levels (Semenov, 1991), will gain advantage over copiotrophs and will proliferate.

Qualitative analysis of the isolates on low-C agar media characterized by 16S rRNA gene comparisons in the four differently-treated soils revealed that there were no major differences in the predominant groups of isolates, i.e. *Bradyrhizobium*, *Mesorhizobium* and *Rhizobium* spp., among these soils, i.e. about 70% of the isolates after the third transfer. These three genera belong to the *Alphaproteobacteria*, which were shown before to dominate the oligotrophic bacterial community in soils and seawater, especially those belonging to *Rhizobium* and *Bradyrhizobium* spp. (Hashimoto et al., 2006; Mitsui et al., 1997; Saito et al., 1998). Two *B. japonicum* strains could be re-isolated from different field soils, 16 and 20 years after they were introduced (Obaton et al., 2002), indicating that members of the *Bradyrhizobium* group of species are well adapted to circumstances prevailing in soil. Most of these genera contain nitrogen-fixing organisms. As our low-C agar medium was low in carbon as well as nitrogen, there could have been a selection for N₂-fixing bacteria on our plates, although we did not check the N₂-fixing activity of the isolates. The ability of many oligotrophic bacteria to fix nitrogen was demonstrated by Japanese researchers a long time ago (Ohta and Hattori, 1983).

Two of our isolates closely matched members of the genus *Rhodococcus*. A representative of the same group was isolated from soil before and characterized as an “extreme oligotroph” (Ohhata et al., 2007). One of our isolates showed a close match to *Collimonas* sp. (*Betaproteobacteria*). *Collimonas* species were also isolated from dune sand, which is an environment extremely low in available nutrients (Hoppener-Ogawa et al., 2009; Leveau et al., 2010). Remarkably, the isolates obtained by Hoppener-Ogawa and coworkers (Hoppener-Ogawa et al., 2009) all were recovered on an agar medium relatively high in nutrients, whereas our *Collimonas* sp. was recovered on low-C agar medium and showed exclusive growth on this medium after three subsequent transfers. However, it did grow on our high-C agar medium after subsequent transfers, indicating that it was a ‘broad range’ organism. A high variation in physiology and taxonomy may be present among members of

this genus.

Further testing for exclusive growth on low-C agar medium by repeated transfers to fresh media revealed an even stronger contribution of the *Alphaproteobacteria* to the total oligotrophic community in these soils (Zavarzin et al., 1991). It must therefore be concluded that the *Alphaproteobacteria* are the most important group among the oligotrophs selected within the constraints of the experimental set up, i.e. by selection for growth on agar media including low concentrations of glucose and casamino acids as sole nutrient sources.

For isolation and quantification of oligotrophic bacteria from any natural sources there are currently no other methods besides cultivation on media with very low concentrations of readily utilized sources of carbon plus some growth factors, which were provided by enzymatic casein hydrolysate in our study. Any cultural method is selective and in our case the main selective factor was the carbon concentration. However, it is clear that the common approach for isolation of oligotrophs is not selective enough for the vast majority of the strains obtained in our study. Semenov (1991) proposed that the vast majority of microorganisms are “polyfunctional”, i.e. they possess a ‘wide reaction range’ for organic carbon concentrations in their surroundings, which is in line with the observations made in our study. Fewer microorganisms possess narrow reaction ranges, either towards low or high organic carbon concentrations (Semenov, 1991). The two isolates, denoted as ‘true’ oligotrophs and identified as *Rhizobium alamii* and *Pedobacter roseus*, persistently showed exclusive growth at low nutrient availability levels and these must belong to the group possessing a so called ‘narrow’ reaction range.

The close match with an uncultured bacterium and a distant match with *Pedobacter roseus* type strain of one of the two ‘true’ oligotrophs indicates that among this group, new or hitherto uncultured bacteria can be found. Recently, isolates identified as *Verrucomicrobia* subdivision 1 and belonging to a hitherto uncultured group present in the potato rhizosphere were obtained on the same low-C agar medium, but then amended with catalase or potato root exudates (Nunes da Rocha et al., 2009). This medium offers great opportunities for the recovery of new species from soil environments, especially among the ones that exclusively grow thereon. The other ‘true’ oligotrophic isolate resembled *Rhizobium sullae* and *R. alamii*, indicating that this isolate may be a plant symbiont. Representatives of *R. alamii* were isolated from the root environment of *Arabidopsis thaliana* (Berge et al., 2009) and those of *R. sullae* originated from *Hedysarum coronarium* L (Squartini et al., 2002). This isolate, and also the ones belonging to the genera *Bradyrhizobium*, *Mesorhizobium* and *Rhizobium* that lost their

exclusive growth on low-C agar medium are important members of the isolates obtained in this study and may play an important role in plant symbiotic N-fixation mentioned above.

It can be concluded that the group of low-C bacteria recovered from the soils in this experiment, whether they could grow at a broad- or narrow range of carbon concentrations, can play important roles in soil functioning. These bacteria may not only be important for nitrogen fixation, but also for biological control of plant pathogens. For example, it was shown that some *Collimonas* species were mycophagous, i.e. they can live on fungi (Leveau et al., 2010) and may be responsible for the control of soil-borne phytopathogenic fungi. It is clear that bacteria isolated on low-C agar media belong to a fascinating group of soil bacteria, possibly affecting plant growth. Thus far, these bacteria have hardly been exploited for improved plant growth and this aspect will be further tested in later studies.

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Chapter 3

Greater *Fusarium* wilt suppression after complex than after simple organic amendments as affected by soil pH, total carbon and ammonia-oxidizing bacteria

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Abstract

A field experiment was conducted to compare effects of four types of organic amendments on soil chemical, microbiological and disease suppression characteristics in an organic farm. The amendments were plant-derived fresh compost (C), steer-derived slurry (S) or slurry plus dung (SD) and slurry, compost and dung (SCD). We hypothesized that amendments with more easily available carbon sources (S and C) might enhance *Fusarium* wilt of flax, while more complex amendments with lower carbon availability might suppress the disease. *Fusarium* wilt development was tested in bioassays with flax growing in *Fusarium oxysporum* f. sp. *lini* Snyder & Hansen inoculated soil samples from previously amended plots in a growth chamber, and area under the disease progress curves (AUDPCs) were determined per pot. Soil chemical and microbial analyses were conducted directly on soil samples taken from the field. Total DNA was extracted from soil and amplified with specific primers for eubacteria, *Pseudomonas* species, ammonia-oxidizing bacteria (AOB) and fungi. The respective microbial compositions were assessed by denaturing gradient gel electrophoresis, and species richness and diversity were calculated from the numbers and intensities of the amplicons on the gels. *Fusarium* wilt was significantly suppressed in soil from SCD treated plots and enhanced in that from C-treated plots. AUDPC values were negatively correlated with pH only. In stepwise and canonical discriminant analyses soil samples with low and high AUDPC values were distinguished based on pH, total carbon content, and diversity of AOB. It is suggested that AOB could be useful indicators for suppression of soil-borne pathogens.

Introduction

Every natural soil possesses some ability to suppress the activity of plant pathogens due to the presence and activity of soil microorganisms (Mazzola, 2002). Soils that are characterized as suppressive are soils where a particular pathogen either does not establish, or establishes but causes little or no damage, or establishes and causes disease which then declines over time even though the pathogen may persist in soil. Thus suppressive soils are capable of reducing a plant pathogen's survival and/or activity (Hiddink et al., 2005). Suppressiveness has sometimes been associated with diverse microbial and faunal communities that have a greater probability to contain antagonistic, competitive, parasitic or predatory species or that can contribute to the activation of induced systemic resistance (Van Bruggen and Semenov, 1999; Workneh et al., 1993).

Two types of disease suppression are recognized: specific and general suppression; the first type is attributed to a particular antagonist or parasite that has a specific interaction with a pathogen often in a mono-cropped system, while the second type is not associated with one organism and may be directed to several pathogens. This second general type of disease suppression is assumed to be present in organically managed soils (Hiddink et al., 2005). Both disease suppression types have been associated with biotic as well as abiotic soil components (Hoper and Alabouvette, 1996; Mazzola, 2002). The extent of disease suppressiveness can be affected by various management practices, including management systems like organic or conventional (Workneh et al., 1993; Van Bruggen, 1995; Hiddink et al., 2005), crop rotation (Leoni et al., 2012), tillage, fertilization, organic amendments and subsequent management (Blok et al., 2000; Bulluck et al., 2002; Goud et al., 2004; Klein et al., 2011) and innate soil characteristics (Hoper and Alabouvette, 1996). The degree of disease suppression of a soil can be quantified in bioassays where symptom development of a pathogen-amended treatment is compared with a non-amended treatment (Hiddink et al., 2005; Grünwald et al., 2000). Usually disease suppression is not complete, and thus it is often studied as a relative characteristic, i.e. one soil or one treatment yielding more disease suppression than another soil or treatment (Termorshuizen et al., 2006).

Fusarium oxysporum is a common soilborne plant pathogen causing wilt (or more seldom root rot) in various hosts. A large number of formae speciales can specifically affect particular hosts, for example *F. oxysporum* f.sp. *lini* W.C. Snyder and H.N. Hansen is pathogenic to flax. Disease suppression of *F. oxysporum* is quite common, and is often associated with non-pathogenic *Fusarium* spp. (Alabouvette et al., 1996), actinomycete populations (Castano et al., 2011), fluorescent pseudomonads or total bacteria (Larkin et al., 1993), but is also affected by soil pH, organic matter content and clay content (Fang et al., 2012; Hoper and Alabouvette, 1996). The effect of various management practices (e.g. crop rotation, organic amendments and tillage) on development of soilborne pathogens in soil has been studied as well (Bulluck et al., 2002; Leoni et al., 2012; Peters et

al., 2003). However, a comprehensive view of management factors affecting suppression of these pathogens is still lacking because of the complexity of interactions between the contributing factors. Therefore, a more holistic approach covering physical, chemical and biological soil attributes is necessary to study suppressiveness (Janvier et al., 2007).

Root diseases are generally suppressed more in organically than in conventionally managed soils (Van Bruggen and Termorshuizen, 2003; Hiddink et al., 2005; Messiha et al., 2007; Workneh et al., 1993). Organic management differs from conventional management in that more organic materials are added to soil, while application of pesticides and fertilizers is limited to those natural and mineral compounds approved by organic certification agencies. As a result, organically managed soils frequently have higher microbial and microfaunal activity and diversity, and a better soil structure (Van Diepeningen et al., 2006). However, it is still unknown which specific organic management practices lead to root disease suppression. It is, for example, not known if application of animal-derived amendments leads to more or less suppression than application of plant-derived amendments. It is also not known if regular applications of low-carbon amendments lead to greater suppression than occasional applications of high-carbon amendments. To solve these questions, a long-term experiment was set up at a certified organic experimental farm, where various organic amendments were applied to plots with an eight-year crop rotation. The original hypothesis was that regular additions of complex, hard to decompose carbon sources with a relatively high C:N ratio to soil would result in greater suppression of soilborne plant pathogens than amendments that are high in nitrogen and easily decomposable carbon sources (Bonanomi et al., 2010).

The objectives of the present study were to gain a better understanding of the influence of various organic amendments on suppressiveness of several soilborne diseases and to evaluate the contribution of biotic (taxonomic and functional groups of microorganisms) and abiotic factors (chemical soil parameters) to this effect. The effects of these amendments on Rhizoctonia damping off of sugar beet has already been published (Van Overbeek et al., 2012). In this paper we present the data on the effects of four types of amendments with different carbon and nitrogen contents on Fusarium wilt of flax caused by *Fusarium oxysporum* f.sp. *lini* and its relationship to an array of microbiological and chemical characteristics.

Materials and methods

Field site and treatments

A field site (field #6) was selected at the mixed organic experimental farm 'de Droevendaal' (Wageningen University, the Netherlands). The soil was loamy sand containing 2-4% organic matter, with $\text{pH}_{\text{CaCl}_2}$ of about 4.5. The farm was converted to certified organic management in 2002. The main

field had an eight-year rotation with wheat (*Triticum vulgare* L.), potato (*Solanum tuberosum* L.), triticale (*Triticosecale* sp. Wittm. ex A. Camus), sugar beets (*Beta vulgaris* L.), a vegetable crop [carrots (*Daucus carota* L.) or beans (*Phaseolus vulgaris* L.)], and 3 years of grass-clover (*Lolium perenne* L. and *Trifolium repens* L.) leys. Soil fertility was maintained by growing winter cover crops and application of manure from steer calves. For a long-term experiment on effects of different amendments on soil health, a section of the field (36 x 28 m) was divided in two blocks with 4 plots each measuring 8 x 9 m. Each plot was randomly assigned to one out of four treatments. The treatments consisted of different types of management with respect to amendment of carbon and nitrogen for three years before samples were collected for the current research: (1) compost (C) - plant-derived amendments (green waste compost, 12 x 10³ kg/ha over 3 years), (2) slurry (S) - low-carbon animal-derived amendments from young bulls (72 m³/ha of liquid manure over 3 years), (3) slurry and dung (SD) - high-carbon animal-derived amendments (43 m³/ha of liquid manure and 26 x 10³ kg/ha of dung during 3 years), and (4) slurry, compost and dung (SCD) - both animal- and plant-derived amendments (37 m³/ha of liquid manure (11 x 10³ kg/ha of plant-derived compost) and dung (27 x 10³ kg/ha) over 3 years. Control plots without amendments were not possible since crop production on certified organic farms cannot be accomplished without some form of organic amendment. Most of the amendments were applied in 2003, when potatoes were cultivated on the selected field; less amendment was applied to the grass-clover in 2004 and 2005. The same crops were grown in the small plots as those in the rest of the field using regular organic practices in accordance with the regulations of the certification agency.

Soil sampling

Field 6 was selected for soil sampling. At the time of sampling, this field had grass clover for the second year (after potatoes). There were two blocks each with 4 plots that had been treated with the 4 amendments (S, C, SSD, and SCD). Soil samples (3 kg, including roots) were collected (September 2005) in duplicate from the top 0-20 cm layer of each plot at two opposite ends of each plot, about 1 m from the edge, resulting in a total of 16 soil samples. All soil samples were sieved to remove grass-clover residues. The sieved soil samples were stored in closed polythene bags at room temperature until the set-up of the bioassays and microbiological and chemical analyses one month later. In addition, soil samples (3kg) were collected from a nearby area at the same farm that was fallow and where organic amendments had not been applied. The samples collected from this field were sterilized for 6 days with γ -irradiation from ISOTRON company in Ede, the Netherlands. These samples were used as non-suppressive controls in the *Fusarium* bioassays.

Bioassay

The suppressiveness levels of the 16 soil samples against *Fusarium oxysporum* f.sp. *lini* (isolate Fohn3, kindly provided by Ph. Lemanceau, INRA, Dijon, France) were determined in a bioassay with flax plants in comparison with Fusarium wilt in conducive sterilized soil samples. Inoculum of *Fusarium oxysporum* f. sp. *lini* (Fol) was prepared as follows. Erlenmeyer flasks with malt extract broth were autoclaved (121°C, 20 min) and closed with a cotton plug, inoculated with three plugs of malt extract agar containing growing mycelium of Fol, and incubated on a shaker for 7 d at 25°C. Fungal cultures were comminuted with a blender (1 min at high speed) and growing medium was removed by centrifugation for 30 min at 4000 rev min⁻¹. The fungal biomass was resuspended in sterile distilled water and was added to talcum powder (2:1, wt:wt) (Blok et al., 2000). This mix was air-dried for 3 wk under sterile conditions to allow the formation of chlamydospores. The air-dried inoculum was passed through a sieve with 0.36 mm mesh size. Inoculum was stored at 4°C until use. The density of the inoculum thus obtained was about 10⁷ propagules g⁻¹ talcum.

The bioassay was carried out in a growth chamber at 20°C, 16 h at 230 μMol m⁻² s⁻² photo-active light (TL28₀HF) and 80% relative humidity, and lasted for 6 weeks. Inoculum of *F. oxysporum* f. sp. *lini* in talcum was applied at 5 × 10⁵ cfu/g of soil to half of 18 soil batches (two times four amendment treatments in duplo and one sterilized control in duplo). The other half remained non-inoculated. Each inoculated and non-inoculated soil sample was divided over five pots (175 ml per pot), resulting in 5 (reps) × 2 (inoculation) × 18 (batches) = 180 pots. In the climate room, the pots were arranged in a completely randomized block design with five blocks of five pots per soil sample.

Flax seeds (*Linum usitatissimum* L. cv. Opaline) were disinfested with 1% sodium hypochlorite, washed for 5 min with tap water, and dried at room temperature overnight. Nine flax seeds per pot were placed on top of the infested and non-infested soil and covered with 25 ml of non-infested soil. Soil infestation, seed disinfection, sowing, and plant care were carried out according to a standard protocol (Blok et al., 2000; Van Rijn et al., 2007). The plants were watered twice a week, at first on the soil surface and later in saucers under the pots. The number of diseased plants per pot was scored based on 0-5 scale (0 = healthy; 1 = slight yellowing of leaves; 2 = pronounced yellowing of leaves; 3 = yellowing and about half of the leaves wilted; 4 = almost all leaves wilted; 5 = dead) and was performed twice a week after the appearance of the first disease symptoms, 3 weeks after seeding. The average disease severity was calculated for each pot. Next, the Area Under the Disease Progress Curve (AUDPC) was calculated per pot from the average disease severities over a 3-week period. In addition, percentages disease suppression (DS) were calculated from the differences between the AUDPC of each sample and the median AUDPC in sterilized control soil divided by the median AUDPC of the sterilized controls.

Culturable bacterial densities

Densities of copiotrophic and oligotrophic bacteria were determined by subsampling 0.5 g of soil, suspending in 5 ml water, sonicating (47 kHz) for 1 min, vortexing for 10 s and serial 10-fold diluting 10^{-1} to 10^{-5} followed by plating of 50 μ l of the 10^{-4} , 10^{-5} dilutions each onto two Petri dishes. For copiotrophic bacteria the so-called S medium was used: 0.5 g $\text{MgSO}_4 \times 7\text{H}_2\text{O}$, 0.5 g KNO_3 , 1.3 g $\text{KH}_2\text{PO}_4 \times 3\text{H}_2\text{O}$, 0.06 g $\text{Ca}(\text{NO}_3)_2 \times 4\text{H}_2\text{O}$, 2.5 g glucose, 0.2 g enzymatic casein hydrolysate, 15 g technical agar (Oxoid nr. 3), 1 l demineralized water and 100 mg sterile cycloheximide after sterilization (Van Bruggen et al., 1990). This medium was later called the copiotrophic medium (Hu et al., 1999; Zelenev et al., 2005). For oligotrophic bacteria the same medium was used at 100-fold dilution of all ingredients except salts and agar (18 g/l Noble Agar, Difco Labs, Detroit) (Senechkin et al., 2010). Plates were incubated in darkness at 25°C for 2 days (copiotrophic bacteria) or 17 days (oligotrophic bacteria). Numbers of obligate oligotrophs were determined by plating the bacteria that were isolated on oligotrophic agar onto copiotrophic and oligotrophic (100 times diluted) medium and calculating colonies that grew up specifically on carbon poor, not on carbon rich medium. The densities of oligotrophic and copiotrophic bacteria were $^{10}\log$ transformed. The ratios of copiotrophic to oligotrophic bacteria were calculated for back-transformed data ($10^{\log\text{-value}}$).

PCR amplification and DGGE analysis

Total DNA was extracted from all 16 soil samples from the differentially amended plots as described previously (Van Overbeek et al., 2012). DNA was purified and checked for purity and quality as described in the same paper. Total bacterial community DNA was amplified using the universal bacterial primers F968 with attached GC clamp (F968-GC) and R1378 (Van Overbeek et al., 2012). For amplification of *Pseudomonas* 16S-rDNA, a semi-nested PCR approach was performed with primers PsF and PsR in the first, and with PsR and F968GC in the second step (Garbeva et al., 2004). Amplification of ammonia-oxidizing β -proteobacteria (AOB) was performed with primers CTO189f-GC and CTO654r (Van Overbeek et al., 2012). Fungal 18S rDNA and ITS regions were PCR-amplified using the primers EF4 and ITS4 as described in Van Overbeek et al. (2012). The PCR products were purified with High Pure PCR Product purification kit (Roche Applied Science, Germany) and used as template in a nested PCR with the primers ITS1-F with GC clamp and ITS2 according to the procedure described in Anderson and Cairney (2004). Although PCR is widely known to be biased towards the more prevalent taxa, PCR-DGGE is widely used to estimate 'species' richness and diversity in soil (He et al., 2012; Van Overbeek et al., 2013).

DGGE analysis was performed with the PhorU2 system (Ingeny, Goes, The Netherlands). PCR products (15 μ l) were applied onto 6% (wt/vol) polyacrylamide gels with a denaturing gradient of 45 to 65% (100% denaturant contains 7M urea and 40% formamide) for bacterial-, *Pseudomonas*-

and AOB DGGE's, and of 30 to 80% for total fungal DGGE. Gel loading was described in detail in Van Overbeek et al. (2012). The gels were subjected to electrophoresis for 15 h at 60°C and 100V. After electrophoresis, the gels were stained for 30 min with SYBR Gold I nucleic acid gel stain (Molecular Probes Europe, Leiden, The Netherlands) and were photographed under UV light by using a SYBR Green gel stain photographic filter (Molecular Probes) and a Docugel V system apparatus (Biozym, Landgraaf, The Netherlands). Digitized finger prints from individual lanes were normalized using a marker loaded at three positions in the gel. This DGGE procedure is highly standardized in our lab, and newcomers obtain extensive training with repeated analyses on the same samples.

Band positions and relative band intensities were treated as 'species' and their relative abundances, respectively. Diversity index (H) and species richness (R) of bacteria, fungi, *Pseudomonas* and AOB communities were calculated based on DGGE profiles (He *et al.*, 2012; Van Diepeningen *et al.*, 2005). The total number of bands per lane represented the species richness of a sample. The Shannon biodiversity index was calculated as $H = - \sum p_i \times \log p_i$, where p_i is the relative intensity of a band (the ratio between the intensity of band i and the sum of all band intensities in one fingerprint). Taking the relative band intensity minimizes any concerns about the quantitative nature of this technique. H and R of oligotrophic bacteria (H_o and R_o) were calculated from the genus-level affiliation of oligotrophic isolates on low-carbon S medium for which 16SrDNA sequences were determined individually (Senechkin et al., 2010).

Chemical characterization of soil

Twelve grams of each soil sample were dried (40°C, 24 h) and ground to be used in all analyses. Total carbon (C_{tot}) was measured by the Dumas method followed by detection by a CHN1110 element analyzer (CE Instruments, Milan, Italy). Total nitrogen (N_{tot}) was determined by the Kjeldahl method. Nitrate (NO₃) content was determined with an Autoanalyzer II (Technicon Instrument Corporation, Tarrytown, NY) after addition of 0.01 M CaCl₂ suspension. Available phosphorus (P) and potassium (K) contents were measured according to Novozamsky protocols. The pH and dissolved carbon (DOC) were determined according to the Houba protocol. Organic matter (OM) content was measured by Ball's method. Details of the soil chemical analyses were given in Senechkin et al. (2010). Analyses of organic matter (OM) content and dissolved organic carbon (DOC) content were repeated on the same soil samples used previously.

Experimental design and statistical analysis

The field experiment had a randomized complete block design with two blocks having four treatment plots with two sampling locations each. For the analysis of AUDPC and DS, the data of five replicate

Table 1. Means and standard errors for microbial and chemical properties of treatment plots (S - slurry; C –compost; SD – slurry an dung; SCD – compost, slurry and dung). Treatments with identical letters in the same row do not differ significantly ($p>0.05$). AUDPC - area under the disease progress curve for *F. oxysporum*; log(oligo), log(copio) – log-transformed counts of oligotrophic and copiotrophic bacteria (CFU per gram dry weight of soil); pH – acidity; OM – organic matter (%); DOC – dissolved organic carbon (mg/kg); Ctot – total carbon (%) ; NO3 – nitrate (%); Ntot – total nitrogen (%); P – phosphorous (%); K – potassium (%); R, H – richness and diversity indexes for (indicated in lower case): b – bacteria, f – fungi, p – *Pseudomonas*, a – ammonia oxidizers, o – oligotrophic bacteria. All percentages are on a soil dry weight basis.

Variable	Treatment								Contrasts (sign. level)		
	S Mean ¹	S St. error	C Mean	C St. error	SD Mean	SD St. error	SCD Mean	SCD St. error	SCD vs rest	C vs all S	D vs no D noD
AUDPC	79.9 ^{ab}	2.0	88.8 ^a	3.7	81.5 ^{ab}	1.8	72.5 ^b	4.2	0.02	0.02	0.05
Log (oligo)	5.13 ^{ab}	0.10	5.32 ^a	0.05	5.28 ^{ab}	0.06	5.10 ^b	0.03	0.06	0.06	ns
Log (copio)	4.92 ^a	0.05	4.95 ^a	0.08	4.80 ^{ab}	0.09	4.65 ^b	0.07	0.01	0.05	0.01
pH	4.57 ^{ab}	0.02	4.50 ^{ab}	0.03	4.48 ^b	0.02	4.58 ^a	0.03	0.07	ns	ns
OM	4.25	0.15	4.10	0.30	4.20	0.10	4.05	0.15	ns	ns	ns
DOC	178.7	85.6	98.7	4.9	176.5	72.6	89.7	6.2	ns	ns	ns
Ctot	15.3	2.9	18.2	1.8	16.8	1.3	17.9	1.3	ns	ns	ns
NO3	0.22	0.02	0.19	0.01	0.17	0.02	0.16	0.02	ns	ns	ns
Ntot	1.68 ^a	0.05	1.58 ^{ab}	0.05	1.51 ^b	0.03	1.53 ^b	0.15	ns	ns	0.03
P	0.68	0.02	0.70	0.05	0.68	0.01	0.74	0.02	0.05	ns	ns
K	0.073	0.003	0.085	0.009	0.073	0.003	0.078	0.003	ns	ns	ns
Rb	33.5 ^{ab}	0.5	34.8 ^a	1.8	31.3 ^b	1.8	34.8 ^a	1.0	ns	ns	ns
Hb	1.41	0.01	1.42	0.02	1.39	0.01	1.43	0.01	ns	ns	ns
Rf	28.8	0.6	30.3	1.3	30.8	1.0	30.3	2.4	ns	ns	ns
Hf	1.29	0.02	1.32	0.02	1.33	0.01	1.31	0.03	ns	ns	ns
Rp	13.8	1.1	17.0	3.8	14.0	1.6	14.8	1.1	ns	ns	ns
Hp	0.98	0.02	1.04	0.09	1.00	0.04	0.97	0.04	ns	ns	ns
Ra	17.0 ^a	0.4	16.0 ^a	0.6	13.0 ^b	0.7	16.5 ^a	1.0	0.09	ns	0.01
Ha	1.06 ^a	0.01	1.06 ^a	0.01	1.00 ^b	0.01	1.05 ^a	0.01	ns	0.07	0.01
Ro ³	13.5	4.1	10.3	3.1	7.8	1.9	8.8	3.5	ns	ns	ns
Ho ³	0.53	0.13	0.40	0.14	0.51	0.17	0.42	0.08	ns	ns	ns

¹ Means are based on four replications

² Means within a row followed by the same letters do not differ significantly ($P>0.05$) according to Duncan's multiple range tests

³ Calculated from the genus-level affiliation of isolates on low-carbon S medium (Senechkin et al., 2010).

pots per soil batch (treatment*block*field rep) were combined. AUDPC and DS data from individual pots that deviated from the other pots in the same treatment were discarded (7% of all pots), but the

number of reps for the analysis remained 4 (2 blocks and 2 reps per plot). Bacterial CFUs, dissolved carbon and potassium contents were normalized by $^{10}\log$, log inverse and inverse transformations, respectively. Other variables did not need to be transformed. Differences in AUDPC and soil chemical and microbiological data between treatments were analyzed by ANOVA with 2 blocks and 2 reps per plot using PROC GLM in SAS 9.1 (SAS Institute, Cary, NC). Residual values were checked for normality. Treatment effects were examined by contrast analysis (Table 1). In addition, treatment means were separated by Duncan's multiple range tests.

Stepwise and canonical discriminant analyses were carried out to determine the relations between abiotic and biotic variables and AUDPCs using the SAS software. The quantitative AUDPC variable was changed into a binomial variable with values 1 (AUDPC<80; 44%) and 2 (AUDPC>80; 56%) and then used as classification variable in the discriminant analyses. All other variables were first standardized before analysis. Discriminant analyses were performed separately for microbial and chemical characteristics to have sufficient degrees of freedom. Variables that were selected in these first analyses were included in the final analysis with eight chemical/ microbial variables (pH, Ctot, Ntot, C-Nratio, Hf, Rf, Ra, and Ha).

Results

Effect of treatments on disease development

All flax plants that were grown in non-infested soil remained healthy. Initial symptoms of Fusarium wilt (yellowing of some leaves) were observed after 3 weeks. Over time, yellowing was accompanied by wilting, finally resulting in plant death. Initially, disease development was variable, but after 34 days, the disease was more homogeneous in most pots, and 40 days after planting, many plants were totally wilted or dead, especially those plants grown in compost-amended soil (C). The AUDPC was the highest for plants grown in this soil (Fig. 1) as well as those grown in sterilized control soil (average AUDPC = 96.7, standard deviation =16.3 severity*days), while AUDPC was the lowest for plants grown in a combination of plant compost, slurry and dung-amended soils (SCD). The AUDPC was significantly ($P=0.02$) higher for plants grown in compost-amended soil (C) than for those in liquid manure or slurry-amended soil (S), slurry and dung-amended soil (SD) and the combination treatment (SCD) (Table 1). The AUDPC was significantly ($P=0.02$) reduced in the SCD treatment compared to all other treatments. Consequently, disease suppression of Fusarium wilt compared to the sterilized control treatment was greater in plants grown in the SCD treatment than in the C, SD and S amended soils, while the disease suppression in compost-amended soil was least (Fig. 1). The significance levels of the differences in disease suppression were similar to those for AUDPC (data not shown).

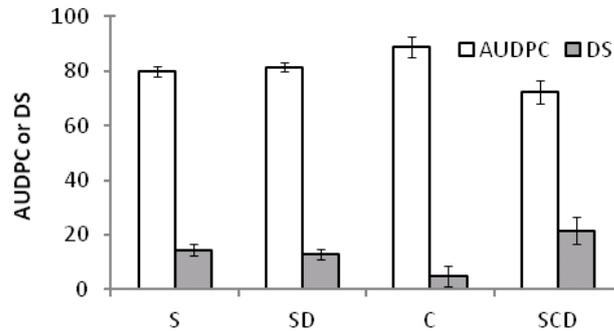


Figure 1. Effect of organic amendments on area under the disease progress curve (AUDPC) and % of *Fusarium* wilt disease suppression (DS) in flax plants grown in a growth chamber. Bars represent standard errors.

Population densities of oligotrophic and copiotrophic bacteria

Densities of oligotrophic bacteria were slightly higher in C-amended soil than in S and SD-amended soil, and lowest in SCD-amended soil (Table 1, Fig. 2). The contrast between C-amended soil and all other amendments was almost significant ($P=0.06$). Similarly, the contrast between SCD-amended soil and all other soils was almost significant ($P=0.06$). The trends in the densities of copiotrophic bacteria were similar to those in oligotrophic bacteria in that they were the lowest in the SCD-treated soil and the highest in S and C-amended soil ($P=0.01$). The average ratio of copiotrophic to oligotrophic bacteria ranged from 0.33 in the SCD treatment to 0.62 in the S treatment but the differences were not significant. There was no direct correlation between AUDPC and the densities of oligotrophic and copiotrophic bacteria, neither with the copiotrophic to oligotrophic ratio.

*Effect of treatments on soil chemical characteristics and their relation to *Fusarium* AUDPC*

Most chemical soil characteristics were not differentially affected by the various amendments (Table 1), probably because few amendments were applied in the year of sampling. The pH was very low in the sandy soil used in this study; it was slightly but significantly higher in the SCD treated plots than in the other plots ($P=0.05$ for the Duncan's test and $P=0.07$ for contrast analysis). The organic matter content was slightly lower in the SCD plots than in the other plots, but the difference was not significant. The total nitrogen content, N_{tot} , was significantly higher in the S-amended soil than in the other soils according to Duncan's test ($P=0.05$), while the contrast between the treatments with dung (SD and SCD) differed significantly from those without dung (S and C) according to contrast analysis

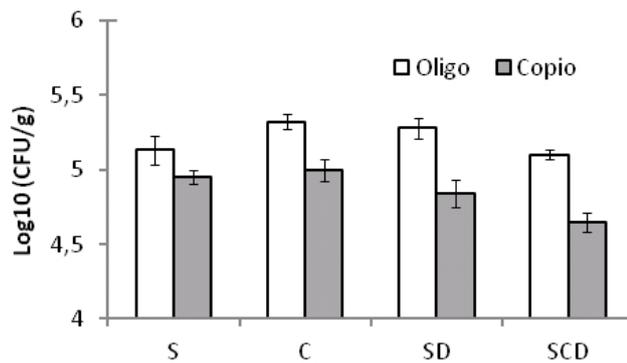


Figure 2. Population densities of oligotrophic and copiotrophic bacterial colonies in soils treated with different amendments: S- slurry; C- compost, SD – slurry and dung, and SCD- slurry, compost and dung. Values are expressed as \log_{10} c.f.u./g dry weight soil. Bars are standard errors.

($P=0.03$). The P content was significantly higher in the SCD-treated soil than in the other soils ($P=0.05$), but there were no differences in P content according to the Duncan's test. The DOC content was very high in some of the soil samples that had been treated with slurry (Table 1), but the differences among treatments were not significant due to the large variability. The AUDPC for Fusarium wilt was negatively correlated with pH ($r = -0.466$; $P = 0.06$), which was highest in the SCD treatment. There were no direct correlations with any of the other variables. However, in stepwise and canonical discriminant analysis, both pH and C_{tot} were selected (Tables 2 and 3). The ratio of N_{tot} to C_{tot} was selected in the canonical discriminant analysis, but the pooled within-class standardized canonical correlation was not quite high enough to be considered significant (Table 3).

Effect of treatments on microbial richness and diversity and their relation to Fusarium AUDPC

The 'species' richness and diversities did not differ very much among the treatments (Table 1), except for the richness of general bacteria, which were lower in the S and SD treatments according to Duncan's test (but did not differ according to contrast analysis), as well as the richness and diversity of the ammonia oxidizers, which were lowest in the SD treatment according to Duncan's test ($P=0.05$) and lower in both treatments with dung (SD and SCD) than in the S and C treatments according to contrast analysis ($P=0.01$). There were no direct correlations between microbial richness or diversity and Fusarium wilt. However, in the stepwise and canonical discriminant analyses, the diversity of the ammonia oxidizing bacterial community (H_a) contributed significantly to the discrimination between soil samples that were less or more conducive to Fusarium wilt of flax, expressed in an $AUDPC < 80$ or > 80 (Tables 2 and 3; Fig. 3). The diversity and richness of fungal communities (H_f and R_f) were also

Table 2. Soil chemical and microbial factors that contributed significantly to the classification of the area under the disease progress curve (AUDPC) of flax Fusarium wilt with values >80 or <80 by stepwise discriminant analysis.

Variable	Part. R ² ¹	Pr>F	Av.sq.can. corr.	Pr>ASCC
Ctot	0.238	0.055	0.238	0.055
pH	0.224	0.075	0.408	0.033
Ha	0.384	0.018	0.636	0.006

¹ Partial coefficient of determination (R²)

² Average squared canonical correlation (ASCC)

Table 3. Soil chemical and microbial factors that contributed significantly to the classification of the area under the disease progress curve (AUDPC) of flax Fusarium wilt with values >80 or <80 by stepwise discriminant analysis. The overall squared canonical correlation was 0.82, and Wilk's lambda for the overall significance of the analysis was 0.042.

Variable ¹	Canonical function 1 ²	Pooled within-class stand. class means ³		
	Stand. Can. Coeff. ⁴	Can. Correlation ⁵	AUDPC<80	AUDPC>80
Ctot	-0.854	0.262	0.593	-0.461
pH	0.903	0.221	0.502	-0.390
Ha	-0.929	-0.219	-0.496	0.386
N/C ratio	-1.896	-0.176	-0.398	0.309
Hf	-3.010	-0.095	-0.215	0.167
Rf	2.697	0.066	0.150	-0.117

¹ Variables selected in canonical discriminant analysis contributing to the discrimination between AUDPC classes

² Canonical function 1 is the linear combination of selected factors that best explain the low/high grouping of Fusarium AUDPC; if there are two groups, there is only one canonical function.

³ Pooled within-class standardized class means are the mean standardized (mean 0, standard deviation 1) values of the selected variables for the two classes of AUDPC.

⁴ Pooled within-class standardized coefficients indicate the direction (positive or negative) and the degree to which each variable contributes to the classification.

⁵ Pooled within-class canonical correlations between each selected variable and the canonical function; variables with a correlation greater than 0.187 [=0.4/√(eigenvalue)] were considered important (Workneh *et al.*, 1993).

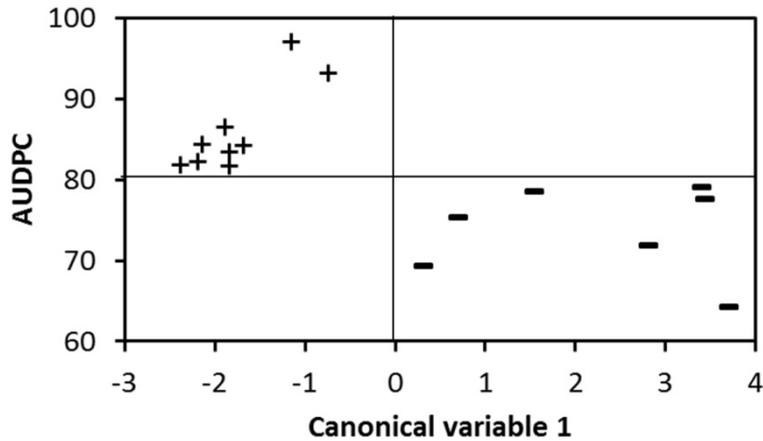


Figure 3. A plot of area under the disease progress curve (AUDPC) of flax Fusarium wilt versus canonical variable 1 as obtained from canonical discriminant analysis for two classes of AUDPC and 8 soil chemical and microbiological variables. Class 1 is depicted by -- ; class 2 is depicted by + .

selected in the canonical discriminant analysis, but their pooled within-class canonical correlations were not high enough to contribute significantly to the distinction between the AUDPC classes.

Nevertheless, the classification of all data points according to the canonical discriminant analysis with eight chemical and microbiological variables coincided perfectly with the original separation based on low and high AUDPC (Fig. 3).

Discussion

The AUDPC for Fusarium wilt on flax was significantly reduced by the application of complex organic carbon sources by a combination of different amendments (SCD). This resulted in significant disease suppression relative to the sterilized control treatment. The single and double amendments (S, C and SD) had only little effect on disease development. Greater improvement in soil quality due to recalcitrant rather than labile amendments has been shown previously (Burke et al., 2013). This result is important for organic and sustainable agriculturists who have to decide on the kinds of organic amendments that will be beneficial in the long run. The differences among the treatments were not very pronounced. This can be attributed to the short period (3 years) since the start of the different treatments and the relatively high organic matter content (about 3.5%) in this sandy soil before the

start of the experiment. Moreover, the amendments were applied as they would be in commercial organic farming systems, with larger quantities for the production of vegetable crops and potatoes and smaller quantities when grass-clover was grown. Samples for this research were taken from second-year grass-clover plots, 3 years after the application of large amounts of the different amendments for potato production. Many more years of differential treatments would be needed to obtain distinct differences in soil quality (Burke et al., 2013) and likely in root disease development (van Bruggen and Termorshuizen, 2003).

Nevertheless, the SCD treatment significantly delayed disease development (confirming our hypothesis), while amendment with plant-derived compost alone enhanced disease development to the same level as that in the sterilized control treatment. One of the reasons for the high AUDPC values in the C-treated plots may be that compost was applied only a few months before the soil was sampled. The effect of compost on the development of root diseases can vary widely: some composts may even enhance disease development (Termorshuizen et al., 2006). Maturation, availability of carbon sources and C/N ratio greatly affect the effect of compost on disease suppression (Bonanomi, 2010). Storage conditions can also affect disease suppressiveness of the compost, as tested for *Fusarium* wilt of flax (van Rijn et al., 2007). Drying under ambient conditions, which occurred to some extent before compost application at the Droevendaal farm, can affect the microbial community as determined by PCR-DGGE, possibly resulting in reduced suppression of flax wilt (van Rijn et al., 2007).

In the vast literature on soil suppressiveness to *Fusarium* wilts and root rots (Borrero et al., 2004; Castano et al., 2011; Klein et al., 2011), suppressiveness has been mainly attributed to biotic factors, evidenced from the elimination of disease suppressiveness after biocidal treatments (Lemanceau et al., 1993). In this study, AUDPC of flax wilt was negatively associated with the diversity of ammonia oxidizing bacteria (AOB) as evidenced from both stepwise and canonical discriminant analyses. Indeed, AOB diversity was significantly lower in treatments with dung than in those without. These bacteria oxidize ammonia to nitrite, the first and rate-limiting step of the nitrification process, and therefore play an essential role in the nitrogen cycle (De Boer and Kowalchuk, 2001; Webster et al., 2005). Previously, we showed that disease severity of *Rhizoctonia solani* on sugar beets was also negatively correlated with the richness and diversity of AOB (van Overbeek et al., 2012). When species richness and diversity of AOB were low, the pH was also low, particularly in the SD treatment (Table 1). It is well known that after addition of fresh organic amendments to soil, both the ammonium concentration and pH increase temporarily, followed by a rise in nitrification and a decline in ammonium and pH (Zelenev et al., 2005). Thus, the activity of AOB and pH are closely associated.

No correlations were found between the various microbial community structures and *Fusarium*

AUDPC as assessed by redundancy analysis (van Overbeek *et al.*, 2012; data for *Fusarium* AUDPC not shown). Thus, there was no relationship between general bacterial diversity (Rb, Hb) and flax wilt development (Table 3). Surprisingly, there was no relationship between *Fusarium* AUDPC and the richness or diversity of *Pseudomonas* populations either, while populations of fluorescent *Pseudomonas* sp. frequently are associated with the suppression of *Fusarium* wilt, at least in conventionally managed soils (Alabouvette 1999). On the other hand, fluorescent pseudomonads, in particular a phloroglucinol-producing *Pseudomonas fluorescens* strain, are suppressed in organically managed soil (Hiddink *et al.*, 2005), and thus, it is understandable that no relationships were found with *Pseudomonas* communities in the organically managed soils at the experimental farm 'de Droevendaal'. In addition, *Pseudomonas fluorescens* populations oscillate in soil after a disturbance, and disease suppression would depend on the time elapsed since the last disturbance (van Bruggen *et al.*, 2008).

In addition, no direct correlations were found between the AUDPC of flax wilt and fungal richness or diversity. Even though fungal richness and diversity were selected to distinguish between high and low AUDPC in canonical discriminant analysis, their canonical correlations with canonical variable 1 were low, and could not be considered significant. Nevertheless, it is well known that populations of non-pathogenic *Fusarium oxysporum* are often associated with suppression of *Fusarium* wilts in disease suppressive soils (Alabouvette, 1999).

Alabouvette *et al.* (1999) attributed soil suppressiveness towards *Fusarium oxysporum* f.sp. *lini* partly to carbon competition among the pathogen and the microorganisms present in soil. Dissolved organic carbon consists partially of easily utilizable carbon sources that are constantly utilized, released upon cell death, and then re-utilized by fast-growing microorganisms (Zelenev *et al.*, 2005; Zelenev *et al.*, 2006) that can compete with plant pathogens in soil and in the rhizosphere, thereby greatly affecting root disease development (He *et al.*, 2012; Hu *et al.*, 1997; Hu *et al.*, 1999). The concentrations of easily available carbon sources, as reflected in the DOC content, was lowest in the most suppressive, SCD-treated soil, but the differences among treatments were not significant due to the large variability in this variable (especially in S and SD amended soils). Apparently, slurry amendments resulted in very high DOC contents in local patches within the S and SD plots, which may have been diminished by high microbial activity in the SCD treated plots. The large variation in DOC contents was likely the reason for the absence of a direct correlation between *Fusarium* AUDPC and DOC content in this study.

Indirect effects of various abiotic variables, such as soil organic matter content, texture, clay type and pH, on microbial populations that contribute to soil suppressiveness is also possible (Alabouvette, 1999; Fang, 2012; Mazzola, 2002). In this study, only soil pH was negatively correlated with AUDPC of *F. oxysporum*. Soil pH was also selected in stepwise and canonical discriminant

analyses, together with the total carbon content C_{tot} , which was higher in soil samples where *Fusarium* wilt was suppressed. Dependency of disease development on pH was expected, as such correlations were reported earlier (Fang et al., 2012). It was also shown that pH can have an indirect effect on microbial populations that enhance soil suppressiveness (Alabouvette 1999). Optimal *Fusarium* wilt suppression can be achieved in more alkaline conditions (Fang et al., 2012; Scher and Baker, 1980). However, the pH in all amended plots used in this study was very low and likely did not influence disease suppression directly. C_{tot} was higher in soil samples where *Fusarium* wilt was suppressed, as expected based on previous studies, although the actual carbon compound greatly matters (Castano et al., 2011). No relationships were found between the AUDPC and any of the other abiotic variables. Involvement of abiotic factors in disease suppression is generally difficult to demonstrate (Alabouvette, 1999) due to the complex interactions of soil properties (Hoper and Alabouvette, 1996). Moreover, an extensive literature review on organic amendments and root disease suppression showed that enzymatic and microbiological parameters are generally more indicative of disease suppression than chemical parameters (Bonanomi et al., 2010), especially if small-scale temporal and spatial effects are not taken into account (He et al., 2012; van Bruggen et al., 2002).

Organic farms generally have a higher soil organic matter content and lower nitrogen losses (from nitrate leaching, nitrous oxide and ammonia emissions) per unit area compared to conventional farms, but crop yields are often lower than in conventional farms (Tuomisto et al., 2012). The long-term goal of our field experiment was to optimize organic management so that soil health would be improved, including a reduction in soil-borne diseases, while maintaining economic yields (van Overbeek et al., 2012). The current study was carried out in the beginning of this long-term experiment. The results point at the importance of soil pH, total carbon content, and ammonia-oxidizing bacteria as potential indicators for soil health (van Bruggen and Semenov, 1999). The soil pH was so low in this experimental field, that a slight increase likely resulted in increased availability of various (micro-) nutrients and improved growth of various microorganisms and plants, resulting in reduced susceptibility to *Fusarium* wilt. Total carbon is difficult to interpret, because different carbon pools can have very different effects on soil quality and health (van Bruggen and Semenov, 1999; Wander et al., 2007; Zelenev et al., 2006). The availability of easily degradable carbon has often been related to the ability of pathogens to survive in soil and a high copiotrophic to oligotrophic ratio (C/O ratio), which has been postulated to be associated with poor soil health and disease conduciveness (Borrero et al., 2004; Semenov et al., 2008; van Bruggen and Semenov, 1999). In particular high densities of oligotrophic actinomycetes relative to copiotrophic bacteria have been associated with disease suppression (Borrero et al., 2004; Castano et al., 2011; Workneh et al., 1993). The application of slurry (S) indeed resulted in the highest C/O ratio and DOC contents, but the differences were not significant due to high variability among samples. On the other hand, the diversity of ammonia-oxidizing bacteria was consistently associated with disease suppression (van Overbeek et al., 2012)

and soil health (Hayden et al., 2010), and additional research is underway to quantify ammonium oxidation by molecular tools (Senechkin et al., unpublished). Thus, despite the short time period since the initiation of differential treatments, this study contributed significantly to our understanding of soil health and disease suppression, and therefore contributes to the overall goal of enhanced agricultural sustainability. To reach this goal, farmers need to be actively involved in on-farm research in sustainable management techniques (Goud et al., 2004; Mancini et al., 2008).

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Chapter 4

Variation in microbial responsiveness and *Rhizoctonia solani* AG2-2IIIB growth in soil under different organic amendment regimes

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Abstract

Organic amendments influence chemical and microbial compositions in soils and also susceptibility to plant diseases. The purpose of this study was to establish different parameters that interfere with pathogen growth in soil. Four different organic-amendment regimes, i.e. slurry, compost, slurry-dung and compost-slurry-dung, were applied to two fields proximate to each other on the same farm and covered with the same grass-clover ley in the last two years preceding sampling. Before that period, there were differences in cropping and management practices between both fields. Chemical analyses of the soils revealed no differences between organic amendments, whereas significant differences were present between fields in all C, N and pH values. Growth of *R. solani* AG2-2IIIB in soil, measured by damping-off in sugar beet plants, was influenced by the interaction of organic amendment with field type. Diversity and evenness values of the microbial communities, studied by PCR-DGGEs specific for bacteria, fungi, *Pseudomonas* and ammonia-oxidizing β -proteobacteria, revealed different patterns, i.e. no differences between organic amendments, but clear differences between fields. Multivariate analyses done on individual species of the four groups, as represented by band location and size in PCR-DGGE fingerprints, and by inclusion of chemical and *R. solani* AG2-2IIIB growth parameters as 'environmental' variables, revealed strong and occasionally significant effects of organic matter content, water-dissolvable organic carbon and pH on microbial communities. It was therefore concluded that different organic amendments had different effects on pathogen growth in soils of both fields and that organic matter content and pH influenced soil microbial compositions most.

Introduction

Microbial community composition of soils is highly complex, and locally differs depending on soil type, land use and plant growth. The structure of microbial communities in agricultural soils are generally governed by soil treatments like tillage (Doran, 2011), amendments with different kinds of organic materials (Hoitink and Boehm, 1999; Pascual et al., 2002; Gorissen et al., 2004; Pérez-Piqueres et al., 2006), chemical fertilizers (Sarniquet et al., 1992 a,b) and crop growth (Kennedy and Smith, 1995; Mazzola, 2002; Garbeva et al., 2006). Agricultural management practices can exert different effects on microbial activities and plant disease control in soils (Larkin et al., 2011). Suppression of plant diseases is an important function of arable soils, but it is difficult to find microbiological parameters that report on antagonism towards phytopathogens in soil (Janvier et al., 2007). Because phytopathogens are integral components of natural soil communities, severe disease outbreaks in agricultural fields can be regarded as indicators for ecosystem distress (Van Bruggen & Semenov, 2000). Nutrient availability is an important criterion for soil-borne facultative saprotrophic phytopathogens to become prevalent in soils and their possible outgrowth can be constrained by soil-indigenous micro-organisms competing for the same nutrients (Hoitink and Boehm, 1999; Grünwald et al., 2000; Termorshuizen et al., 2006).

Most important microbial groups found to be responsive to various soil treatments and crop rotations were: Pseudomonads (Sarniquet et al., 1992 a, b; Hoitink & Boehm, 1999; Mazzola, 2002; Garbeva et al., 2006), fungi (Smit et al., 1999; Mazzola, 2002; Pascual et al., 2002; Anderson & Cairney, 2004; van Overbeek et al., 2008), *Lysobacter* (Postma et al., 2010) and *Burkholderia* species (Salles et al., 2004), and α - and β -proteobacteria (Schönfeld et al., 2003; Senechkin et al., 2010). Members of these groups are often associated with the suppression of phytopathogens in soils and it is generally assumed that these groups play important roles in disease suppression.

The purpose of this study was to find correlations between chemical and microbiological parameters in soil and susceptibility to a typical soil-borne phytopathogen like *Rhizoctonia solani* Kuhn. Therefore, we applied four different organic amendments to the same soil in two adjacent fields with identical grass-clover leys in the two years preceding soil sampling. The organic amendments are realistic for practice and differed from each other in quality and quantity of total organic carbon and nitrogen input. We determined chemical and microbiological parameters in the different plots, the last by virtue of group-specific molecular fingerprinting on soil DNA extracts. Fungi and *Pseudomonas* were considered to be microbial groups involved in antagonism towards *R. solani* AG3 (Garbeva et al., 2006), whereas chemolitho-autotrophic ammonia oxidizing β -proteobacteria (Kowalchuk et al., 2000; Kowalchuk and Stephen, 2001) were considered to be bacterial groups responsive to differences in available nitrogen. Acquired information will be relevant for the control of plant diseases caused by soil-borne pathogens in sustainable agriculture.

Materials and methods

Origin of the soils

Fields, denoted as 1 and 6, were located at the Droevendaal experimental farm, Wageningen, The Netherlands (coordinates, 5° 39' 36.69'', 51° 59' 30.30''). The farm was converted to organic farming in 2002 and accredited as an 'organic farm' in 2004. Before 2002, field 1 was cultivated by crop rotation (rye, potato, grass-clover) and managed under conventional farming practices, whereas field 6 was covered with grass and managed under organic agricultural practices. From 2002 onwards, four replicate plots (8 x 9 m in area) in randomized order, were established over both fields (each with a total area of 36 x 28m) with the following treatments: liquid cattle manure, slurry (S), green waste compost (C), combination of slurry and solid cattle manure, dung (SD), and combination of compost, slurry and dung (CSD) (for details see Table 1). During this period, plots in both fields were either covered with a grass-clover ley for four years (field 1) or cropped with rye (2002), potato (2003) and then covered with a grass-clover ley for two years (field 6) (Table 1). In September 2005, mixed samples were taken at two locations per plot. Each sample consisted of soil from four bore cores (10 cm in diameter), taken within an area of 40 x 40 cm to a depth of 20 cm each. Samples within the same plot were taken at a distance of 6 m from each other along the diagonal transect of the plot, keeping a minimal distance of 2 m from the borders of the plot. A total of 32 samples were thus taken and soils were stored for not longer than 1 week at 15°C. Chemical analyses were applied on all soil samples as described in Senechkin et al. (2010), with the exception of the organic matter (OM) content which was performed as described in Ball (1964).

Soil DNA extraction, PCR amplification and denaturing gradient gel electrophoresis

Total DNA was extracted from all soil samples using the MoBio Ultraclean soil DNA extraction kit (MO Bio Laboratories, Biozym TC, Landgraaf, the Netherlands). Therefore, 0.5 mL of sodium pyrophosphate solution containing 0.5 g soil and 50 mg of glass beads were added to microtubes and cells were dislodged from soil particles by bead beating (Hybaid Ribolyser, Hybaid, Middlesex, United Kingdom) for 60 s. Cell lysis and DNA purification was performed according to the protocol provided by the manufacturer. An additional DNA purification step was performed using the Wizard DNA clean-up kit (Promega, Leiden, the Netherlands). DNA quality was checked upon electrophoresis in 0.8% agarose gels stained with ethidium bromide.

Table 1. History and management of the field soils from experimental farm ‘De Droevendaal’.

	Field history and treatment	
	Management	Crop history
Before 2002		
Field 1	Conventional	Grass, potato, maize rotation
Field 6	Organic	Grass pasture
2002 - 2005		
Field 1	Organic	4 x grass-clover
Field 6	Organic	Rye, potato, 2 x grass-clover
After 2003		
Slurry (S)	72 m ³ / ha liquid cattle manure (slurry)	
Compost (C)	12 ton/ ha green waste compost (compost)	
Slurry, dung (SD)	43 m ³ / ha slurry, 27 ton/ ha solid cattle manure (dung)	
Compost, slurry, dung (CSD)	37 m ³ / ha slurry, 27 ton/ ha dung, 11 ton/ ha compost	

Bacterial PCR amplification with primers F968 with GC clamp (Muyzer *et al.*, 1993) and R1378 (Heuer *et al.*, 1997) was performed according to Van Elsas and Wolters (1995). *Pseudomonas*-specific PCR amplification was performed with the primers PsF and PsR (Widmer *et al.*, 1998) in the first, and with PsR and F968GC in the second step, according to the procedure described in Garbeva *et al.* (2004). Ammonia-oxidizing β -proteobacteria (AOB) PCR amplification with CTO189f-GC and CTO654r primers was performed according to Kowalchuk *et al.* (1997). Fungal PCR amplification was performed using primers ITS4 (White *et al.*, 1990) and EF4 (Smit *et al.*, 1999) in a first PCR, and primers ITS1-F (Gardes and Burns, 1993) with GC clamp and ITS2 (White *et al.*, 1990) in a second, nested, PCR according to the procedure described in Anderson and Cairney (2004).

For DGGE, polyacrylamide gels (6%) were prepared with denaturing gradients of 45–65% (100% denaturant consists of 7M urea and 40% formamide) for total bacterial-, *Pseudomonas*- and AOB-DGGE's and of 30 to 80% for total fungal DGGE. Gels were loaded with 15- μ L volumes prepared by mixing 10 μ L PCR product (c. 200 ng) with 5 μ L loading buffer (0.25% bromophenol blue, 0.25% xylene cyanol FF and 30% glycerol). All gels were placed in a PhorU2 apparatus (Ingeny, Goes, NL) set at 60°C and run at 100 V for 16 h. Then, gels were stained with SYBR Gold (Molecular Probes, Leiden, NL) and digitized fingerprints from individual lanes were normalized using a marker loaded at three different positions in the gel with Molecular Analyst software (version 1.61, BioRad, Veenendaal, NL).

Plant disease development tests

Spread of *Rhizoctonia solani* AG2-2IIIB in soil was recorded as damping-off in sugar beet (*Beta vulgaris* ‘Aligator’) seedlings growing at different positions from the *R. solani* AG2-2IIIB-point-of-inoculation in soils of all 32 samples, according to the experimental design described in Postma *et al.*

(2008). In short, 64 trays of 4 x 25 x 30 cm in size were filled with each of the 32 soils in duplicate, one for treatment with *R. solani* AG2-2IIIB and the other served as untreated control. Sugar beet seeds were planted in two rows of 11 seeds, keeping a distance of 20 mm between each seed in a row. After seedling emergence (after approximately 7 d), soils were treated by placing oat kernels with *R. solani* AG2-2IIIB 20 mm in front of the first seedling in each row (treatment with *R. solani* AG2-2IIIB), or soil in trays were kept untreated (non-inoculated control). Then, treated and control trays were checked for damping-off in sugar beet seedlings at days 7, 14, 21 and 25. Numbers of diseased plants from the two rows per tray were added and expressed as percentage of total number of plants per tray (22). From these data, the 'area under the disease progress curve' (AUDPC) and the total percentage of diseased plants after 25 d (cumulative disease incidence, DI) were calculated.

Experimental design and statistical analysis

The experimental fields were designed according to a split-plot scheme, consisting of two fields, separated into two blocks, each consisting of four plots (Senechkin et al., 2010). One treatment was applied for each plot and two samples per plot were independently analyzed. Values averaged from duplicate samples of each plot of chemical, microbiological evenness and diversity, and *R. solani* AG2-2IIIB growth parameters were used for making comparisons between organic amendment and field types. For multivariate analysis, values of all 32 samples were separately taken into consideration.

Distance matrices of PCR-DGGE banding patterns were calculated with GelcomparII software (version 4.5; Applied Maths, Woluwe, Belgium) using Pearson correlation, and clustering was performed using the unweighted-paired group mathematical averages (UPGMA) algorithm. Location and relative intensity of individual bands in fingerprints were taken into account, assuming that these represent single populations within each community. The correlation was calculated using Dice coefficient of similarity and a relatedness tree was produced with the algorithm of the Molecular Analyst software.

Multivariate analysis on PCR-DGGE fingerprints was performed using CANOCO 4.53 (Biometris, Wageningen University and Research Centre, NL). Hill Evenness (E) and Shannon diversity (H') values were calculated from the fingerprints made with the four different microbial-group-specific primer systems. Bands in PCR-DGGE fingerprints were used as 'species' variables, whereas nominal values for organic amendment and field, and quantitative values for all chemical and *R. solani* AG2-2IIIB growth parameters (AUDPC and DI) were used as 'environmental' variables. Indirect gradient analysis between variables was performed by correspondence analysis (CA) and direct gradient analysis by redundancy analysis (RDA). In order to judge for significance of effects of environmental parameters on species compositions, a Monte Carlo permutation test based on 499

permutations, was included. Effects were considered to be significant at levels of $P \leq 0.05$, whereas trends in effects were considered to be present at P levels of between 0.05 and 0.1.

Significance of differences between average E and H' values of total bacteria, fungi, *Pseudomonas* and AOB, and of AUDPC and non-transformed and arcsine-transformed DI values for *R. solani* AG2-2IIIB growth, were calculated by ANOVA (Genstat 10th edition, Rothamsted Experimental Station, Harpenden, UK). Differences were considered to be significant at levels of $P \leq 0.05$.

Results

Effect of organic amendment and field types on the chemical composition of soil

The average values of all chemical parameters calculated per organic amendment for each of the two fields were published before (Senechkin et al., 2010), except for the organic matter fraction in the different soil samples. No significant differences between the four organic amendments over the two fields were found for all six chemical parameters. When the data of the two fields were combined, significant differences between organic amendments were found only for OM, i.e. the percentage OM was lowest in CSD (2.90) and similar for S (3.12), C (3.12) and SD (3.29). When the data of all organic amendment treatments were combined per field, there were significant differences between both fields for all six parameters. The highest values in field 1 were found for pH (4.86 in field 1 and 4.53 in field 6) and $\text{NO}_3\text{-N}$ (0.23 g/ kg in field 1 and 0.184 g/ kg in field 6), whereas the highest values in field 6 were found for OM (2.49 % in field 1 and 3.71 % in field 6), water-dissolvable organic carbon (DOC) (71 mg/ kg in field 1 and 95 mg/ kg in field 6), total nitrogen (Ntot) (1.28 g/ kg in field 1 and 1.57 g/ kg in field 6) and total carbon (Ctot) (10.6 g/ kg in field 1 and 17.0 g/ kg in field 6).

Effect of organic amendment and field types on the microbiological composition of soil

Bacterial, fungal, *Pseudomonas* and AOB PCR-DGGEs, performed on soils of all 32 soil samples revealed the presence of between 34 and 51 bands in bacterial, 34 and 48 bands in fungal, 10 and 33 bands in *Pseudomonas*, and 34 and 51 bands in AOB fingerprints. Dendrograms constructed on the basis of intensity and position of these bands in the gels revealed strongest clustering per field for bacterial, *Pseudomonas* and AOB fingerprints (not shown). No consistent clustering per organic amendment within each field was found among all fingerprints made with the four different primer systems.

Two ecologically distinct parameters, Hill evenness (E) and Shannon diversity (H'), calculated from all fingerprints revealed E values of between 0.514 and 0.784 for bacteria, 0.617 and 0.845 for

fungi, 0.623 and 0.848 for *Pseudomonas* and 0.750 and 0.908 for AOB and H' values of between 2.875 and 3.501 for bacteria, 3.003 and 3.364 for fungi, 1.837 and 2.826 for *Pseudomonas* and 2.023 and 2.573 for AOB. No significant differences between average E values were found among organic amendments over the two fields, indicating that there were no interactions between organic amendment and field types. Also, no significant differences were present among values of the same organic amendments averaged over the two fields, indicating that there was no observable effect of the organic amendment type on the evenness of the studied microbial communities. However, E values for bacteria and AOB averaged per field significantly differed between both fields: for bacteria it was 0.657 in field 1 and 0.744 in field 6 and for AOB, 0.797 in field 1 and 0.838 in field 6. E values for fungi and *Pseudomonas* averaged per field did not differ significantly between both fields. Average Shannon diversity H' values of the organic amendments over both fields, and per organic amendment of both fields together, never significantly differed from each other. This indicates that diversities of the four studied microbial groups are not influenced by the interaction between organic amendment and field types and also not by organic amendment alone. However, average H' values for *Pseudomonas* and AOB per field significantly differed between fields: for *Pseudomonas* it was 2.295 in field 1 and 2.524 in field 6 and for AOB, 2.199 in field 1 and 2.375 in field 6. No significant effects between average H' values from both fields were observed for bacteria and fungi. There were, thus, significant effects of field type on the evenness of bacteria, on the diversity of *Pseudomonas* and on evenness and diversity of AOB.

Effect of organic amendment and field types on R. solani AG2-2IIIB growth in soil

Growth of *R. solani* AG2-2IIIB in all 32 soils, recorded as post-emergence damping-off in sugar beet plants (blackening of the roots followed by root rot and damping off of the plantlets), was monitored within a period of 25 days. Damping-off in sugar beet plants was not observed in plants grown in the same, but non-inoculated (control) soils within the same time period. *R. solani* AG2-2IIIB growth rate, measured as the progress in the number of diseased plants over time and expressed as 'area under the disease progress curve' (AUDPC) and *R. solani* AG2-2IIIB growth after 25 days, expressed as cumulative disease incidence (DI), differed per organic amendment and field type.

Average AUDPC and DI values, calculated per organic amendment for each of the two fields, are presented in Table 2. There was a statistical interaction between organic amendment and field types on the *R. solani* AG2-2IIIB growth rate in soil. AUDPC value for CSD amendment was found to be highest in field 1, whereas it was lowest in field 6, when compared with all other organic amendments in both fields. The AUDPC value of CSD amendment in field 1 was statistically different from all other organic amendments in both fields, except for C amendment in field 1 and SD amendment in field 6. There was no statistical interaction between organic amendment and field type

Table 2. *R. solani* AG2-2IIIB growth in soils treated with different organic amendments.

Disease parameter	Field 1#				Field 6			
	S	C	SD	CSD	S	C	SD	CSD
AUDPC	286 ^a	400 ^{ab}	277 ^a	563 ^b	260 ^a	264 ^a	426 ^{ab}	250 ^a
<i>Average per field</i>		381				300		
DI (%)	47.7	50.0	44.3	64.8	33.0	39.8	47.7	38.6
<i>Average per field</i>		51.7 ^b				39.8 ^a		

S, C, SD and CSD denote soils treated with, respectively, slurry, compost, slurry-dung, and compost-slurry-dung. Average values are based on four replicates per treatment in each field.

AUDPC, area under the disease progress curve; DI, cumulative disease incidence, number of diseased plants 25 d after inoculation with *R. solani* AG2-2IIIB.

Values with different letters indicate significant difference ($P \leq 0.05$), where $b > a$. LSD values: 191 (AUDPC), 8.04 (DS).

on *R. solani* AG2-2IIIB growth after 25 days. However, after averaging DI values from all organic amendment treatments per field, it revealed that there was a significant effect of field type on *R. solani* AG2-2IIIB growth after 25 days; i.e. DI values were higher in field 1 (51.7) than in field 6 (39.8). ANOVA performed on arcsin-transformed DI values revealed the same pattern; i.e., no statistical interaction between organic amendment and field type and a significant difference between average values from field 1 and 6. There is thus an interaction between organic amendment and field types on the *R. solani* AG2-2IIIB growth rate in soils, whereas a measurable effect of field type was found on *R. solani* AG2-2IIIB growth after 25 days.

Cross comparison between chemical, microbiological and R. solani AG2-2IIIB growth parameters

Biplot diagrams made by redundancy analyses (RDA) on individual bands in the 32 fingerprints from each of the four microbial groups as ‘species’ variables, and on nominal values of organic amendment and field types and on quantitative values of chemical and *R. solani* AG2-2IIIB growth parameters as ‘environmental’ variables, always revealed a correlation of field type with the first axis for all four microbial groups (Fig. 1). The effect of field type on microbial community structures in soils was significant for bacteria, fungi and AOB (P values of between 0.002 and 0.008) and just above the arbitrary level of significance for *Pseudomonas* ($P=0.07$). Overall, the second axis was correlated with organic amendment types, but the effects of organic amendments on species composition in all four communities were never significant. At three occasions there were effects just above the arbitrary level of significance, i.e. C treatment on the bacterial ($P=0.056$), SD treatment on the fungal

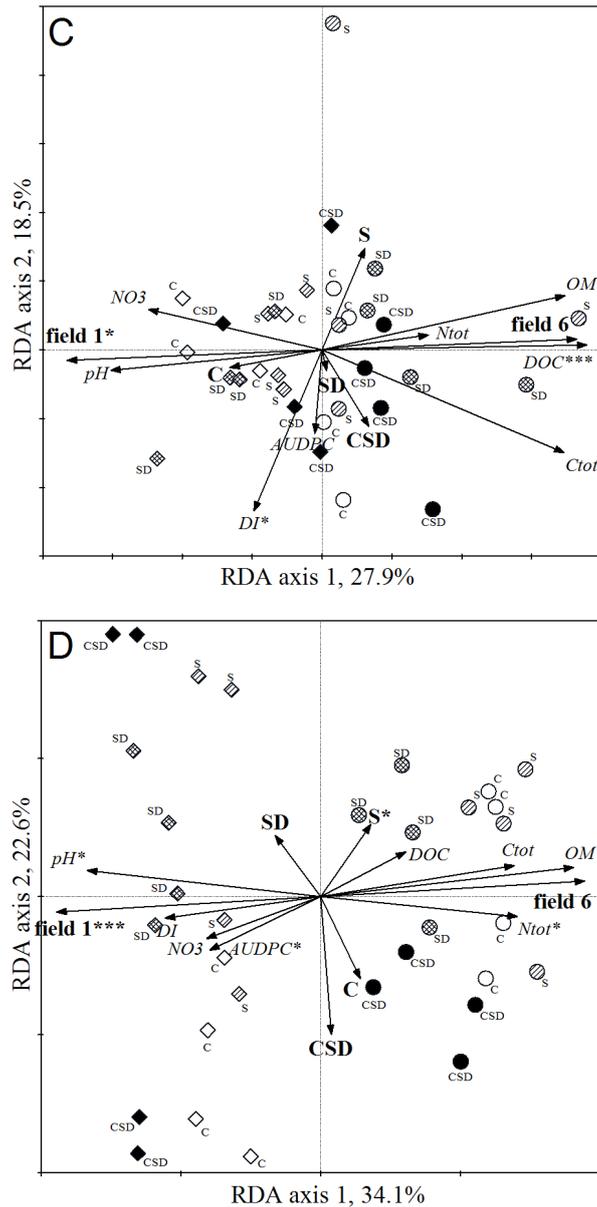


Figure 1. Biplots calculated by redundancy analysis (RDA) on a) bacterial b) fungal c) *Pseudomonas* and d) ammonia-oxidizing β -proteobacterial communities, determined by group-specific PCR-DGGEs on DNA extracts from 32 soil samples. Soils were taken from plots located in fields 1 and 6. For RDA, nominal values of organic amendment (S, C, SD, CSD) and field type (1, 6) and quantitative values of C (OM, DOC and Ctot) and total N, NO₃-N, pH, and *R. solani* AG2-2IIIB growth (DI and AUDPC) were used as ‘environmental’ variables. Explanation of symbols: diamonds, field 1; circles, field 6; symbols with diagonal lines, S; grid lines, SD; solid, CSD; open, C. Values at both axes indicates the percentage of the variation explained by each of the axes. Vectors are indicative for the contribution of each of the environmental variables on the community structure; longer sizes and smaller angles with the axis indicate higher correlation with the axis. The level of statistical significance of the interactions with the community structures are indicated by ***, $P \leq 0.01$; **, $0.01 > P \leq 0.05$; *, $0.05 > P \leq 0.1$; nothing, $P > 0.1$.

Vectors of the measured chemical parameters pH and OM always pointed into opposite directions and were, at most occasions, longest and showing the smallest angle with the first axis. This indicates that both parameters had an inverse relationship with each other and that both explained highest variation in all four microbial communities. However, significant effects of OM ($P=0.002$) and pH ($P=0.008$) were only observed on the fungal community structure. One other significant effect was found for DOC on the *Pseudomonas* community structure ($P=0.008$). Effects just above the arbitrary level of significance were only found for total nitrogen content ($P=0.086$) and pH ($P=0.088$) on the AOB community structure.

Vector lengths of the *R. solani* AG2-2IIIB growth parameters AUDPC and DI were in general smaller than vectors representing quantitative chemical, and nominal field parameters. This indicates that the variation that can be explained by these two parameters is relatively low in comparison with the other parameters. Significant relationships were not found, although at two occasions interactions just above the arbitrary level of significance were found between DI and *Pseudomonas* ($P=0.056$), and between AUDPC and AOB ($P=0.054$) communities. This indicates that trends in relationships exists between *R. solani* AG2-2IIIB growth parameters and the *Pseudomonas* and AOB species compositions.

Discussion

The combinations of organic amendment with field types, by virtue of differences in organic carbon and nitrogen input into two soils that differed in cropping and agricultural management histories, led to large variations in the measured soil chemical and microbiological parameters and two parameters describing growth of *R. solani* AG2-2IIIB in soil. No obvious effects of soil organic amendment on all measured parameters were found, with the exceptions of small, but non-significant effects of compost amendment on the bacterial community structure, slurry on the ammonia-oxidizing β -Proteobacteria (AOB) community structure and slurry and dung amendment on the fungal community structure. The effect of field type, however, was evident in three of the four studied communities.

Field type was the factor that was discriminative for most parameters and in field 1 soils, pH and $\text{NO}_3\text{-N}$ were higher and total C, organic matter content, water dissolvable C, total N, evenness of bacterial and ammonia-oxidizing β -proteobacterial communities and diversities of *Pseudomonas* and ammonia-oxidizing β -proteobacterial communities were lower than in field 6 soils. Across the board, pH, organic matter content and water-dissolvable organic carbon were the strongest chemical parameters determining the structures of the bacterial, fungal, *Pseudomonas* and ammonia-oxidizing β -proteobacterial communities in all 32 soils, although their effects were only significant for fungi (pH and organic matter) and *Pseudomonas* (water dissolvable organic carbon). How the chemical

composition influences the microbial community structure in soils, and how both factors influence pathogen growth in soil are questions that still need to be addressed.

The soils of the two studied fields were extreme with respect to pH and organic matter content. Both fields were located proximate to each other on the same experimental farm and there are no structural or textural differences between the soils at both locations. Also, grass-clover ley and organic management practice were the same for both fields during the two years preceding sampling. The chemical and microbiological diversification must have occurred in the period before and short after conversion to organic farming. It has been shown before that cropping history is a major driver behind changes in microbial community structures and increased suppression of plant diseases (Garbeva et al., 2004; Garbeva et al., 2006; Larkin et al., 2010; Larkin et al., 2011). In the studies done by Larkin and coworkers (2010, 2011), it was demonstrated that cropping histories may have long-lasting effects on disease incidences and crop yields. Also, in the studies done by Garbeva et al. (2004, 2006), the effect of crop history was evident and the soil used in these studies was almost identical with that of ours (the location of the experimental site was not further than 500 m from our sites). There, it was shown that damping-off in cauliflower caused by *R. solani* AG3 was lower in soil with a history of permanent grass coverage than in soil under crop rotation. Also, the *Pseudomonas* community structure differed between soils with different cropping histories. It can be concluded that the over-two-years difference in cropping histories between our fields was responsible for major differences between chemical and microbiological parameters.

The amendment of compost, slurry and dung to field 1 soil resulted in the highest rate of *R. solani* AG2-2IIIB growth in soil, whereas this rate was lowest in the same treatment applied to field 6 soil. Opposite effects in *R. solani* AG2-2IIIB growth thus resulted from the same treatment in different fields. We did not anticipate such a large variation in effects of organic amendments between both fields. This can be a finding of importance for agricultural practices where organic amendments are applied for control of soil-borne diseases, as the effect of organic amendment on susceptibility to plant diseases even can fluctuate within the same soil, depending on differences in crop rotations, soil amendments and agricultural management practices.

The structure of the *Pseudomonas* community was influenced by the size of the water-dissolvable organic carbon fraction in soil. *Pseudomonas* species generally are considered to be opportunists, rapidly responding to available nutrients in soil (Grünwald et al., 2000). Also, individual *Pseudomonas* species often act as antagonists towards plant pathogens and there was a small, at the level of $P=0.056$, relationship between the structure of the *Pseudomonas* community and cumulative disease incidence after 25 days. Via multivariate analyses, only correlations between measured parameters can be calculated and significance of effects do not necessarily indicate existence of a direct relationship between measured parameters. Still, it is an intriguing observation that lowest *R.*

solani AG2-2IIIB growth was measured in the soil that contained the highest dissolvable organic carbon fraction and the highest diversity of *Pseudomonas* species (field 6 soil). Most likely, dissolvable organic carbon influence the *Pseudomonas* community structure in soil by increasing the number of dominant *Pseudomonas* species. There might be an effect of resident *Pseudomonas* species on *R. solani* AG2-2IIIB growth in the studied soils.

One of the groups not expected to be responsive to any variation in carbon availability in soil are the chemolitho-autotrophic ammonia oxidizing β -proteobacteria. These ammonium-oxidizing β -proteobacteria are chemolithotrophes and do not necessarily need organic substrates for growth. Next to field type, no other significant interactions with any of the measured parameters were found. However, remarkable was the interaction, just above $P=0.05$, with *R. solani* AG2-2IIIB growth rate in soil. To the best of our understanding, there is no direct relationship between ammonium-oxidizing β -proteobacteria and *R. solani* AG2-2IIIB. Presumably, the same factors affected both the ammonium-oxidizing β -proteobacteria and *R. solani* AG2-2IIIB in soil. Two of these factors may be total nitrogen content and soil pH, because interactions of these two parameters with ammonium-oxidizing β -proteobacteria were at levels below $P=0.1$. The fact that there was also an interaction between the ammonium-oxidizing β -proteobacteria and slurry amendment at the level of $P=0.096$ indicates that ammonia present in slurry, or in the total nitrogen pool in soil, can be drivers behind the species composition of this group. Further, it has been shown before that soil pH had an effect on the ammonium-oxidizing β -proteobacterial community structure, mostly comprised of *Nitrospira* species (Kowalchuk et al., 2000). Especially one subgroup of *Nitrospira* species, denoted as cluster 2 in Kowalchuk et al. (2000), became more dominant in soils with lower pH values. We did not perform further attempts to identify these shifted groups of ammonium-oxidizing β -proteobacteria in our community fingerprints.

The combination of the different agricultural measures applied to our experimental soil plots changed soil chemical and microbiological parameters and it is difficult to assign their causal relationships in soil. Most likely, it is the chemical composition of the soil that drives the microbial community structure; however, it is unknown how the input of micro-organisms associated with the different amendments might change the soil microbial community structure. Further, it may be the soil micro-organisms that interact with the pathogen, but we did not measure effects directly on *R. solani* AG2-2IIIB mycelium in soil, but indirectly via development of damping-off in sugar beet plants. Decrease in susceptibility for damping-off most likely result from suppression of the pathogen, but changes in the physiology of sugar beet plants by the different circumstances prevailing in the differently treated soils also can play a role. We, however, can conclude that particular chemical parameters like pH, organic matter and water-dissolvable organic carbon contents are responsible for structural changes in communities of particular groups of soil micro-organisms. Growth of *R. solani* AG2-2IIIB in soil may be influenced by these community shifts. Interactions between particular

groups of micro-organisms and *R. solani* AG2-2IIIB either can be direct, as proposed for the *Pseudomonas* group of species, or in concert affected by the same factors, which most likely is the case for soil pH and total N on both the ammonium-oxidizing β -proteobacteria and *R. solani* AG2-2IIIB. To demonstrate direct effects of soil micro-organisms on soil-borne pathogens, it is better to focus on interactions between pathogen and antagonizing micro-organisms in soil, or under simulated circumstances representing the soil environment. This ultimately will provide information on the specific microbial groups that are responsible for suppression of phytopathogens and that can report on differences in susceptibility to plant diseases upon application of organic amendments to soil.

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Chapter 5

Interaction of *Collimonas* Strain IS343 with *Rhizoctonia solani* at low carbon availability in vitro and in soil

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Abstract

Collimonas sp. IS343, isolated from an organically-farmed arable soil and characterized as a broad-range oligotrophic bacterium, was shown to degrade chitin and to suppress *R. solani* mycelium growth under in vitro conditions at high and low carbon availabilities. In contrast to *C. fungivorans* Ter331, strain IS343 did not respond with an increase in growth rate to higher carbon levels in liquid medium, it reached higher cell numbers in carbon-poor media and it showed better survival in bulk soil. Therefore, it was concluded that strain IS343 cells are better adapted to circumstances of low carbon availability as present in bulk soils than strain Ter331 cells. Further, strain IS343 cells were more suppressive towards *R. solani* than strain Ter331 cells *in vitro*. When introduced into soil, strain IS343 cells delayed disease development caused by *R. solani* AG2-2IIIB in sugar beet plants. These results suggest that strain IS343 cells are able to tentatively suppress *R. solani* AG2-2IIIB mycelium growth in soil. Potential mechanisms behind the observed suppressive effects can be competition for available nutrients between strain IS343 cells and *R. solani* mycelium in soil or the production of chitinase as shown for this and other *Collimonas* species.

Introduction

Soils can be considered as environments that are generally low in carbon availability with occasional peaks in available carbon sources (He et al., 2010; He et al., 2012; van Bruggen and Semenov, 1999; van Bruggen et al., 2006; Van Veen et al., 1997; Zelenev et al., 2000; Zelenev et al., 2005b; Zelenev et al., 2006). Soil microbes respond to these circumstances and quickly utilize all available nutrients, thereby maintaining fluctuating, low levels of available nutrients in soils (van Bruggen and Semenov, 1999; van Bruggen et al., 2000; van Diepeningen et al., 2005; Zelenev et al., 2006). Among the various elements needed for bacterial growth in soils, carbon is most often limiting (Hu et al., 1997; Hu et al., 1999; Demoling et al., 2007). Part of the organic carbon fraction, mostly constituting dissolvable organic carbon (except for humic and fulvic acids), is readily available for microbial growth (Semenov et al., 1999), whereas the remaining fraction, mostly consisting of lignin- and cellulose-containing or humified organic matter, is rather unavailable and only becoming available after degradation by slow-growing micro-organisms (Van Elsas et al., 1997; Zelenev et al., 2005a; Zelenev et al., 2006). Therefore, to be successful in terms of competitiveness towards other microorganisms in soil, it is needed to cope with circumstances of low carbon availabilities (Semenov, 1991). Bacterial cells able to utilize carbon under low carbon availability levels thus can occupy important ecological niches in soil habitats.

Many processes relevant for agricultural production like phytopathogen suppression and nutrient cycling are taking place under conditions of low carbon availability in soils (Hiddink et al., 2005; Hu et al., 1997; Hu et al., 1999; Van Bruggen et al., 2002; Van Elsas and Van Overbeek, 1993). The role of bacterial oligotrophy in agricultural soils has been investigated on several occasions, revealing the importance of obligate and broad-range (facultative) oligotrophic bacteria in long-term low nutrient input soil systems (Semenov, 1991), which are characterized by their relatively high levels of stable organic matter, low levels of easily available carbon, and high microbial diversities (Van Bruggen et al., 2006; Van Diepeningen et al., 2006; Senechkin et al., 2010). These are the conditions typical for low-nutrient input (organic) farming systems where solid manure and/ or compost are regularly applied to field soils (Grünwald et al., 2000; Semenov et al., 2008; Semenov et al., 2009; Van Diepeningen et al., 2006; Van Overbeek et al., 2012). In soils under these farming practices, soil-borne phytopathogens generally are more suppressed (Hiddink et al., 2005; Van Bruggen & Termorshuizen, 2003) than in soils under (conventional) farming practices with high inputs of mineral nutrients and/or liquid manure (Semenov et al., 2009). The mechanisms behind these suppressive effects of soils on phytopathogen growth and plant infection, often called general suppressiveness, are not well understood.

Those bacteria that are well adapted to low carbon-availability levels and still are able to grow under these circumstances are called oligotrophs (Kuznetsov et al., 1979; Semenov, 1991). Cell

numbers of oligotrophic bacteria are fairly constant over different soils and often approach the carrying capacities for bacterial activity and growth in soils that have not been disturbed recently (Fierer et al., 2007; Zelenev et al., 2006). Oligotrophs are distinguishable from copiotrophs by their sustained growth even at low available carbon levels, whereas copiotrophs quickly respond in their activities and growth at circumstances of high nutrient availability, while they become arrested in their growth under growth limiting (i.e. low carbon availability) circumstances (Van Overbeek et al., 1994; Whang & Hattori, 1988; Hu et al., 1999). Oligotrophs can be isolated from soils on low-carbon agar media, so called oligotrophic media like the one that was used by Semenov et al. (1999) and Hu et al. (1999). On this medium *Collimonas* sp. strain IS343 was isolated from an organically-farmed arable soil (Senechkin et al., 2010) and was used in this study.

Collimonas species can be of relevance in general disease suppression in arable soils because these species prefer sites low in carbon availability and are able to predate on fungi (Leveau et al., 2009). Bacteria belonging to the genus *Collimonas* were isolated for the first time by De Boer and coworkers (1998) and later were recognized as a monophyletic group among the β -*Proteobacteria* (De Boer et al., 2001). Currently, the genus *Collimonas* comprises three different species: *C. fungivorans*, *C. arenae* and *C. pratensis* (De Boer et al., 2004; Höppener-Ogawa et al., 2008). Bacterial strains identified as *Collimonas* sp., based on their 16S rRNA gene sequence, have been isolated from many different environments (Ikeda et al., 2006; Opelt & Berg, 2004; Männistö & Häggblom, 2006; Schmidt et al., 2006; Uroz et al., 2007; Offre et al., 2007) and representatives of this genus are versatile in their occupation of ecological niches. *Collimonas* spp. generally are characterized by their slow growth rates, the ability to degrade chitin and to use living fungi as their sole food source (De Boer et al., 2001). Invasion of field soils by fungal hyphae has stimulated growth of indigenous *Collimonas* spp. (Höppener-Ogawa et al., 2009) and antifungal activity towards several phytopathogenic fungi that are common in the rhizosphere soil of Marram grass was demonstrated in *Collimonas* spp. isolates (De Boer et al., 1998). Tomato foot and root rot caused by *Fusarium oxysporum* f.sp.*radicis-lycopersici* was suppressed by *C. fungivorans* strain Ter331 (Kamilova et al., 2007). Cells of this strain colonized tomato roots and from there they could reduce further infection of the fungus into the plant roots. Although antifungal activities are common among *Collimonas* spp., the mechanisms behind these are rather obscure. Production of antifungal compounds like chitinases and competition for nutrients are often proposed to be key mechanisms behind suppression of fungal growth.

Based on the expectation that antifungal activity commonly occurs among *Collimonas* spp., our hypothesis is that *Collimonas* sp. strain IS343 is able to reduce *R. solani* growth in arable soil at a low carbon availability level. For that purpose we measured *Collimonas* sp. strain IS343 growth rates at high and low available carbon levels and also its interaction with *R. solani* under the same circumstances. The potential of *Collimonas* sp. strain IS343 to reduce post-emergence damping off

and stem blackening caused by *R. solani* AG2-2IIIB on sugar beet plants was evaluated in a plant-soil experimental set up.

Materials and Methods

Bacterial strains and standard conditions for bacterial growth

Collimonas spp. strains used in this study are shown in Table 1. The taxonomical relationship of *Collimonas* strain IS343 to other *Collimonas* strains, its physiology and its potential to suppress *R. solani* growth and disease development were investigated in this study, whereas *Collimonas* sp. strain 8.2.7, *C. arenae* strain Ter10, *C. pratensis* strain Ter91 and *C. fungivorans* strains Ter331 and Ter6 were used as reference strains. *Collimonas* sp. strain 8.2.7 originated from the same region ('De Veluwe', NL) and soil type (glacial sand deposit) as *Collimonas* sp. strain IS343. For standard growth of all strains, one-tenth strength trypticase soy broth agar ($0.1 \times$ TSB agar), consisting of 1 g liter⁻¹ KH₂PO₄, 5 g liter⁻¹ NaCl, 3 g liter⁻¹ trypticase soy broth and 20 g liter⁻¹ agar, at 25°C was used.

Table 1. *Collimonas* spp. and *Rhizoctonia solani* strains used in this study.

Species	Strain	Origin	Reference
<i>Collimonas</i> sp.	IS343	organic farmland soil	Senechkin et al., 2010
<i>Collimonas</i> sp.	8.2.7	organic farmland soil	Postma et al., 2008
<i>C. fungivorans</i>	Ter331	dune soil	De Boer et al., 2004
<i>C. fungivorans</i>	Ter6 ^T	dune soil	De Boer et al., 2004
<i>C. arenae</i>	Ter10 ^T	dune soil	Höppener-Ogawa et al., 2008
<i>C. pratensis</i>	Ter91 ^T	dune soil	Höppener-Ogawa et al., 2008
<i>R. solani</i>	AG2-2IIIB	Sugar beet	Postma et al., 2008
<i>R. solani</i>	AG2-1/21,	Cauliflower	Postma et al., 2010

^T Type strains.

Taxonomic comparisons between strain IS343 and other Collimonas spp. Strains

Strain IS343, described as a typical 'broad-range oligotroph' (Semenov, 1991) and originating from an organically-managed sandy soil, was identified as *Collimonas* sp. (Senechkin et al., 2010). Near complete 16S rRNA gene (1465 bp) comparison with SILVA database entries revealed a nearest match at non-type strain level with strain *Collimonas* sp. wged41 and a nearest match at species level was with type strain *C. fungivorans* Ter331. The exact taxonomical identity of strain IS343 remained inconclusive and therefore the taxonomic relationship between *Collimonas* sp. strains IS343 and other strains belonging to the genus *Collimonas* was determined by 16S rRNA gene sequence comparisons and BOX-PCR whole genome fingerprinting (Rademaker et al., 1997).

Genomic DNA of strains IS343, 8.2.7, Ter331, Ter6, Ter91 and Ter10 was extracted with a Pure Gene Genomic DNA isolation kit (Gentra System, Minneapolis, USA). For sequencing of the near entire 16S rRNA gene, PCR amplifications with 10 ng genomic DNA of each strain as templates were performed as described in Senechkin et al. (2010). Briefly, PCR amplification was carried out with primers 27F and 1492R; the PCR products were purified using a Sephadex plate (Senechkin et al., 2010) and then used as templates for further PCR reactions with primers P027F, R530 and 968R (Senechkin et al., 2010). Sequencing was done with an ABI Prism automatic sequencer by Greenomics (Wageningen, the Netherlands). Contiguous fragments of approximately 1400 bp were assembled using SeqMan software (DNASTAR Inc., USA). A neighbor joining dendrogram based on 16S rRNA gene sequences of all six strains mentioned above and of 17 *Collimonas* spp. strains and 10 *Herbaspirillum* species (as an outgroup), both from SILVA release 94 (Pruesse et al., 2007) database entries, was constructed using ARB software (<http://www.arb-home.de/>). The 16S rRNA gene sequence of strain IS343 was deposited in the EMBL database and is available under accession number FR729923.

BOX-PCR fingerprinting was done according to the procedure described by Rademaker et al. (1997) using genomic DNA from all strains as templates (100 ng). PCR products were run on a 2 % agarose gel containing 0.5× TBE buffer for 3.5 hours at 35 V and then gels were stained with ethidium bromide for visualization under UV light using Imago Compact Imaging System (Isogen Life Science, Maarssen, the Netherlands).

Growth rates of strains IS343 and Ter331 at different carbon levels

Growth rates of strains IS343 and Ter331 were determined in liquid media with high or low carbon availability. Cells of each strain were grown in copiotrophic medium (Semenov et al., 1999) overnight at 25°C with shaking. This medium has a carbon content of about 1000 mg/l. Then, cells were 10⁷-fold diluted in copiotrophic medium, oligotrophic medium (similar as copiotrophic medium except that 0.025 g liter⁻¹ glucose and 0.002 g liter⁻¹ casein was used, leading to a 100-fold lower carbon concentration in comparison with copiotrophic medium) or mineral medium (the same mineral composition as copiotrophic medium, but without glucose and casein hydrolysate); and 1 ml of each diluted suspension was added to 100 ml of the respective media in sterile Erlenmeyer flasks, leading to a final cell density of about 10 cells per 100 ml. Flasks were incubated at 25°C under shaking and a total of four replicates per treatment (medium x strain) were used for growth rate measurements.

Bacterial cultures were sampled after 12 hours and subsequently every 6 hours until the stationary growth phase was reached. The samples were serially diluted, plated onto 0.1× TSB agar, and plates were incubated at 25°C for two days after which bacterial colonies were counted using a stereo microscope (Leica Wild M5, Switzerland). Growth rates (μ) of bacterial strains were calculated using the following equation: $\mu = 3.322 \times (\log_{10} C_2 - \log_{10} C_1) / (T_2 - T_1)$, where C_2 and C_1 , respectively,

are the final and initial CFU numbers at time points T_2 and T_1 (Monod, 1949). C_2 and C_1 values were always taken from the exponential phase in the growth curves.

In vitro chitinase production of Collimonas sp. strain IS343

For determination of chitinolytic activity, chitin-yeast agar was used (1 g/l KH_2PO_4 , 5 g/l NaCl, 2 g/l colloidal chitin, 0.05 g/l yeast extract and 20 g/l agar) (De Boer et al., 2004). Droplets of 10 μl containing 10^7 cells of strain IS343 or of *C. fungivorans* strain Ter331, as a reference, were placed on three chitin-yeast extract agar plates each. Eventual appearance of clearance zones were recorded after five days of incubation at 25°C.

In vitro interactions between Collimonas and R. solani strains

In vitro competition between *Collimonas* strain IS343 and strain Ter331 with two *R. solani* strains (i.e. AG2-2IIIB, pathogenic for sugar beet and AG2-1/21, pathogenic for cauliflower; Table 1) was tested on R2A (Difco, Inc., Detroit, USA), serving as a relatively nutrient-rich reference medium (about 1000 mg C / L), and then on copiotrophic and oligotrophic agar media (Semenov et al., 1999) solidified with 20 g liter⁻¹ Difco Noble agar (Detroit, Mi).

Ten microliter drops with 10^6 cells of *Collimonas* sp. strains IS343 and Ter331 were placed at three locations on R2A, copiotrophic and oligotrophic agar surfaces (in Petri dishes of 9 cm in diameter), each at 0.5 cm from the edge of the plate. The plates were then incubated at 25°C for two days, mycelial plugs of either of the *R. solani* strains (growing on R2A plates) were placed in the center, and plates were incubated for seven more days at the same temperature. There were four replicates for each treatment (*Collimonas* sp. x *R. solani* strains). Eventual appearance of fungal inhibition zones surrounding the bacterial colonies were recorded from day four onwards. The distances between the hyphal tips and the edge of the plates at the location of the bacterial colonies were used as measures for the inhibition zones and averages of three inhibition zones per plate were calculated.

Inhibition of *R. solani* mycelium growth by strains IS343 and Ter331 (as a reference) was investigated in a second in vitro competition experiment using copiotrophic and oligotrophic agar plates. Two parallel straight lines were drawn on the bottom of the plates at 2 cm distance from the centers of all Petri dishes (14 cm diameter). One line served as control without *Collimonas*. Along the other line, a loop with bacterial cells of strain IS343 or Ter331 grown on copiotrophic agar medium was smeared over the copiotrophic and oligotrophic agar surface. Plates without *Collimonas* spp. smears, but with parallel marked lines at both sites (2 cm) of the center served as additional controls. The number of replicates per treatment (with or without *Collimonas* sp. strain x agar medium) was

three, and all experiments were repeated three times. Plates were incubated at 25°C for one day to allow bacteria to grow first and then an agar plug of *R. solani* AG2-2IIIB mycelium was placed in the center of each plate. The plates were then incubated at 25°C for one more day after which *R. solani* hyphal densities were measured at crossings on both lines on the plates. Hyphal densities were expressed as the number of hyphae crossing a 0.5 cm transect in the middle of the lines with and without *Collimonas* sp. cells. Measurements were repeated every day, up to day four after incubation at the same temperature. Ratios between hyphal densities on the lines with and without bacteria on the same plate were calculated and were used as measure for the inhibitory effect on *R. solani* hyphal branching. Averages per plate were calculated for all three experiments coming to a total of nine independent measurements per treatment. At the end of each experiment, hyphae on the marked lines were stained with cotton blue, and pictures were taken with a digital AxioCam MRc camera attached to a compound microscope (Zeiss Axioskop, Germany) at 100 times magnification and were processed using AxioVision 4 imaging software (Carl Zeiss vision GmbH, Germany).

Survival of Collimonas sp. IS343 and C. fungivorans Ter331 cells in soil

Survival of *Collimonas* sp. IS343, and of *C. fungivorans* Ter331 as a reference, was studied in soil collected from the experimental farm 'De Droevendaal' (51°59'N, 5°39'E) near Wageningen, The Netherlands. The soil was a loamy sand containing 2 % organic matter, with a water holding capacity of 25 % and a pH (KCl) of 4.8. Dried (pF 4.2) and sieved (1 mm mesh) soil portions (20 g dry weight) were inoculated with sterile water (control), or 10⁶ or 10⁸ cells of either strain per g of dry soil. Sterile distilled water was added so that the final matric potential of all soil portions was at pF 2 (equivalent to 18 % soil moisture for this soil). Soil samples were stored at 20 °C in plastic petri dishes. Subsamples (1 g) were taken on days 0, 1, 3, 5 and 11, and frozen at -20 °C for later DNA extraction, using three replicates per treatment (*Collimonas* spp. strain x cell density). Soil DNA was extracted from all frozen subsamples using the Power Soil DNA extraction kit (Mo Bio Lab, USA) and the *Collimonas* spp. genome equivalents were quantified by qPCR using the same *Collimonas* spp.-specific primers and running the qPCR under the same conditions as described in Höppener-Ogawa et al. (2007). For preparation of the standard curve, genomic DNA of strain IS343 was serially 10-fold diluted and all diluted samples were run in triplicate under the same qPCR reaction conditions. The resulting standard curve ($Ct = -4.51 \times \text{Log}(\text{cell equivalent}) + 50.61$; $R^2 = 0.99$) was used for conversion of Ct values into log₁₀ cell equivalents, under the assumption that one genome equivalent was present per cell.

Effect of Collimonas sp. strain IS343 on sugar beet damping off caused by R. solani AG2-2IIIB

The effect of *Collimonas* sp. strain IS343 on post-emergence damping off and stem blackening, caused

by *R. solani* AG2-2IIIB, was studied on young sugar beet plants. The following treatments were applied: (1) strain IS343 and *R. solani* AG2-2IIIB added to soil (experimental treatment), (2) *R. solani* AG2-2IIIB added to soil (control for disease development in the absence of strain IS343), and (3) noninoculated soil (control for background disease development, caused by *R. solani* indigenously present in the soil). Every treatment consisted of 20 replicates completely randomized on a greenhouse bench.

A total of three experiments were performed at different stages of sugar beet seedling development. In experiment 1, seven-day old sugar beet (*Beta vulgaris* cultivar Alligator) seedlings (in the four-leaf stage) were used, whereas in experiments 2 and 3 ten-day old seedlings (at the six-leaf stage) were used. Seedlings had been grown in standard potting mix. Pots (16 cm diameter and 18 cm height for experiment 1; 12 cm diameter and 14 cm height for experiments 2 and 3) were filled with non-inoculated conventional field soil collected next to the Droevendaal experimental farm. Holes (3 cm dia, 5 cm deep) were made in the soil in the center of each pot. For the experimental treatment, soil inoculated with strain IS343 (10^8 cells per g dry soil) was put into the central hole, one sugar beet seedling per pot was planted into the strain IS343-inoculated soil, and four oat kernels infected with *R. solani* AG2-2IIIB (Postma et al., 2008) were symmetrically placed at 1 cm from the border of each pot. In the control for disease development, all holes were filled with noninoculated soil, one seedling was planted in each hole, and four *R. solani* AG2-2IIIB-infected oat kernels were placed as described for the experimental treatment. For the control treatment for background disease development, the holes were filled with noninoculated soil and planted with sugar beet seedlings; no *R. solani* AG2-2IIIB-infected oat kernels were placed in the pots. Although the seedlings in experiment 1 were 3 days younger than those in experiments 2 and 3 when planted, the ages were likely the same when *R. solani* reached the plants, because the pathogen had to grow 3 cm further in the larger pots to reach the plants (assuming that *R. solani* grows 1 cm per day).

All pots were placed in the greenhouse and maintained under a light / darkness and temperature regime of 16 h at 23°C / 8 h at 18°C, at a constant air humidity of 90 %. Disease symptoms, i.e. post emergence damping-off or stem blackening typical for *R. solani* infection on sugar beet plantlets were recorded over a four-week period in all treatments during the three experiments. The areas under disease progress curves (AUDPC), including plants with lesions and those that were damped off, were calculated using the trapezoidal method (Campbell, 1990) for all treatments in the three different experiments.

Statistical analyses

Two-tailed student t tests were used to compare differences between: (1) average growth rates ($n = 3$) between strain IS343 and Ter331 in copiotrophic, oligotrophic and mineral media, (2) average ($n = 9$)

R. solani AG2-2IIIB hyphal densities between control (no *Collimonas* spp. applied), strain IS343 and strain Ter331 treatments, separately on oligotrophic and copiotrophic agar media, (3) average ($n = 3$) Log *Collimonas* spp. cell equivalent numbers per g of dry soil measured on day 0 (3 h after introduction into soil) and on day 11 in soils inoculated with strain IS343 and strain Ter331 cells at levels of 0, 6 and 8 log cell equivalents per g dry soil, (4) average ($n=3$) number of diseased sugar beet plants and AUDPC values in control (no *R. solani* AG2-2IIIB) and *R. solani* AG2-2IIIB-treated soils, with and without strain IS343 cells. Additionally, differences in numbers of sugar beet plants diseased by *R. solani* AG2-2IIIB in soils with (experimental treatment) and without strain IS343 cells (control) were analyzed by Wilcoxon matched pairs test using Statistica software (Version 9, StatSoft Inc., Tulsa, USA). Calculations were based on pairs of corresponding numbers of diseased plants per time point from experimental and control treatments and the number of time points were 14 in experiment 1, nine in experiment 2, and 12 in experiment 3.

Results

Taxonomic relationship between strain IS343 and other Collimonas spp.

The dendrogram constructed on the basis of 16S rRNA gene sequences of different *Collimonas* spp. strains revealed a cluster consisting of strains IS343, 8.2.7 and of *Collimonas* sp. strain wged41 that was distinguishable from three other clusters (Fig 1). This cluster was most closely related to the cluster containing sequences of all *C. arenae* strains, whereas the two clusters, containing the sequences of *C. pratensis* and of *C. fungivorans* strains, were more distinctly related. Representative strains of different *Collimonas* spp., i.e. strain Ter10 (*C. arenae*), Ter91 (*C. pratensis*) and Ter331 and Ter6 (both *C. fungivorans*), were used for comparison with strains IS343 and 8.2.7 at a whole genome level using BOX-PCR fingerprinting. Fingerprints of all six strains were clearly distinguishable from each other and the one of strain IS343 showed closest resemblance with that of Ter6 (84 % similarity), whereas the one of strain 8.2.7 showed closest resemblance with that of Ter331 (85 % similarity). In spite of the fact that strains IS343 and 8.2.7 had identical 16S rRNA genes, they were clearly distinguishable from each other at a whole genome level. Therefore, strain IS343 was selected to represent a hitherto undescribed group of *Collimonas* species and its physiology and interaction with *R. solani* was studied. Strain Ter331 was chosen as a reference because it is a well described strain belonging to the *Collimonas* group of species.

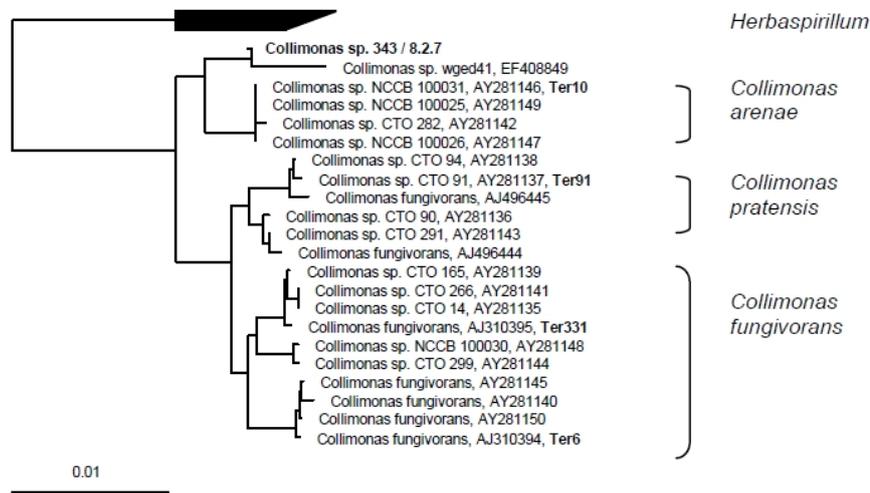


Figure 1. Phylogenetic tree of a subset of representative *Collimonas* spp. 16S rRNA gene sequences including *Collimonas* sp. IS343 sequence.

Growth of strains IS343 and Ter331 in liquid media with different carbon levels or with and without chitin

Growth rates of strains IS343 and Ter331 in three liquid media, all differing in initial carbon concentration (mineral, oligotrophic, and copiotrophic medium) were measured. Cells of strain IS343 in mineral medium started to grow from 12 h onwards and continued to grow up to a final CFU density (expressed in Log_{10} CFU ml^{-1}) of 6.22 ± 0.10 after 78 h. Cells of strain Ter331 in the same medium also started to grow from 12 h on, but the growth curves leveled off after 38 h, reaching final densities of 5.22 ± 0.14 after 78 h. In oligotrophic medium, cells of both strains immediately started to grow and curves of both strains leveled off after 78 h, reaching final CFU densities of 9.36 ± 0.32 for strain IS343 and 6.84 ± 0.91 for strain Ter331. In copiotrophic medium, cells of both strains also immediately started to grow and the growth curve of strain IS343 leveled off after 78 h, reaching a final CFU density of 10.20 ± 0.48 . At 78 h, strain Ter331 had not leveled off yet, and had a CFU density of 10.48 ± 0.57 at that time.

Average growth rate values (in log CFU per h) of strain IS343 calculated from the growth curves in oligotrophic and copiotrophic media, were not significantly different, respectively, 0.64 ± 0.14 and 0.60 ± 0.06 ($P = 0.68$). However, for strain Ter331 average growth rate values were significantly different between media, 0.62 ± 0.04 for oligotrophic, and 0.76 ± 0.10 for copiotrophic

medium. The physiologies of the two strains were different; where growth of strain IS343 cells remained largely unaffected by the available amount of carbon present in the medium, growth of strain Ter331 was affected resulting in higher growth in copiotrophic than in oligotrophic medium.

Similar to strain Ter331, colonies of strain IS343 grown on chitin-yeast extract agar formed clear halos that became apparent after 6 days of incubation. Therefore, cells of strain IS343 must produce chitinolytic enzymes degrading chitin in the medium and suggesting the same potential as strain Ter331 to parasitize on fungi.

In vitro competition between Collimonas and R. solani strains at different carbon levels

Rhizoctonia solani inhibition zones surrounding colonies of strains IS343 and Ter331 on R2A became apparent after three days. On day four, fungal inhibition zones surrounding colonies of strain IS343 were at a maximum with diameters of 1.20 ± 0.30 cm for *R. solani* AG2-2IIIB and 0.95 ± 0.06 cm for *R. solani* AG2-1/21. Thereafter, some hyphae crossed the inhibition zones. Fungal inhibition zones surrounding colonies of strain Ter331 were 0.58 ± 0.34 cm in diameter for *R. solani* AG2-2IIIB, whereas no inhibition zones surrounding strain Ter331 colonies were registered in all four replicates for *R. solani* AG2-1/21. Both *Collimonas* sp. strains thus were able to suppress *R. solani* mycelium in vitro on R2A, except that strain Ter331 was only capable to suppress growth of one *R. solani* anastomosis group, whereas strain IS343 did that for both tested anastomosis groups.

No clear fungal inhibition zones of both *R. solani* strains were registered along the streaks of strains IS343 and Ter331 on copiotrophic and oligotrophic agar media. However, there were differences in mycelium growth in the neighborhood of colonies of both strains on both copiotrophic and oligotrophic agar at 20 times magnification. Hyphae most proximate to the streaks of both *Collimonas* sp. strains were thinner and less branched than those on the side of the plate without bacteria (Fig 2). Differences in hyphal densities expressed as the ratio of hyphal densities near *Collimonas* spp. cells and at the same distance from the center, but in the absence of *Collimonas* spp. cells on the same plates, were calculated for the combinations of *R. solani* AG2-2III without (control), or with *Collimonas* spp. strains IS343 or Ter331 (experimental treatments) on copiotrophic and oligotrophic agar media. After one day on copiotrophic agar medium, all ratios were between 0.8 and 1.0 and not statistically different between control and experimental treatments. After two days, a significant difference ($P = 0.006$) occurred between the control and strain IS343 treatments and the ratio in hyphal density was 0.54 in the treatment with strain IS343 and 0.97 in the control treatment (Fig 3A). On day three, the difference in ratios was still present although not significant ($P = 0.075$) anymore, 0.76 in the treatment with strain IS343 and 0.99 in the control treatment. Between strain Ter331 and control treatments on the same copiotrophic medium, no difference between ratios was present on day two (ratios were 0.97 for Ter331 and 1.01 for control treatment), whereas a significant

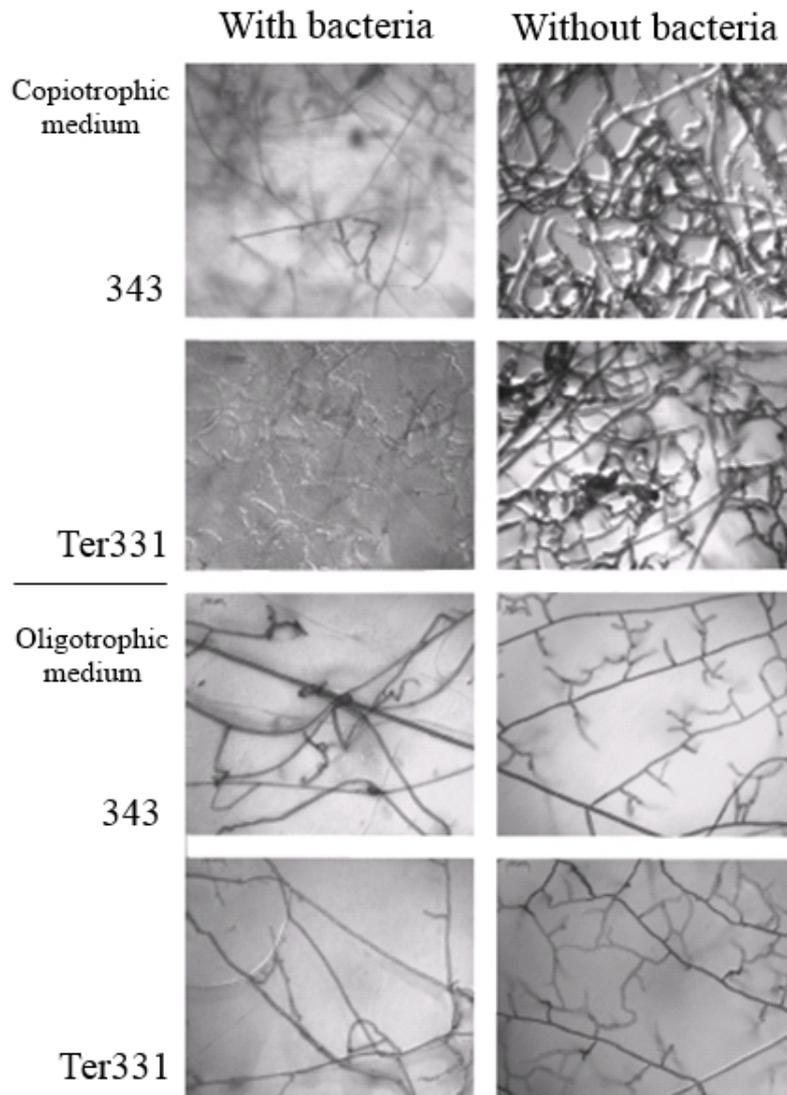


Figure 2. Cotton blue stained hyphae of *Rhizoctonia solani* AG2-2IIIB coming into contact with *Collimonas* sp. IS343 or *Collimonas fungivorans* Ter331 cells or growing in the absence of bacteria on copiotrophic and oligotrophic agar media.

difference ($P = 0.026$) was present on day three, viz. 0.67 for treatment with stain Ter331 and 1.02 for the control treatment (Fig 3A). From day four on, ratios were around 1 again and no significant differences between treatments were present, indicating that strains IS343 and Ter331 reduced *R. solani* AG2-2III mycelium growth on copiotrophic agar medium when hyphae passed through the bacterial streaks, but were not suppressive anymore once some *Rhizoctonia* hyphae had passed the bacterial colonies.

On oligotrophic agar medium, the ratios ranged again from 0.8 to 1.0 on day one for all

treatments and differences were not significant. However, on days 2 and 3 the ratio between the number of hyphae crossing the line with strain IS343 and the line without bacteria differed significantly from that of the control plates (both lines without bacteria), being respectively, 0.16 and 1.02 ($P < 0.001$) on day 2 and 0.62 and 1.08 ($P = 0.038$) on day three (Fig 3B). The ratio between the hyphae crossing strain Ter331 and the line without bacteria differed significantly from the control treatment on day two only, being 0.67 and 1.02 ($P = 0.026$), respectively, while no difference between treatments was present on days three and four (ratios were around 1 in both treatments on both days) (Fig 3B). This indicates that the effect of the two *Collimonas* spp. strains on *R. solani* AG2-2IIIB mycelium growth was also temporary on oligotrophic agar. Moreover, strain IS343 had a stronger effect on mycelium growth on the oligotrophic than on the copiotrophic agar medium.

In the experiments with strain Ter331, the hyphal density ratio was significantly lower than that of control plates without bacteria only on day 2 on oligotrophic agar medium. Comparing treatments with strain IS343 and strain Ter331, the ratios were significantly lower for strain IS343 on day two on both copiotrophic agar ($P = 0.035$) and on oligotrophic agar medium ($P = 0.026$), whereas on day one, three and four no differences were present. When repeated (3 times), the experiments gave similar results. Thus, the suppressive effect of strain IS343 on *R. solani* AG2-2III mycelium growth was stronger than that of strain Ter331 on both agar medium types. Most importantly, both *Collimonas* spp. strains were able to reduce *R. solani* AG2-2IIIB mycelium density under conditions low in carbon availability suggesting that strain IS343 can retard development of disease caused by this phytopathogen in soils where available carbon is low.

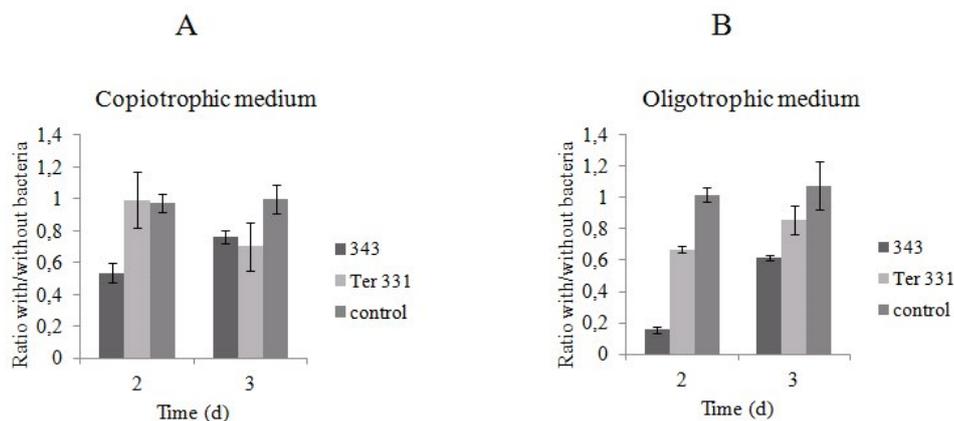


Figure 3. Ratio of hyphal density of *Rhizoctonia solani* AG2-2IIIB, growing through a line of *Collimonas* sp. IS343 or Ter331 cells, to that in the absence of bacterial cells on copiotrophic (a) and oligotrophic (b) agar media.

Survival of Collimonas strains IS343 and Ter331 cells in soil

Survival of cells of strains IS343 and Ter331, introduced into soil at two density levels, was monitored over a period of 11 days. In noninoculated soils, the numbers of *Collimonas* spp. cell equivalents (expressed in Log number of cell equivalents per g of dry soil) were between 4.51 and 5.97 during the entire monitoring period of 11 days. When introduced into soil at density levels of 8.70 (strain IS343) and 8.85 (strain Ter331), *Collimonas* spp. cell equivalent numbers in bacteria-treated soils dropped to 8.52 (strain IS343) and 6.47 (strain Ter331) after 11 days. Differences in *Collimonas* spp. cell equivalent numbers between days zero and 11 were not significant for strain IS343-treated soils, but were for strain Ter331-treated soils ($P < 0.001$). *Collimonas* spp. cell equivalent numbers in treated soils were not significantly different between the IS343 and the Ter331 treatments, on day zero (3 h after introduction), whereas they differed significantly on day 11 ($P < 0.001$).

When introduced into soil at a density of 6.46 (for both strains), cell equivalent numbers dropped to 6.12 (strain IS343) and 5.30 (strain Ter331) on day 11. Differences in cell equivalent numbers between day zero and 11 were not significant in strain IS343-treated soils, whereas they were in strain Ter331-treated soils ($P = 0.002$). Moreover, measured *Collimonas* spp. cell equivalent numbers in strain IS343-treated soils were still above the natural *Collimonas* spp. background level in non-inoculated Droevendaal soil on day 11, whereas numbers in strain Ter331-treated soils were indistinguishable from the background level at the same sampling time. Differences in *Collimonas* spp. cell equivalent numbers between strain IS343- and strain Ter331-treated soils were marginally significant at $P = 0.098$ on day 11. Clear differences in survival in Droevendaal soil were thus present between strains IS343 and Ter331 in soil; strain IS343 cell numbers remained about constant over the entire monitoring period of 11 days, whereas strain Ter331 cells dropped over two orders in magnitude. Because of the better survival of introduced strain IS343 cells in Droevendaal soil, it was decided to study the effect of this strain on disease development caused by *R. solani* AG2-2IIIB in a sugar beet plant-soil experiment.

Post-emergence damping-off and stem blackening by R. solani AG2-2IIIB on young sugar beet plants

Three experiments were conducted where the effect of strain IS343 cells introduced into conventional Droevendaal soil at levels between Log 8.01 and 8.30 cell equivalents per g of dry soil on *R. solani* AG2-2IIIB disease development (combination of post-emergence damping off and stem blackening) in sugar beet plants was followed over time.

In control treatments (without added *Collimonas* or *R. solani* AG2-2IIIB), the numbers of diseased sugar beet plants (out of 20 in all experiments) at the end of the experiments were 2 on day 25 for experiment 1, zero on day 12 for experiment 2 and four on day 16 for experiment 3. *R. solani* indigenously present in Droevendaal soil was responsible for the disease observed in experiments 1

and 3, as determined from the appearance of typical root symptoms and isolations on water agar. In the treatment without *Collimonas* sp. strain IS343 cells in soil, but with the addition of *R. solani* AG2-2IIIB, the total numbers of diseased plants were higher than in the treatment with strain IS343, namely 20 on day 25 (experiment 1), 20 on day 12 (experiment 2) and 13 on day 16 (experiment 3) (Table 2). Introduced *R. solani* AG2-2IIIB was responsible for the disease observed in sugar beet plants in this treatment in all three experiments. In the treatment where *Collimonas* sp. strain IS343 cells were introduced into soil and where *R. solani* AG2-2IIIB was added, the numbers of diseased sugar beet plants were 18 on day 25 (experiment 1), 18 on day 12 (experiment 2) and 9 on day 16 (experiment 3). Comparing the average number of diseased plants over the monitoring periods in the three experiments between strain IS343-treated and untreated soils using Wilcoxon matched pair test, the number of diseased plants in treated soils was significantly higher: at $P = 0.007$, $P = 0.027$, and $P = 0.003$ for experiments 1, 2 and 3, respectively. Thus, introduced strain IS343 cells had a small but consistent suppressive effect on *R. solani* disease incidence in sugar beet plants.

When expressed as AUDPC values, disease development in sugar beet plants caused by *R. solani* AG2-2IIIB was more reduced in IS343-treated, than in untreated soils in all three experiments (Table 2). However, AUDPC values varied among the three experiments. The values were larger in experiment 1 than in experiments 2 and 3, and this can be explained by the difference in experimental set up. In spite of the fact that plants were presumed to be at the same age when coming into contact with mycelium of *R. solani* AG2-2IIIB, the time interval between the introduction of strain IS343 into soil and root infection caused by the introduced fungus was three days longer in experiment 1 than in experiments 2 and 3. Moreover, observations in experiment 1 were continued for 25 days, while they were stopped after 12 and 16 days in experiments 2 and 3.

Average AUDPC values over all three experiments were lower in the treatment with strain IS343 (589.2) than in the one without (805.8), and this difference was marginally significant at $P = 0.09$. This relatively high P value is due to the high variation among AUDPC values calculated for the three experiments, resulting from differences in experimental set up. There was a clear tendency towards slower *R. solani* disease development in sugar beet plants in the presence of strain IS343 cells in all three experiments. The ratio in AUDPC values over the treatments without and with strain IS343-inoculated soils was 1.38 for experiment 1, 1.22 for experiment 2 and 1.54 for experiment 3, coming to an average value of 1.38 over all three experiments (Table 2).

Table 2. *R. solani* AG2-2IIIB disease progress (AUDPC for post-emergence damping-off or black lesions) on sugar beet plants and maximum number of diseased plants (between brackets) in soils non-inoculated and inoculated with *Collimonas* sp. strain IS343 cells.

	With strain IS343	Without strain IS343	AUDPC ratio ¹
Exp 1	1130 (18)	1555 (20)	1.38
Exp 2	370 (18)	450 (20)	1.22
Exp 3	267.5 (9)	412.5 (13)	1.54
Average	589.2 (15)	805.8 (17.7)	1.38²

¹ The ratio of AUDPC without strain IS343 over that with IS343.

² Significance levels calculated over the three experiments by Student t test: for AUDPC, $P = 0.09$ and for the maximum number of diseased plants, $P = 0.03$.

Discussion

Collimonas sp. strain IS343 growth and interaction with *R. solani* was investigated at carbon levels comparable with those in (bulk) soils, viz. about 10 mg C / dm³. Concentrations of dissolved organic carbon ranged from 65 to 100 mg C / dm³ in the organic Droeendaal soil after various amendments (Senechkin et al., 2010), and without amendments these would have been even lower. It was hypothesized that oligotrophic Collimonads would actively grow in bulk soil devoid of high concentrations of directly utilizable carbon sources and in principle would be capable of suppressing fungal phytopathogens under these circumstances, at least partially as a result of chitinolytic activity. Indeed, cells of strain IS343 grew at low carbon availability and these cells were able to temporarily reduce mycelium density of two *R. solani* strains, especially at low carbon levels. Moreover, these cells sustained in bulk soil for at least 11 days at about the same density. We therefore conclude that *Collimonas* sp. strain IS343 can antagonize *R. solani* in bulk soil and by virtue of this activity, may contribute to general suppressiveness against *R. solani* and possibly other soil-borne fungal phytopathogens. The precise role of *Collimonas* sp. strain IS343 types in suppression of *R. solani* in agricultural fields need to be further explored, especially in relation with soil treatments by organic amendments.

Soil low in easily mineralizable carbon was shown to be the preferred site for oligotrophic bacteria (Hu et al., 1999). Strain IS343 is a broad-range (facultative) oligotrophic bacterium and originates from arable soil kept under a low input (organic) management regime (Senechkin et al., 2010), the same soil as used in this study. Slightly acidic soils, low in nutrients and hardly influenced by human interferences, generally are considered to be the preferred niches for Collimonads (Leveau et al., 2009). Collimonads were found to be present in higher numbers in the mineral, than in the organic fraction of soil (Höppener-Ogawa et al., 2007) and species belonging to this genus were

shown to be involved in mineral weathering (Uroz et al., 2009). Like for other *Collimonas* spp. strains, the preferred niche of our strain, IS343, is soil low in easily available organic matter. However, the physiology of strain IS343 differed somewhat from that of strain Ter331, used as a reference in this study. In contrast to strain Ter331, strain IS343 did not adapt its growth rate to a higher level of utilizable carbon in the medium, it reached higher cell levels at low carbon availabilities (in mineral and oligotrophic media) and it showed better survival in bulk soil. It may therefore be concluded that cells of strain IS343 are better adapted to conditions prevailing in bulk soil than those of strain Ter331.

Strain IS343 cells also appeared to be better suppressors of *R. solani* than strain Ter331 cells. Fungal inhibition zones surrounding IS343 colonies were larger than the ones surrounding Ter331 colonies on R2A (relatively copiotrophic) agar medium; strain IS343 cells reduced mycelium density of two *R. solani* anastomosis groups on copiotrophic and oligotrophic agar medium, whereas Ter331 cells reduced that of only one *R. solani* isolate. *R. solani* hyphal growth on oligotrophic agar medium was more suppressed by IS343 than by Ter331 cells. Therefore, it was concluded that cells of strain IS343 are better suited to suppress *R. solani* in soil than those of strain Ter331. This conclusion is supported by the better growth and survival of strain IS343 than strain Ter331 at low available carbon concentrations, the greater inhibition zone and reduced mycelial density on oligotrophic medium, and the known increased chitinase production at low glucose concentrations due to catabolite repression of the production of this enzyme (Ingram and Westpheling, 1995; Lavrent'eva et al., 2009).

Fungal suppression by Collimonads by chitinase activity was demonstrated by De Boer and coworkers (1998). Later, suppression of *Fusarium oxysporum* f.sp. *radicis-lycopersici* by strain Ter331 in tomato plants was demonstrated by Kamilova et al. (2007). Although colonization of *F. culmorum* hyphae by strain Ter331 was already demonstrated (De Boer et al., 2001), colonization of the *F. oxysporum* hyphae on tomato roots by Ter331 cells was demonstrated in the study by Kamilova and coworkers (2007). From the latter study it was concluded that the mechanism behind *F. oxysporum* suppression was not antibiosis or fungal cell wall degradation, but competition for available nutrients.

Competition for available nutrients between *R. solani* mycelium and IS343 cells on oligotrophic agar medium and in soil may also be one of the mechanisms behind the observed reduction in mycelium density and disease development in this study. This mechanism may partially explain why the inhibitory effect of strain IS343 cells on *R. solani* was only temporal on copiotrophic and oligotrophic agar media. Local consumption of available nutrients by *Collimonas* cells can cause mycelium growth retardation, but lost nutrients easily can be replenished by the fungus by the virtue of its extensive mycelium network on agar media and in soil. The fungus possibly takes up nutrients from elsewhere and transports these to sites where it is in competition with strain IS343 cells. This will result in growth retardation, but not in complete killing of the fungus. An additional explanation could

be that diffusion of chitinase is restricted, so that individual hyphae that pass colonies of strain IS343 could form new branches again when out of reach of the enzyme.

Whatever the mechanism, the question is whether soil-indigenous Collimonads can reach cell numbers adequate for control of fungal phytopathogens under realistic field conditions. Strain IS343 cell numbers applied under the experimental conditions were higher than the indigenous Collimonads cell numbers found in natural Droevendaal soil. From the current study we can conclude that under natural circumstances *Collimonas* strain IS343-like cells have the potential to retard *R. solani* growth in soil. *Collimonas* spp. cell numbers vary among different soils (Höppener-Ogawa et al., 2007) and it is possible that different soil treatments affect *Collimonas* spp. cell numbers in soils; in particular, long-term organic treatments may favor *Collimonas* spp. and induce root disease suppression (van Overbeek et al., 2012). Further, *Collimonas* spp. cell numbers can fluctuate very locally (Semenov et al., 1999), e.g. at the tomato root surface where *C. fungivorans* Ter331 microcolonies were observed (Kamilova et al., 2007). Oscillations in bacterial densities and counter-oscillations in easily available carbon sources along the length of a root were shown to result in oscillations in infections by *R. solani* (van Bruggen et al., 2002). Sampling location can thus influence the ability to predict whether possible interactions of *Collimonas* spp. cells with phytopathogens will occur in plant-soil environments (van Bruggen et al., 2006).

In conclusion, we describe strain IS343 as a member of the *Collimonas* genus that thrives under circumstances low in carbon availability commonly occurring in soil. Under these circumstances, cells of this strain presumably are actively competing for scarcely available nutrients with fungi, as well as breaking down the cell wall of these fungi, including the ones that are pathogenic for plants. Because of these activities, *Collimonas* sp. IS343, and presumably many other members of the *Collimonas* genus, are responsible for fungal phytopathogen suppression in soils. The Collimonad group is one of the fractions of the soil-indigenous community that may be involved in general disease suppression in arable soils. *Collimonas* community sizes thus could be a measure for general disease suppressiveness present in soils and tools designed to quantify (local) Collimonad cell numbers could then be predictive for the disease-suppressive status of arable soils.

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Chapter 6

Key genes responsible for the conversion of nitrogenous compounds in soil as potential indicators for root disease suppressiveness of soils

Senechkin, I.V., Bara, R, Van Bruggen, A.H.C. and Van Overbeek, L.S. (2013) Key genes responsible for the conversion of nitrogenous compounds in soil as potential indicators for root disease suppressiveness of soils.

Abstract

Cycling of nitrogenous compounds in soil is essential for plant growth and microbial life. Availability of N could possibly play a role in development of soil-borne plant diseases. Therefore, the quantities of key genes involved in nitrogen cycling, ammonium monooxygenase (*amoA*) and nitrite reductases (*nirS* and *nirK*) genes, and their effects on the disease suppressive status in soil were investigated using real-time PCR. No correlation was found between gene quantities and disease intensity values (disease incidence and area under the disease progress curve) determined for sugar beet and flax plants inoculated with, respectively, *Rhizoctonia solani* AG2-2IIIB and *Fusarium oxysporum* f.sp. *lini*. However, significant curvilinear relationships between *nirS* and *nirK* gene quantities and the total soil nitrogen fraction were found. Although a direct interaction between N cycling genes and disease suppression was not found, an indirect relationship via the total N fraction in soil may exist.

Introduction

Soils managed under organic farming practices are considered to be lower in easily degradable nutrients, more diverse in soil species and more resistant against stresses and diseases than conventionally managed soils (Van Diepeningen et al., 2006). However, there are large differences among organically managed soils (Franz et al., 2008). To investigate potential differences in soil health, a field experiment was performed on a certified organic farm where four different organic amendments were applied for fertilization and enhancement of root disease suppression (Van Overbeek et al., 2013; Senechkin et al., 2010; Senechkin et al., 2013). In these studies it was found that soil microbial communities involved in nitrogen cycling and plant health were shifted, either depending on the type of organic amendment, or on the natural variation in chemical parameters occurring between fields differing in crop history. One of the key questions arising from these studies was whether there would be a link between mineralization of essential elements (specifically N) and disease suppression (Van Overbeek et al., 2013; Senechkin et al., 2013) in arable soils.

In the current study we postulate that processes important in cycling of N that make this element available for plants and soil micro-organisms is also important for suppression of soil-borne pathogens. It is well known that soil borne plant pathogens and the root disease they cause are affected by the amount and type of nitrogen compounds in soil and in plants (Knopp and Martensson, 2010; Rowaished, 1981). Microbial populations responsible for the nitrification and denitrification processes could be, in principle, indicators for the disease suppressive status of soils (Van Bruggen and Semenov, 2000; Van Overbeek et al., 2013). Genes coding for enzymes in key steps of denitrification and ammonium-oxidation could serve as indicators for the 'health status' of soils and could be measured with quantitative (real-time) PCR (Braker et al., 1998; Chapman et al., 2012; Hannig et al., 2006; Hayden et al., 2010; Rothauwe et al., 1998; Sims et al., 2012, 2013; Throbäck et al., 2004; Wallenstein and Vitgalys, 2005). Bacteria responsible for the last step in the denitrification process (possessing *nir* genes) were more abundant in organically than in conventionally managed soils, so that denitrification was more complete in organically managed soils and less nitrite was emitted from those soils (Kramer et al., 2006). Combining this information with the well-established facts on enhanced root disease suppression in organically managed soils (van Bruggen and Termorshuizen, 2003) led to the formulation of the hypothesis that bacterial populations responsible for (de)nitrification could possibly be used as indicators for soil health and disease suppression. Therefore, a molecular, real-time PCR-based approach was applied with the purpose to establish potential relationships of nitrification and denitrification genes with disease suppression and other measurable soil characteristics, like pH, soil organic matter and nitrogen content. Real-time PCR measurements on soil extracted DNA samples were carried out using the same soils as in Van Overbeek et al. (2013) and Senechkin et al. (2013) with primers directing genes coding for ammonium

monooxygenase (*amoA*) and nitrite reductase (*nirS* and *nirK*) genes.

Materials and Methods

Field site description and sampling

The experimental field where soil samples were collected was located at the certified organic experimental farm 'De Droevendaal', Wageningen, The Netherlands. For experimentation, the same 16 soils from field 6 were used as those in the studies done by Senechkin et al. (2010), Van Overbeek et al. (2013) and Senechkin et al. (2013). In short, four different organic amendments were applied in duplicate on field 6 soil: liquid cattle manure (slurry, S), green waste compost (C), combination of slurry and solid cattle manure (slurry and dung, SD), and combination of compost, slurry and dung (CSD). Two soil samples were collected per plot. Soil sample collection and chemical soil analyses were done as described in Senechkin et al. (2010), Van Overbeek et al. (2013) and Senechkin et al. (2013).

Soil DNA extraction and real-time PCR

DNA was extracted from all 16 soil samples as described in Van Overbeek et al. (2013) and Senechkin et al. (2013). Real-time PCR was performed on all 16 samples using primer systems directing the following genes: *amoA*, *nirS* and *nirK* (Table 1). Melting curves derived from each individual reaction were inspected in order to ascertain that the signals obtained originated from specific PCR reactions and not from primer dimer formation or any other artifact. Further, PCR amplicons made with all three primer systems with DNA from three different soils were checked in 1.5% agarose gels for presence of bands of the expected sizes and absence of any secondary or false-positive products.

Soil DNA extracts were diluted in sterile demineralized water to set soil DNA concentrations at 1 ng per μl for each sample. Soil DNA samples were 10-times further diluted for real time PCR with *amoA* primers only. Real time PCR mixtures (25 μl) were then prepared containing the following ingredients: 12.5 μl of 2x concentrated SYBR Premix Ex Taq (TAKARA Bio Inc., Japan), 0.5 μl of each primer (10 μM ; Biolegio, NL), 0.5 μl of 50x concentrated ROX Reference Dye II (TAKARA Bio Inc., Japan), 6.0 μl H₂O and 5.0 μl template DNA (0.5 ng for *amoA* and 5 ng for *nirS* and *nirK*). All reactions were run, in duplicate, in a 96-well microtiter plate format in an ABI Prism 7500 Sequence Detection System (Applied Biosystems). Applied PCR conditions were set at 2 min at 50°C for one cycle; 10 s at 95°C for one cycle and 5 s at 95°C and 35 s at 60°C for 35 cycles. The number of cycles was limited to 35 to avoid occurrence of false positive signals (Sipos et al., 2007). Average Ct values were calculated from duplicate reactions for each soil sample, separately amplified with each of the

three primer systems (Table 1).

Table 1. Quantitative PCR systems for detection of ammonium oxidizing (*amoA*) and nitrite reduction (*nirS*/ *nirK*) genes in organically amended Droevendaal soils.

Target gene (gene product)	Primer set	Amplicon length	References
<i>amoA</i> (ammonium monooxygenase)	amoA-1F/	491 bp	Rotthauwe et al. (1997)
	amoA-2R		Wallenstein and Vilgalys (2005)
<i>nirS</i> (nitrite reductase)	nirS1F/	900 bp	Braker et al. (1998)
	nirS6R		Wallenstein and Vilgalys (2005)
			Hannig et al. (2006)
<i>nirK</i> (nitrite reductase)	nirK1/	520 bp	Throback et al. (2004)
	nirK5R		Braker et al. (1998)

Data analyses

Analysis of variance (one-way ANOVA; Genstat 15th edition, Hemel Hempstead, UK) was performed with the purpose to calculate significance of differences in average Ct values, separate for each primer system, among the four soil treatments applied on field 6 soil. All differences were considered to be significant at levels of $P \leq 0.05$.

Multivariate analysis (CANOCO for Windows version 4.5, Biometris, Plant Research International, The Netherlands) was performed using average Ct values per soil sample of the three different primer systems as the ‘species’ variables and measured chemical (pH, phosphorous [P], Potassium [K], total Kjeldahl Nitrogen [Ntot], organic matter [OM], nitrate [NO₃], total Carbon [Ctot], dissolvable Carbon [DOC]) and disease intensity (disease incidence [DI] and area under the disease progress curve [AUDPC] values of *Rhizoctonia solani* AG2-2IIIB in sugar beet and *Fusarium oxysporum* f. sp. *lini* in flax) parameters as the ‘environmental’ variables. Gradient lengths were calculated by detrended correspondence analysis (DCA) in a first step, and correlations between ‘environmental’ and ‘species’ variables in following steps by redundancy analysis (RDA). A Monte Carlo permutation test (499 permutations) was included to calculate significance of effects of individual environmental parameters on species variables.

Cubic polynomial regression equations were calculated in MS Excel using the eight measured chemical parameters in soils as explanatory variables and average Ct values per soil sample for each of the three primer systems separately as response variables.

Results

Confirmation of amoA, nirS and nirK amplicons derived from Droevendaal soils

Melting curves of the amplicons, made with three primer systems, revealed that no primer dimerization and no formation of aspecific products had occurred under the applied real-time PCR circumstances (melting temperature of 60°C), using DNA from Droevendaal soil as template. Real-time PCR conducted on 10-fold diluted target DNA samples revealed three to four-fold higher Ct values, indicating linearity in the relationship between the target DNA concentration and the appearance of the first amplicons during PCR. Analysis of the amplicons in agarose gels revealed the expected sizes for all three primer systems. It was therefore concluded that the three primer systems, *amoA*, *nirS* and *nirK*, were reliable tools for quantification of, respectively, ammonium monooxygenase, multi heme and copper containing nitrite reductase genes in Droevendaal soils.

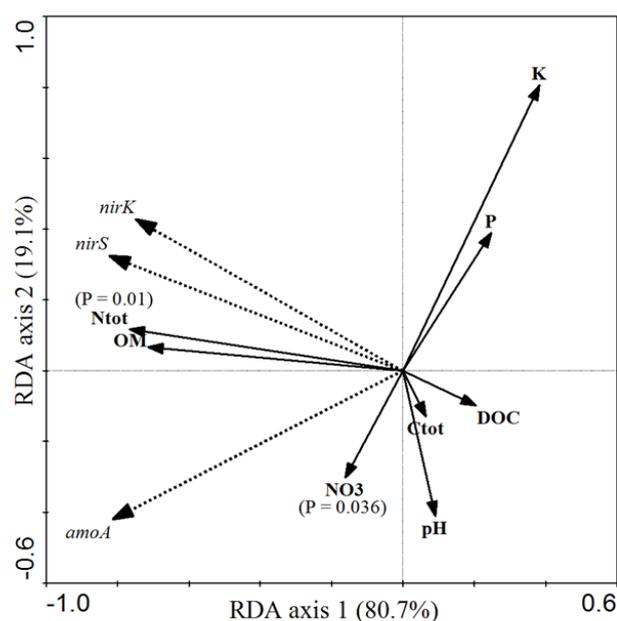


Figure 1. Biplots calculated by redundancy analysis (RDA) on *amoA*, *nirS* and *nirK* gene quantities (expressed in Ct values), determined by real-time PCR on DNA extracts from 16 soil samples from differentially amended Droevendaal soils, as species variables and quantitative values of OM, DOC, Ctot, Ntot, NO₃, P, K and pH as ‘environmental’ variables. Probability values indicating significance of effects of chemical parameters on species composition are indicated between brackets. Values on RDA axes indicate the percentage of the variation in the dataset accounted for.

Effect of organic amendment on amoA, nirS and nirK gene quantities

Cycle threshold values averaged per treatment revealed no significant differences for each of the three systems ($P > 0.05$, ANOVA), indicating that there was no measurable effect of soil treatment on the abundance of *amoA*, *nirS* and *nirK* genes in Droevendaal soil. Multivariate analysis using eight chemical and four plant disease parameters as ‘environmental’ variables and the Ct values measured over all 16 soil samples using the three primers systems as ‘species’ variables revealed no correlation between plant disease parameters and the species variables (not shown). However, significant effects of total Kjeldahl nitrogen (N_{tot}) and nitrate (NO₃) contents in the soils on the species variables were present (Fig.1). The vector representative for the nitrate content in these soils was correlated with the second RDA axis (representing 19.1% of all variation) and was not correlated with any of the three genes in particular. This contrasted with the vector representing the total nitrogen content in Droevendaal soils, which was correlated with the first RDA axis (representing 80.7% of all variation) and pointed into the same direction as the vectors representing *nirS* and *nirK*. The vector representing the organic matter (OM) fraction in soil pointed into the same direction as the one of total nitrogen content and it was also strongly correlated with the first RDA axis. Although the effect of organic matter on the species variables was not significant, it would imply that a relationship existed between total nitrogen and organic matter contents and *nirS* and *nirK* gene quantities in Droevendaal soils.

Polynomial regression analysis with total nitrogen content as input variables and the prevalence of *amoA*, *nirS* and *nirK* genes in the 16 soils as output variables indicated that *nirS* and *nirK* prevalence were non-linearly related to the total nitrogen content (Fig 2). The linear, quadratic and cubic terms were significant ($P = 0.001$), and about 61% of the variation was explained by these regression models. Total nitrogen content was positively correlated to organic matter content ($r = 0.61$; $P = 0.02$). Other regression models, with nitrate or organic matter content as input variables or with *amoA* as response variable were not significant. This confirmed the multivariate analyses, indicating that bacterial populations carrying *nirS* and *nirK* genes were significantly responsive to total nitrogen, but not to nitrate in these Droevendaal soils. Bacteria carrying *amoA* genes were not responsive either to the total nitrogen or to the nitrate contents in the same soils.

Discussion

Bacteria carrying nitrite reductase genes (detected with *nirS* and *nirK* primers) were shown to be responsive to variations in the total Kjeldahl nitrogen content in the different organically-amended soils of field 6 at Droevendaal. Significant differences in *amoA*, *nirS* and *nirK* gene copy numbers or in total nitrogen content were not found among the four treatments applied at this location, probably because the application of the different amendment had been initiated only three years before this

study. Variation naturally occurring in the total nitrogen content among the 16 soil samples must have led to the observed variations in *nirS* and *nirK* gene copy numbers. The relationship between the total nitrogen content and $^2\log$ copy numbers (expressed as Ct values) of these genes in the sampled soils were demonstrated to be non-linear with an optimum total nitrogen content of about 1.6–1.7 g kg⁻¹. Thus, bacteria carrying *nirS* (*Alcaligenes*, *Azospirillum*, *Paracoccus*, *Pseudomonas* and *Roseobacter* species) and *nirK* (*Alcaligenes*, *Hyphomicrobium*, *Ochrobactrum*, *Blastobacter* and *Rhizobium* species) (Braker et al., 1998) responded quantitatively but non-linearly to the amount of total available nitrogen in soil. Absence of a clear correlation between total nitrogen and *amoA* carrying bacteria, or between nitrate and *nirS* and *nirK* carrying bacteria would indicate that the total nitrogen content in Droevendaal soil may be the source of nitrogenous substrates for *nirS* and *nirK* carrying bacteria. The association between the total nitrogen and organic matter contents in Droevendaal soils may imply that it is the immobile rather than the mobile (mineralized) nitrogen fraction of the soil that must be held responsible for the build-up of a nitrite reducing community in Droevendaal soil.

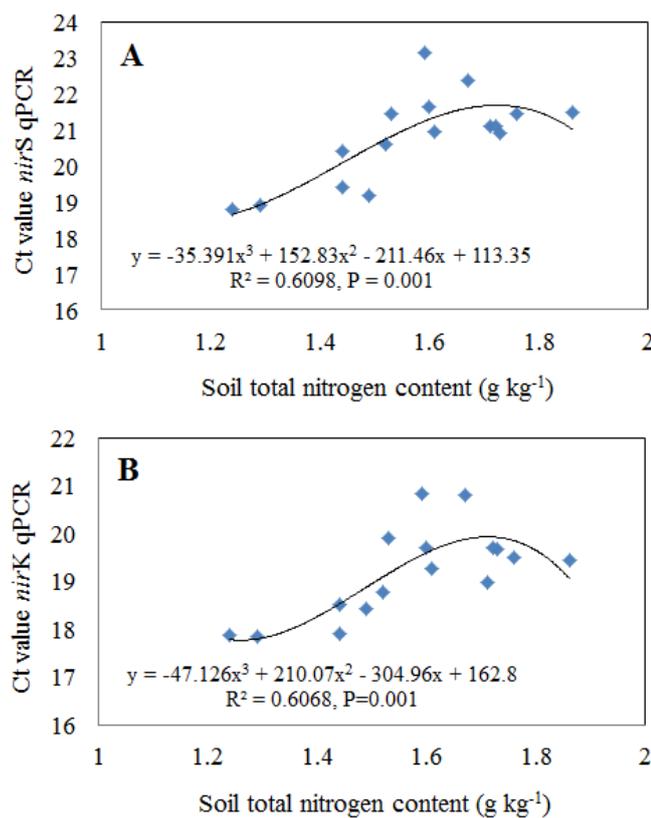


Figure 2. Relationship between the total nitrogen fraction and Ct values for *nirS* (A) and *nirK* (B) in 16 Droevendaal soil samples calculated by polynomial regression analysis. P values, percentages of the variation accounted for and equations are presented within the borders of each graph.

The *nir* genes studied here are responsible for the last step in denitrification under low oxygen conditions and are essential for limiting the emission of the greenhouse gas nitrite. Significant differences in denitrifier activity were observed between organically and conventionally managed soils; this was associated with higher organic matter contents in the organically farmed soils (Kramer et al., 2006). Ammonia oxidation to nitrite and nitrate, on the other hand, takes place under aerobic conditions, and may be more limited in organically managed soils, so that less nitrate is formed and leached in organically than in conventionally managed soils (Kramer et al., 2006). However, there is tremendous variation among organically managed soils depending on organic matter management practices (Franz et al., 2008; van Diepeningen et al., 2006), and it takes many years of consistent practices before relative stability sets in (Leifeld et al., 2009; Tonitto et al., 2010). Similarly, it can take many years before disease suppression and natural pest control sets in (Birkhofer et al., 2008; Grantina et al., 2011). Although we hypothesized that complex organic amendments would lead sooner to soil health and disease suppression than simple amendments, differences among amendment treatments were hardly found (van Overbeek et al., 2013; Senechkin et al., 2013). In this study, no differences in *nirK*, *nirS* and *amoA* genes were found, even though ammonia oxidizing bacteria and archaea have been suggested as indicators of soil and wetland health (Chapman et al., 2012; Hayden et al., 2010; Sims et al., 2012, 2013). Apparently three years of consistent organic matter management was too short a time period for the development of pronounced differences in soil health and disease suppression.

Using the same Droevendaal soil samples, an intriguing interaction between the community composition of ammonium oxidizing β -proteobacteria and the incidence of damping-off in sugar beet plants caused *R. solani* AG2-III2B was found (van Overbeek et al., 2013). However, differences in disease incidence were primarily due to the long-term crop history differences among main fields. On the other hand, significant differences among amendment treatments were found for disease severity and area under the disease progress curves of Fusarium wilt of flax (Senechkin et al., 2013). Fusarium wilt was more suppressed in soil amended with complex amendments, and less in that amended with compost. Similarly, fungistasis of four different fungi was more released by lignin-poor but labile C-rich substrates than by lignin-rich substrates (Bonanomi et al., 2013). The differences in Fusarium wilt were associated with the diversity of ammonium-oxidizing bacteria. Because there was no evidence for a direct interaction between ammonium oxidizing β -proteobacteria and *R. solani* AG2-III2B or *Fusarium oxysporum* f.sp. *lini* in soil, it seemed plausible that variation in the contents of different nitrogen sources might be responsible for variation of both the oxidizing β -proteobacteria and suppression of *R. solani* AG2-III2B or *Fusarium oxysporum* f.sp. *lini* in Droevendaal soils. In the current study we found responses of *nirS* and *nirK* carrying bacteria to the total nitrogen content in soil, but not to disease suppressive parameters measured with *R. solani* AG2-III2B in sugar beet and with *F. oxysporum* f. sp. *lini* in flax. We, therefore, found no evidence for a direct interaction between

nitrite reducing bacteria in soil and soil-borne pathogens, but most likely it is the amount of available nutrients (N) that is the driving force behind shifts in the abundance and/ or activity of the different microbial groups in Droevendaal soils.

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Chapter 7

General discussion

Introduction

Maintaining soil health is fundamental to successful crop production and ecosystem sustainability. The concept of soil health refers to the biological, chemical, and physical features necessary for long-term, sustainable agricultural productivity with minimal environmental impact. Healthy soils maintain a diverse community of soil microorganisms that help to: (1) recycle plant nutrients and improve soil structure with positive repercussions for its water- and nutrient-holding capacity; (2) form beneficial symbiotic associations with plant roots such as nitrogen fixing bacteria; (3) control plant diseases as well as insect pests; and (4) improve crop production (Arias et al., 2005). In particular, the ability of the biological community to decrease disease incidence and severity by suppressing plant pathogens is characteristic of soil health. In addition, a healthy soil has been defined as a stable soil, resilient to disturbances, with high biological diversity and high levels of internal nutrient cycling (Elliott and Lynch, 1994; van Bruggen and Semenov, 2000). With respect to microbial diversity, stability and root disease suppression organically managed soils frequently are healthier than conventionally managed soils (van Diepeningen et al., 2006).

For the assessment of soil health it is necessary to establish measurable indicators. According to the definition of soil health, we hypothesized that several microbial characteristics could be attractive soil quality indicators. Soil microorganisms are considered important components of a healthy soil, due to their central role in organic matter decomposition, nutrient cycling, disease suppression, maintenance of soil structure and their sensitivity to changes in the soil ecosystem (Birkhofer et al., 2008; Fliessbach et al., 2007; Nannipieri et al., 2003; Romaniuk et al., 2011). It was assumed that the composition and diversity of soil microbial populations, their ability to suppress plant diseases and their resistance and resilience to disturbances can be considered indicators of a stable and healthy soil ecosystem (van Bruggen and Semenov, 1999, 2000). It was also hypothesized that, in a healthy soil a more stable, slow-growing community of primarily oligotrophic microorganisms would form a 'buffer' against oscillations of copiotrophic microorganisms after a disturbance (van Bruggen and Semenov, 2000; Zelenev et al., 2006). Thus, after a disturbance, there would be a microbial succession which would be characteristic for the health status of a soil (van Diepeningen et al., 2005; He et al., 2012).

Microbial communities can partially be quantified and characterized by culture-dependent methods, especially when low-nutrient media (about 10 µg C/liter) are used for isolation. Under these conditions, at least 30% of the microscopically visible bacterial cells can be cultured (Nunes da Rocha et al., 2010), and their DNA can be extracted for characterization of the community. In addition, DNA extraction directly from soil is indispensable to characterize the non-culturable components of microbial communities; however, this includes DNA from dead and inactive cells. Therefore, a polyphasic approach needs to be used to characterize the potentially active microbial community in

soil.

The availability of nutrients, in particular easily useable carbon sources, is an important factor affecting the health status of a soil, because it will determine the relative numbers and activity of oligotrophic micro-organisms. Oligotrophs are adapted to exploit ecological niches characterized by low substrate concentrations and low energy flows (Semenov, 1991). They have highly effective systems for the uptake of inorganic and organic nutrients that occur in nano- and picomolar concentrations, due to their efficient carbon transport and utilization systems characterized by unique metabolic regulation (Semenov, 1991). The ecological significance of oligotrophs is that they not only grow at low nutrient concentrations, but their activities are also responsible for maintaining low concentrations of nutrients in their environment. Thus, oligotrophic bacteria can contribute to the regulation of copiotrophic activity by lowering the concentration of low molecular weight substances to such levels that they become inaccessible for the majority of eutrophs, including plant pathogens. Oligotrophs are particularly numerous in virgin soils, and are present in smaller numbers in conventionally managed than in organically managed soils. Therefore, the ratio of copiotrophic to oligotrophic bacteria has been proposed as one of the indicators of disease suppressiveness and soil health (van Bruggen and Semenov, 1999). In addition, unique transporter systems of oligotrophic bacteria for nutrient uptake could possibly provide molecular markers for the trophic as well as health status of soils (Lauro et al., 2009).

Two types of root disease suppression are distinguished: specific and general disease suppression. The former is frequently induced after monoculture of crops and caused by antibiotics producing *Pseudomonas* species (Weller et al., 2002; Mendes et al., 2011), while the latter is often found in natural and organically managed soils and can be caused by a variety of bacterial genera, such as *Pseudomonas*, *Bacillus*, *Burkholderia*, and actinomycetes as well as unavailability of essential nutrients (Workneh and van Bruggen, 1994; Larkin and Fravel, 1998). General root disease suppression is often greater at organic than at conventional farms due to the greater emphasis on maintenance of the organic matter content and biological diversity in organically managed soils (Mondelaers et al., 2009; van Bruggen and Termorshuizen, 2003; van Bruggen et al., 2006). The use of synthetic fertilizers and pesticides in conventional farms may reduce microbial diversity and evenness (Krauss et al., 2011; Liu et al., 2007; Seghers et al., 2003; Sugiyama et al., 2010). However, there is a tremendous variation in organic management practices, ranging from substitution of synthetic fertilizers and pesticides by approved quick-release organic fertilizers and pesticides to a complete management overhaul with lengthy crop rotations, integration of plant and animal production, the application of complex mixtures of organic amendments and minimization of the application of any specific pest control measures (Letourneau and van Bruggen, 2006; Stone et al., 2004). During the transition to organic agriculture soil management strategies can also vary (Tu et al., 2006). These differences in soil management among organic farms likely lead to differences in soil

health and root disease suppression (Bonanomi et al., 2013; Grantina et al., 2011; Liu et al., 2007; Stone et al., 2004; van Bruggen and Termorshuizen, 2003). To develop strategies that would lead to a build-up of organic matter, soil health characteristics and root disease suppression a long-term experiment was set up at the experimental farm Droevendaal at Wageningen, comparing four different types of organic amendments: (1) plant derived compost, (2) cattle slurry, (3) cattle slurry and dung, and (4) compost, slurry and dung. Soils of this field experiment were used to examine the relative trophic and health status and root disease suppressiveness after these four differential treatments. -

Thesis objectives

The objectives of this thesis were:

- to determine the relationship between carbon availability and population density of bacteria isolated on media low in carbon available for bacteria from soils treated with different organic amendments and partially characterize the isolated bacteria based on 16S rRNA gene sequences;
- to analyze the effects of different organic amendments on suppression of disease development of *R. solani* on beets and *F. oxysporum* f. sp. *lini* on flax, and relate this suppression to soil chemical characteristics and bacterial communities as determined by polymerase chain reaction (PCR) and denaturing gradient gel electrophoresis (DGGE) on DNA directly isolated from soil;
- to investigate in vitro and in vivo interactions between a selected oligotrophic bacterial isolate, *Collimonas* sp., and *R. solani*;
- to investigate the relationships between number of genes involved in nitrogen cycling, amount of nitrogen available for micro-organisms in soil and suppression of *R. solani* and *Fusarium oxysporum* f.sp. *lini*.

Main results and discussion

Thus, the research for this thesis was based on four pillars: organic farming, soil health, oligotrophy, and suppression of soilborne pathogens. Organic farming, soil health and root disease suppression have been studied intensively, but oligotrophy is rarely considered due to the difficulties involved in studying oligotrophic microorganisms. Moreover, the abundance and composition of oligotrophic bacteria and their potential functions in soil are poorly understood. Therefore, the emphasis of the research described in this thesis has been on oligotrophic bacteria (chapter 2) and their relation to soil health (chapter 5). Estimation of the soil health status was focused on quantification of soil suppressiveness against two different soilborne pathogens, *Rhizoctonia solani* and *Fusarium*

oxysporum f.sp. *lini* (chapters 3 and 4). In addition, an attempt was made to select functional genes of the nitrogen cycle that could be related to soil health and disease suppression. Genes for nitrogen fixation, in particular *nifH* (Bürgmann et al. 2005), were included in preliminary tests, because many oligotrophic bacteria belong to the *Rhizobiaceae* (chapter 2). However, problems were encountered with amplification of these genes from Droeendaal soil DNA extracts, in particular primer-dimer formation and lack of specificity (Senechkin and Barra, unpublished results). Therefore, the focus was on genes that could be amplified reliably, namely ammonium monooxygenase (*amoA*) and some denitrification genes (*nirS* and *nirK*) (chapter 6).

First, the abundance and taxonomic identity of oligotrophic bacteria were determined in soil samples from plots treated with the four different organic amendments mentioned above. We isolated 720 potential oligotrophic bacteria on a low-carbon medium (chapter 2), and identified the genera to which these bacteria belonged by extracting their DNA and amplifying and sequencing the 16S rRNA genes. Predominant groups were the actinobacteria and members of the α -subclass of proteobacteria. Subsequent transfers onto low and higher carbon media to select oligotrophs that would not grow on high carbon media resulted in a limited number of genera, mainly *Bradyrhizobium*, *Mesorhizobium* and *Rhizobium* spp. After ten such transfers, only two isolates were considered strict oligotrophs, because they could not grow on the richer medium; these isolates were classified as *Rhizobium alamii* and *Pedobacter roseus*, both belonging to the α -subclass of proteobacteria. *R. alamii* was originally isolated from the rhizosphere of *Arabidopsis thalianae* on RCV medium containing a relatively high concentration of malate (Berge et al., 2009). *P. roseus* was originally isolated on R2A medium (containing various carbon compounds at relatively low concentrations) from a hypertrophic pond (Hwang et al., 2006). Thus, our isolates may be more oligotrophic than the original isolates of these species. *Pedobacter* belongs to the *Sphingobacteriaceae*, which also contains the only plant pathogenic broad-range oligotrophic bacterium, *Rhizomonas suberifaciens* (van Bruggen et al., 1990). The results obtained for this thesis confirmed that *Alphaproteobacteria* dominate among oligotrophic bacteria in soil, as they do in seawater (Hashimoto et al., 2006; Mitsui et al., 1997) and that many oligotrophic bacteria have nitrogen-fixing properties (Ohta and Hattori, 1983). Thus, they may play an important role in soil.

However, isolation and characterization of oligotrophic bacteria was a major challenge due to the time consuming cultivation technique, which was the only possible method when this research was carried out. Oligotrophy is a functional characteristic that can be found in many taxa (Semenov, 1991), and for the development of indicators for soil health, it would be useful to use functional genes. Recently, several functional genes were identified that were able to distinguish copiotrophic from oligotrophic bacteria isolated from oceans, in particular genes associated with transporter systems for the uptake of carbon sources (Lauro et al., 2009). This presents an opportunity for future research identifying similar functional genes associated with oligotrophic bacteria isolated from soils differing

in health status. Once these genes have been identified they could be included in multiplex, real-time detection systems, targeting genes related to oligotrophy, efficient nutrient cycling and disease suppression, that could report on the health status of soils.

One of the most interesting findings from the work on oligotrophs (chapter 2) was the identification of an oligotrophic *Collimonas* sp. strain. Some *Collimonas* species exhibit chitinolytic activity (Leveau et al., 2009). Further research on the isolated *Collimonas* strain (IS343) was conducted in order to reveal a direct link between oligotrophy, soil health and disease suppression (chapter 5). Strain *Collimonas* sp. IS343 was able to degrade chitin and reduce mycelial growth of the root pathogen *Rhizoctonia solani* in vitro at high and low carbon availabilities. In contrast to *C. fungivorans* Ter331, a well characterized *Collimonas* strain (De Boer et al., 2004), strain IS343 did not respond with an increase in growth rate to higher carbon levels in liquid medium and showed better survival in bulk soil. It was better adapted to circumstances of low carbon availability and was more suppressive towards *R. solani* than strain Ter331 cells in vitro and in vivo. The suppressive effect was seen as a reduction in hyphal branching and mycelium density of *R. solani* indicative of competition for available nutrients and/or decomposition of hyphal tips by chitinase. Strain IS343 also delayed disease development on beets inoculated with *R. solani*. Thus, the presence of oligotrophic *Collimonas* sp. might be a potential indicator for general disease suppression in soils. Comparison of the genomes of *Collimonas* IS343 and Ter331 could be useful to reveal genes responsible for the better adaptation of the oligotrophic strain to low-carbon conditions in soil. Genes that differentiate *Collimonas* IS343 from Ter331 could then be compared to the genes that were found to differentiate copiotrophic from oligotrophic bacteria in seawater (Lauro et al., 2009).

The same soil samples as used for the isolation of oligotrophic bacteria were also used to estimate the effects of different management practices on soil suppressiveness against *R. solani* on sugar beets and *F. oxysporum* f. sp. *lini* on flax in bioassays. A large number of microbial and chemical properties of the same soils were analyzed to discover parameters related to disease suppression. Among others, the ratio of copiotrophic and oligotrophic bacteria isolated on C-rich and C-poor media and the compositions of bacterial and fungal communities were determined. Total microbial community DNA was extracted and amplified with primers for different taxonomic groups and operational taxonomic units were separated by denaturing gradient gel electrophoresis (DGGE). Multivariate analyses were performed to identify factors that distinguished soils differing in soil health as determined by diseases suppression. The combinations of organic amendments to two fields that differed in cropping and agricultural management histories, led to large variations in the measured soil chemical and microbiological parameters (chapters 3 and 4).

Fusarium wilt of flax was significantly suppressed by the slurry-compost-dung (SCD) treatment and enhanced by compost in one field at the Droevendaal organic farm (chapter 3). Other

variables were only marginally affected by the different amendments, except for soil pH which was highest in the SCD treatment. The area under the disease progress curve (AUDPC) was negatively correlated with pH, but not with any of the other variables including the ratio of copiotrophic to oligotrophic bacteria. Discriminant analyses of soils with high versus lower AUDPCs selected pH, total carbon contents and the diversity of ammonia-oxidizing β -Proteobacteria (AOB) as variables contributing to the difference in AUDPC. AOB oxidize ammonia to nitrite, the first and rate-limiting step of the nitrification process and, therefore, play an essential role in nitrogen cycling (De Boer and Kowalchuk, 2001). Diverse ammonia-oxidizing communities were associated with high total nitrogen levels and a lower AUDPC. This is in line with previous reports that *Fusarium* root disease can be reduced at relatively high ammonium concentrations compared to nitrate (Borrero et al., 2012).

Results obtained for *R. solani* suppression were different from those for *F. oxysporum* f.sp. *lini* in that no significant overall effects of the amendment treatments were found (chapter 4). Also for the other measurements, no significant effects of soil organic amendments were found, with the exceptions of small, but non-significant effects of compost amendment on the bacterial community structure, slurry on the AOB community structure and slurry and dung amendment on the fungal community structure. This was probably due to the fact that two fields were sampled, and effects of amendments were sometimes opposite in the two fields. For example, amendment with compost-slurry-dung in one field resulted in the highest growth rate of *R. solani* in soil, whereas this rate was lowest in the same treatment applied to the other field. This finding can be of importance for agricultural practices where organic amendments are applied for control of soil-borne diseases, as the effect of organic amendment on susceptibility to plant diseases can vary within the same soil, depending on differences in crop rotations, soil amendments and agricultural management practices. On the other hand, there were strong effects of pH, organic matter content, and water-dissolvable organic carbon on the bacterial, fungal, *Pseudomonas* and AOB communities in soil.

Since the pH and the diversity and community composition of AOB seemed to play a role in the suppression of both pathogens, these factors could be useful indicators for general suppression of soil-borne pathogens (Chapman et al., 2012; Fang et al., 2012). The absence of common effects of the treatments on soil chemical properties, bacterial groups or disease suppression points at the very complex and indirect interaction between various chemical and biological soil parameters. Moreover, the treatments were initiated only three years prior to the research carried out for this thesis. Nevertheless, we can conclude that particular chemical parameters like pH, organic matter or total carbon content and water-dissolvable organic carbon content are responsible for structural changes in the communities of particular groups of soil micro-organisms. Growth and infection by *R. solani* and *F. oxysporum* f.sp. *lini* in soil may be influenced by these community shifts, in particular of AOB, after application of different amendments. Interactions between particular groups of micro-organisms and the pathogens can be direct, as proposed for *Pseudomonas* or *Collimonas* species (Mendes et al.,

2011; Senechkin et al., 2013), or in concert affected by the same environmental factors, such as pH and total N, which were related to both the AOB community structure and *R. solani* or *F. oxysporum* f.sp. *lini*.

Because bacteria contributing to the nitrogen cycle were associated with the suppression of both *R. solani* and *Fusarium oxysporum* f.sp. *lini*, several genes involved in nitrogen cycling were quantified by real-time PCR and related to the amount of nitrogen available for micro-organisms and suppression of both pathogens in the same soils (chapter 6). The choice fell on the *amoA* gene of chemolithotrophic ammonium-oxidizing bacteria (AOB), which encodes the active site of ammonium monooxygenase that effectuates the transformation of ammonia to nitrite that is then further oxidized to nitrate (Kuo et al., 2006). In addition, denitrification genes were selected, because it had been demonstrated that denitrification was more complete in organic than in conventional soils (Kramer et al., 2006). Denitrification takes place in anaerobic pockets in soil. It involves the reduction of nitrate via nitrite to the gaseous end products of nitric oxide (NO), nitrous oxide (N₂O) and molecular nitrogen (N₂) (Wolsing and Prieme, 2004) with the aid of four sets of different enzymes produced by different microorganism. The reduction from nitrate into nitrite is encoded by *nar* genes while that from nitrite to nitric oxide is encoded by *nir* genes, from nitric oxide into nitrous oxide by the *nor* gene, and finally from nitrous oxide into dinitrogen by the *nosZ* gene (Zumft, 1997). Many bacterial taxa can respire oxygen from nitrate into nitrite via reduction. When the oxygen level in soil becomes a limiting factor, facultative anaerobic bacteria will accomplish these processes. Nitrite reductase is a fundamental enzyme for the denitrification process. Two types of these enzymes have been distinguished, namely copper nitrite reductase encoded by the *nirK* gene and cytochrome cd1-nitrite reductase encoded by *nirS* genes (Zumft, 1997). Besides the *amoA* gene, *nirK* and *nirS* were selected as potential indicators for soil health and disease suppression. However, no correlation was found between gene quantities (as determined by q-PCR) and disease incidence or area under the disease progress curves for sugar beet and flax plants inoculated with *Rhizoctonia solani* AG2-2IIIB and *Fusarium oxysporum* f.sp. *lini*, respectively. Significant curvilinear relationships between *nirS* and *nirK* gene quantities and the total soil nitrogen fraction were found. Although a direct interaction between N cycling genes and disease suppression was not found, an indirect relationship via the total N fraction in soil may exist.

Conclusion and need for additional research

In conclusion, only slight effects of the different amendments on root disease suppression and various chemical and microbiological variables were found, probably because the treatments had been initiated only three years before the start of this thesis research. Nevertheless, the most complex

amendment (slurry-dung-compost) suppressed Fusarium wilt on flax, in support of the original hypothesis underlying the set-up of a long-term experiment, which was meant to help organic farmers to choose a strategy to develop long-term soil health and ecological sustainability. Suppression of *R. solani* on beets was more affected by past management and cropping pattern rather than the recent amendments. However, in both cases, the community structure or diversity of ammonia-oxidizing bacteria as determined from DGGE analysis was associated with disease suppressiveness. On the other hand, the quantity of the *amoA* gene as determined by qPCR was not associated with either of the two root diseases in bioassays. Although oligotrophic bacteria play important roles in soil stability, carbon cycling and nitrogen fixation, densities of oligotrophic bacteria and the ration of copiotrophic to oligotrophic bacteria were not related to suppression of *R. solani* on beets or *F. oxysporum* f.sp. *lini* on flax. Nevertheless, the oligotrophic strain IS343 of *Collimonas* sp. was capable of suppressing the development of *R. solani* both in the lab and in the greenhouse with beet plants. Thus, suppression of the pathogens tested here may be more specific than is often assumed. Altogether, the research conducted for this thesis pointed at potential indicators for soil health and disease suppression, but specific indicators could not be selected. The number of indicators in this study, especially the quantification of functional genes was limited to a few genes. Other researchers have suggested using the ratio of archaeal and bacterial ammonia-oxidation genes (Sims et al., 2013), and *amoA* plus *nifH* genes (Chapman et al., 2012; Hayden et al., 2010). However, in an extensive literature review Kibblewhite et al. (2008) came to the conclusion that “measurement of individual groups of organisms, processes or soil properties does not suffice to indicate the state of the soil health”. Instead, they suggested considering four fundamental ecological functions and quantifying the flows of energy and carbon among those functions. Similarly, Kremen and Miles (2012) suggested to make an inventory of ecological functions, which are consistently improved by biological diversity (Birkhofer et al., 2008; Kremen and Miles, 2012; Romaniuk et al., 2011). Thus, more research will be needed to understand the processes determining the health status of soils and to select indicators that could be applied universally. This thesis has contributed to our understanding of some aspects of soil health, in particular the potential contribution of oligotrophic bacteria, which have been grossly understudied thus far.

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Summary

Maintaining soil health is fundamental to successful crop production and ecosystem sustainability. A healthy soil is characterized by high biological diversity, stability and resilience to disturbances, efficient internal nutrient cycling, low concentrations of easily available nutrients (oligotrophy) and suppression of pests and diseases. Organically managed soils are often considered healthier than conventionally managed soils, and the former are considered good model systems for studying soil health indicators. Plant disease suppressiveness is a quantifiable soil health characteristic that has been related to many other soil quality characteristics. However, the relationship between a predominant group of soil micro-organisms, oligotrophic bacteria, and root disease suppression has not been studied intensively. Therefore, this thesis is focused on soil health, organic farming, oligotrophic bacteria and suppression of soilborne pathogens. The main goal of this thesis was to assess the soil health status of organically managed soils with different organic amendments in terms of oligotrophy, plant disease suppression and components of the carbon and nitrogen cycles.

Soil samples for this study originated from a certified organic farm 'De Droevendaal' at Wageningen, the Netherlands, where a long-term experiment was set up in two fields with different crop history. Experimental treatments consisted of four different types of organic amendments in two replicated blocks per field - (1) plant derived compost, C, (2) cattle slurry, S, (3) cattle slurry and dung, SD, and (4) compost, slurry and dung SCD. This range of amendments provided different amounts and types of introduced carbon and nitrogen. Two soil samples per plot were subjected to detailed analyses of oligotrophic bacteria, soil suppressiveness against *Rhizoctonia solani* on sugar beets and *Fusarium oxysporum* f. sp. *lini* on flax, abundances and community composition of eubacteria, fungi, *Pseudomonas* and ammonia-oxidizing β -proteobacteria, and abundances of functional genes involved in nitrogen cycling (*amoA*, *nirS*, *nirK*) (chapters 2-7).

Oligotrophic bacteria thrive in low-carbon environments and are hampered by high carbon concentrations. Although oligotrophic bacteria are well known in marine environments, their taxonomic identities and functions in soil are poorly understood (chapter 2). A total of 720 bacterial strains were isolated on a low organic carbon agar medium (10 $\mu\text{g C/ml}$). Repeated transfers (up to ten times) of the isolates from low-C agar medium to fresh low- and high-C agar media were done to test for exclusive growth of isolates under oligotrophic conditions. The number of isolates exclusively growing under oligotrophic conditions dropped after each subsequent transfer from 241 after the first, to 98 after the third transfer step. Identification on the basis of partial 16S rRNA gene sequences revealed that most of the 241 isolates (as well as the subset of 98 isolates) belong to wide-spread genera such as *Streptomyces*, *Rhizobium*, *Bradyrhizobium* and *Mesorhizobium*, and the taxonomic composition of dominant genera changed from the first transfer step to the third. A selected subset of 17 isolates were further identified and characterized for exclusive growth on low-C agar medium. Two

isolates continued to grow only on low-C agar medium up to the tenth transfer step and matched most closely with *Rhizobium alamii* and *Pedobacter roseus* on the basis of the almost full length 16S rRNA gene. It was concluded that the vast majority of strains which are isolated on low-C agar media belong to the trophic group of microorganisms adapted to a 'broad range' of carbon concentrations, including well-known and wide-spread bacterial genera. Oligotrophy is a physiological, not a taxonomic property, and it is hard to isolate and identify oligotrophic bacteria from soil. However, recent research on marine bacteria shows that they can be distinguished from copiotrophic bacteria by means of the quantity and type of carbon transporter genes. This bodes promise for quantification of oligotrophic bacteria in soil.

In chapters 3-4 we estimated the effects of different soil amendments on suppressiveness against *R. solani* on sugar beets and *F. oxysporum* f. sp. *lini* on flax in bioassays. A large number of soil microbial and chemical properties were analyzed to discover variables related to disease development. Among others, the ratio of copiotrophic and oligotrophic bacteria isolated on C-rich and C-poor media and the compositions of bacterial and fungal communities were determined. Total microbial community DNA was extracted and amplified with primers for different taxonomic groups and operational taxonomic units were separated by denaturing gradient gel electrophoresis (DGGE). Multivariate analyses were performed to identify soil variables that distinguished soils differing in soil health as determined by diseases suppression.

Fusarium wilt of flax was significantly suppressed by the slurry-compost-dung (SCD) treatment and enhanced by compost in one field at the Droevendaal organic farm (chapter 3). Other variables were only marginally affected by the different amendments, except for soil pH which was highest in the SCD treatment. The area under the disease progress curve (AUDPC) was negatively correlated with pH, but not with any of the other variables including the ratio of copiotrophic to oligotrophic bacteria. Discriminant analyses of soils with high versus lower AUDPCs selected pH, total carbon contents and the diversity of ammonia-oxidizing β -Proteobacteria (AOB) as variables contributing to the difference in AUDPC. Diverse ammonia-oxidizing communities were associated with high total nitrogen levels and a lower AUDPC.

Results obtained for *R. solani* suppression were different from those for *F. oxysporum* f.sp. *lini* in that no significant overall effects of the amendment treatments were found (chapter 4). Also for the other measurements, no large, significant effects of soil organic amendments were found. This was probably due to the fact that two fields were sampled, and effects of amendments were sometimes opposite in the two fields. For example, amendment with compost-slurry-dung in one field resulted in the highest growth rate of *R. solani* in soil, whereas this rate was lowest in the same treatment applied to the other field. This finding can be of importance for agricultural practices where organic amendments are applied for control of soil-borne diseases, as the effect of organic amendment on

susceptibility to plant diseases can vary within the same soil, depending on differences in crop rotations, soil amendments and agricultural management practices. On the other hand, there were strong effects of pH, organic matter content, and water-dissolvable organic carbon on the bacterial, fungal, *Pseudomonas* and AOB communities in soil.

One of the most interesting findings from our work on oligotrophic bacteria was the identification of an oligotrophic *Collimonas* sp. strain. Some *Collimonas* species are known to exhibit chitinolytic activity. Therefore, further research on the isolated *Collimonas* strain (IS343) was conducted to reveal a direct link between oligotrophy and disease suppression (chapter 5). *Collimonas* sp. IS343 was capable of degrading chitin and suppressing *R. solani* mycelium growth in vitro at high and low carbon concentrations. In contrast to *C. fungivorans* Ter331, strain IS343 did not respond with an increase in growth rate to higher carbon levels in liquid medium; it reached higher cell numbers in carbon-poor media and survived better in bulk soil. Thus, strain IS343 is better adapted to low carbon availability as present in bulk soils than strain Ter331 cells. Strain IS343 was also more suppressive towards *R. solani* than strain Ter331 *in vitro*. When mixed into soil, strain IS343 delayed disease development caused by *R. solani* AG2-2IIIB in rows of sugar beet plants, suggesting that strain IS343 suppressed *R. solani* AG2-2IIIB mycelium growth in soil. Potential mechanisms underlying the observed suppressive effects could be competition for available nutrients between strain IS343 cells and *R. solani* or the production of chitinase as shown for this and other *Collimonas* species.

Because bacteria contributing to the nitrogen cycle were associated with the suppression of both *R. solani* and *F. oxysporum*, several genes involved in nitrogen cycling were quantified by real-time PCR and related to the amount of nitrogen available for micro-organisms and suppression of both pathogens in the same soils (chapter 6). The quantities of ammonium monooxygenase (*amoA*) and nitrite reductases (*nirS* and *nirK*) genes, and their effects on the disease suppressive status in soil were investigated using real-time PCR. No correlation was found between gene quantities and disease intensity values (disease incidence and area under the disease progress curve) determined for sugar beet and flax plants inoculated with, respectively, *R. solani* and *F. oxysporum* f. sp. *lini*. However, significant curvilinear relationships between *nirS* and *nirK* gene quantities and the total soil nitrogen fraction were found. Although direct interactions between N cycling genes and disease suppression were not found, we suggested that indirect relationships via the total N and available N fractions in soil may exist.

This research has given insight in potential indicators for soil health. However, more research will be needed to understand the processes determining the health status of soils and to select indicators that could be applied universally. Also, this thesis revealed some aspects of the relation between soil health and oligotrophic bacteria, a very important but poorly studied group of soil microorganisms.

Samenvatting

Het behoud van bodemgezondheid is essentieel voor verduurzaming van teelt- en ecosystemen. Een gezonde bodem wordt gekenmerkt door hoge biologische diversiteit, stabiliteit, veerkracht na verstoring, efficiënte interne nutriënten omzettingen, lage concentraties van beschikbare nutriënten (oligotrofie) en onderdrukking van ziekten en plagen. Biologisch beheerde bodems worden vaak als 'gezonder' beschouwd dan conventioneel beheerde bodems, en worden om die reden gezien als goede modelsystemen voor de studie naar bodemgezondheidsindicatoren. Weerbaarheid tegen plantenziekten is een kwantificeerbare eigenschap van bodemgezondheid, die is gerelateerd aan veel andere bodemkwaliteitkenmerken. Echter, de relatie tussen een dominante groep van bodemmicroorganismen, de groep van oligotrofe bacteriën, en onderdrukking van wortelziekten is tot op heden nog niet intensief onderzocht. Daarom richt dit proefschrift zich op de belangrijkste onderwerpen die verband houden met bodemgezondheid: biologische landbouw, bodemgezondheidsindicatoren, oligotrofe bacteriën en onderdrukking van bodemgebonden ziekteverwekkers. Het doel van dit proefschrift was het evalueren van de gezondheidstoestand van biologisch beheerde bodems met verschillende organische toevoegingen op basis van oligotrofie, microbiële gemeenschappen, onderdrukking van plantenziekten en aanwezigheid van moleculaire indicatoren van koolstof-en stikstofkringlopen.

De bodemmonsters die zijn gebruikt voor deze studie waren afkomstig van de gecertificeerde biologische boerderij 'De Droevendaal' in Wageningen (Nederland) waar een langdurig experiment was uitgezet, verspreid over twee velden die verschilden in historie van gewasteelt. Experimentele behandelingen bestonden uit vier verschillende soorten van organische toedieningen aan grond in twee herhaalde blokken per veld, namelijk, (1) plantaardige compost, C, (2) runderdrijfmest, S, (3) runderdrijfmest en stalmest, SD, en (4) compost, runderdrijfmest en stalmest, SCD. Deze verschillende toevoegingen resulteerden in een variatie in aard en hoeveelheid van koolstof en stikstof verbindingen over de verschillende behandelingen. Twee bodemmonsters per perceel werden geanalyseerd op basis van aanwezigheid van oligotrofe bacteriën, bodemweerbaarheid tegen *Rhizoctonia solani* en *Fusarium oxysporum* f. sp. *lini*, met respectievelijk suikerbiet en vlas als modelgewassen, dichtheid en samenstelling van bacteriële, schimmel, *Pseudomonas* en ammonium-oxiderende β -proteobacteriële levensgemeenschappen en dichtheden van functionele genen die betrokken bij stikstofkringlopen (*amoA*, *nirS*, *nirK*) (hoofdstukken 2-7).

Oligotrofe bacteriën groeien goed in koolstofarme milieus en worden beperkt in hun groei bij hogere concentraties beschikbare koolstof. Hoewel er veel bekend is over oligotrofe bacteriën in mariene milieus, is hun taxonomische identiteit en functioneren in de bodem nog onvoldoende begrepen (hoofdstuk 2). In totaal werden er 720 bacterie isolaten willekeurig geselecteerd van agar medium met laag beschikbare organische koolstof (10 μ g / ml) waarop grondsuspensies waren

uitgeplaat. Herhaald overzetten (maximaal tien keer) van deze isolaten, van agar medium met een laag C gehalte naar dat met een hoog C gehalte, werd uitgevoerd om te toetsen of deze isolaten exclusief zouden kunnen groeien onder voedselarme omstandigheden. Het aantal isolaten dat uitsluitend kon groeien onder voedselarme omstandigheden daalde na iedere volgende stap van 241 na de eerste, tot 98 na de derde stap. Uit identificatie op basis van de DNA volgorde van een variabel deel van het 16S rRNA gen bleek dat de meeste van de 241 isolaten (alsmede een deel van de 98 isolaten verkregen na de derde stap) tot alomtegenwoordige taxonomische geslachten als *Streptomyces*, *Rhizobium*, *Bradyrhizobium* en *Mesorhizobium* behoorden, en dat de verhouding tussen de dominante geslachten veranderde tussen de eerste en derde overdrachtstap. Een geselecteerd aantal van 17 isolaten werd verder geïdentificeerd op basis van het volledige 16S rRNA gen en gekarakteriseerd voor eventuele exclusieve groei op agar medium met een lage hoeveelheid beschikbare koolstof. Twee isolaten konden uitsluitend groeien op agar medium met een lage hoeveelheid beschikbare koolstof, tot aan de tiende overdrachtstap, en deze isolaten bleken nauw verwant te zijn aan collectiestammen behorend tot de soorten *Rhizobium alamii* en *Pedobacter roseus*. Er werd geconcludeerd dat de overgrote meerderheid van de isolaten, verkregen door kweek op agar medium met een lage hoeveelheid beschikbare koolstof, behoort tot de trofische groep van micro-organismen die zijn aangepast aan een breed spectrum van beschikbare koolstof-concentraties in het milieu en dat tot deze groep bekende en wijdverbreide bacteriële geslachten behoren. Oligotrofie blijkt een fysiologische en geen taxonomische eigenschap te zijn en daarom is het moeilijk om oligotrofe bacteriën uit de bodem te kunnen isoleren en te identificeren. Echter, uit recent onderzoek in mariene milieus is gebleken dat oligotrofe bacteriën kunnen worden onderscheiden van copiotrofe bacteriën op basis van hoeveelheid en type koolstof transport genen. Dit biedt in de toekomst een goede mogelijkheid om, op basis van deze genen, oligotrofe bacteriën in de bodem te kunnen kwantificeren.

In de hoofdstukken 3-4 hebben we de effecten van verschillende bodemverbeteraars op weerbaarheid tegen *R. solani* en *F. oxysporum* f. sp. *lini* bepaald met behulp van biologische toetsen met, respectievelijk, suikerbiet en vlas als modelgewassen. Een aantal bodemmicrobiële en chemische eigenschappen werden geanalyseerd om variabelen te kunnen identificeren die mogelijk een effect hebben op ziekteontwikkeling. Onder andere werd de verhouding bepaald tussen aantallen copiotrofe en oligotrofe bacteriën, die respectievelijk waren gemeten op koolstofrijke en arme media, alsmede de samenstelling van de verschillende bacterie en schimmel gemeenschappen. Hiervoor werd DNA afkomstig van de totale microbiële levensgemeenschap uit de bodem geëxtraheerd en werd het aantal operationele taxonomische eenheden bepaald na PCR vermeerdering met behulp van specifieke primers gericht tegen de verschillende taxonomische groepen, gevolgd door scheiding op basis van denaturerende gradiënt gelelektroforese (DGGE). Multivariate analyses werden uitgevoerd om variabelen te kunnen vaststellen op basis waarvan bodems die variëren in bodemgezondheid, bepaald op basis van ziekte onderdrukking in biologische toetsen, onderscheiden kunnen worden.

Fusarium verwelkingsziekte in vlas werd significant onderdrukt na behandeling met de combinatie van compost, runderdrijfmest en stalmest (SCD), maar versterkt na behandeling met compost in een veld van de biologische proefboerderij ‘de Droevendaal’ (hoofdstuk 3). Andere gemeten variabelen werden slechts marginaal beïnvloed door de verschillende behandelingen, met uitzondering van de zuurgraad (pH) van de bodem, die het hoogst was in grond behandeld met SCD. Het gebied onder de curve van ziekteverloop uitgezet tegen de tijd (AUDPC) was negatief gecorreleerd met de gemeten pH, maar niet met één van de andere variabelen, zoals de verhouding tussen copiotrofe en oligotrofe bacteriën. Uit discriminant analyse van bodems met hoge ten opzichte van lage gemeten waarden voor AUDPC bleek dat pH, totaal koolstofgehalte en de diversiteit van ammonium-oxiderende β -Proteobacteriën (AOB) de meeste invloedrijke variabelen waren op het verloop van verwelkingsziekte door Fusarium in de tijd. Ammonium-oxiderende bacteriële levensgemeenschappen in de bodem werden vooral geassocieerd met een hoog gehalte aan totaal stikstof, en een laag meetbare waarde voor de AUDPC.

Verkregen resultaten voor onderdrukking van *R. solani* waren zodanig verschillend van die van *F. oxysporum* f.sp. *lini* dat er geen significante effecten van bodembehandelingen op algemene weerbaarheid werd gevonden (hoofdstuk 4). Ook voor de andere metingen werden er geen grote significante effecten van bodembehandelingen door organische verrijking vastgesteld. Dit was waarschijnlijk te wijten aan het feit dat er twee velden waren bemonsterd, en dat de effecten van deze behandelingen op deze velden vaak tegenovergesteld bleken te zijn. Bijvoorbeeld, behandeling met compost, runderdrijfmest en stalmest (SCD) in het ene veld resulteerde in de hoogste groei van *R. solani* in grond, terwijl in het andere veld met dezelfde behandeling de gemeten groeisnelheid van dit pathogeen het laagst was. Deze bevindingen zijn relevant voor de landbouwpraktijk waarbij organische verrijkingen van bodems dikwijls worden toegepast om bodem-gebonden ziekten te kunnen beheersen. Namelijk, organische stof verrijking van dezelfde grondsoort kan een wisselend effect hebben op de gevoeligheid voor plantenziekten dat afhangt van de verschillen in vruchtwisseling en behandeling met bodemverbeteraars en andere landbouwkundige maatregelen die in het verleden hebben plaatsgevonden op verschillende percelen. Echter, er werden wel sterke effecten van pH, organische stof en water-oplosbare organische koolstof op bacterie, schimmel, *Pseudomonas* en AOB gemeenschappen in alle bodemmonsters gemeten.

Een van de meest fascinerende bevindingen van ons werk met oligotrofe bacteriën was de identificatie van een oligotrofe *Collimonas* sp. stam. Sommige *Collimonas* soorten staan bekend om hun chitinolytische activiteit. Daarom is de *Collimonas* sp. stam IS343 verder onderzocht om een eventueel direct verband te kunnen leggen tussen oligotrofie en ziekte onderdrukking in de bodem (hoofdstuk 5). *Collimonas* sp. IS343 bleek in staat te zijn om chitine af te breken en om in vitro *R. solani* mycelium groei te onderdrukken bij zowel hoge als lage concentraties aan beschikbare koolstof in agar media. In tegenstelling tot *C. fungivorans* stam Ter331 groeide stam IS343 niet harder bij een

hogere aanbod van koolstof in vloeibaar medium, bereikte deze hogere celaantallen in medium dat arm is in beschikbaar koolstof en overleefde beter in bulkgrond. Cellen van stam IS343 bleken dus beter te zijn aangepast aan lage beschikbaarheid van koolstof in bulkgrond dan die van stam Ter331. Stam IS343 was tevens beter in staat om *R. solani* in vitro te onderdrukken dan stam Ter331. Gemengd in grond vertraagde stam IS343 de ziekte ontwikkeling die werd veroorzaakt door *R. solani* AG2-2IIIB, gemeten in een rij van opeenvolgende suikerbietplanten, wat suggereert dat stam IS343 in staat was om *R. solani* AG2-2IIIB mycelium groei in grond te kunnen onderdrukken. Het mogelijke mechanisme achter het waargenomen onderdrukkend effect van stam IS343 op *R. solani* is concurrentie om beschikbare nutriënten tussen cellen van stam IS343 en het mycelium van het pathogeen, of productie van chitinase zoals eerder werd aangetoond bij andere *Collimonas* soorten.

Omdat gebleken was dat vooral de bacteriën die betrokken zijn bij het omzetten van stikstof verbindingen in de bodem geassocieerd waren met de onderdrukking van zowel *R. solani* en *F. oxysporum*, werden verschillende genen, verantwoordelijk voor de kringloop van stikstof, gekwantificeerd met behulp van real-time PCR. Gemeten waarden werden vervolgens gerelateerd aan de hoeveelheid stikstof die beschikbaar is voor micro-organismen en aan parameters (ziekte-incidentie en AUDPC) die het onderdrukkend effect op ziekteverwekkers beschrijven; allemaal gemeten in dezelfde bodemmonsters (hoofdstuk 6). Er werd geen correlatie gevonden tussen de hoeveelheden aan ammonium monooxygenase (*amoA*) en nitriet reductase (*nirS* en *nirK*) genen en hun effecten op bodemweerbaarheid tegen ziekten in suikerbiet en vlas planten ten gevolge van respectievelijk *R. solani* en *F. oxysporum* f. sp. *lini*. Er werd wel een significant curvi-lineair verband vastgesteld tussen de hoeveelheden van *nirS* en *nirK* genen enerzijds en de totale fractie aan beschikbare stikstof in de bodem anderzijds. Hoewel er geen direct verband werd aangetroffen tussen genen die betrokken zijn bij stikstofomzettingen in de bodem en ziekte onderdrukking, vermoeden we toch dat er indirecte verbanden kunnen bestaan tussen beide parameters.

Samenvattend geeft dit onderzoek meer inzicht in potentiële indicatoren voor bodemgezondheid. Er zal echter meer onderzoek voor nodig zijn om de processen die gezondheid in de bodem bepalen te kunnen begrijpen en om universeel toepasbare indicatoren te kunnen selecteren. Daarnaast toonde dit proefschrift een aantal aspecten aan in de relatie tussen bodemgezondheid en oligotrofe bacteriën; een belangrijke maar slechts beperkt onderzochte groep van micro-organismen in de bodem.

Резюме

Забота о здоровье почв необходима для успешного растениеводства и поддержания устойчивого развития экосистем. Для здоровых почв характерно большое биоразнообразие, стабильность, устойчивость к воздействию внешних факторов, сбалансированный обмен веществ, низкие концентрации легкодоступных питательных веществ (олиготрофность) и способность подавлять развитие патогенов растений (супрессивность). Считается, что органические почвы являются более здоровыми, чем традиционные, вследствие чего первые в большей степени подходят для изучения индикаторов качества почв. Супрессивность является надежной количественной характеристикой здоровых почв, и коррелирует со многими иными почвенными критериями качества. Однако, на сегодняшний день взаимоотношение между доминирующими почвенными микроорганизмами, олиготрофами, и супрессивностью почв изучено мало. Таким образом, в данной работе основное внимание уделяется здоровым почвам, органическому земледелию, олиготрофным бактериям и подавлению почвой патогенов растений. Основная цель исследования заключается в оценке влияния различных органических удобрений на здоровье органических почв в аспектах олиготрофии, супрессивности и углеродного и азотного циклов почвы.

Образцы исследуемых почв были взяты с двух экспериментальных полей, имеющих разную историю выращиваемых с/х культур, и расположенных на экспериментальной ферме 'De Droevendaal' рядом с г. Вагенинген (Wageningen), Голландия. Каждое из полей было разделено на два экспериментальных блока, которые подвергались воздействию четырех типов органических удобрений – (1) компостированные остатки растений, С, (2) жидкий навоз, S, (3) жидкий и твердый навоз, SD, (4) компост, жидкий и твердый навоз, SCD. Вследствие этого было достигнуто разнообразие внесенных типов удобрений и количества углерода и азота. Собранные образцы почв (по два с каждого удобренного участка) были подробно изучены – проведено детальное исследование присутствующих олиготрофных бактерий, определены супрессивность к *Rhizoctonia solani* и *Fusarium oxysporum*, численность и состав сообществ зубактерий, грибов, *Pseudomonas* и аммонийоокисляющих бета-протеобактерий; также было определено содержание генов, функционально задействованных в цикле азота (*amoA*, *nirS*, *nirK*) (главы 2-7).

Олиготрофы адаптированы к существованию в условиях низких концентраций углерода, а повышение концентрации углерода не способствует увеличению их жизненной активности и может приводить к гибели. Олиготрофы водной среды достаточно хорошо изучены, однако о таксономическом разнообразии и функциональности олиготрофов почв известно очень мало (глава 2). С использованием низкоуглеродной питательной среды (10 мкг/мл С), были выделены 720 бактериальных штаммов. Многократный (до 10 раз)

последовательный пересев каждого штамма на богатую и бедную углеродом среды позволил выявить те штаммы, которые избирательно растут только на низкоуглеродной среде. Таким образом, после первого посева, количество олиготрофных изолятов снизилось до 241, а после третьего – до 98. На основании частичного секвенирования 16S rRNA было показано, что большинство штаммов, предпочитающих низкоуглеродную среду, принадлежало к широко распространенным родам *Streptomyces*, *Rhizobium*, *Bradyrhizobium* и *Mesorhizobium*, а таксонометрический состав доминирующих родов менялся при последующих посевах. Выборка из 17 штаммов была охарактеризована более подробно, в результате чего на основании практически полного секвенирования 16S rRNA гена было идентифицировано 2 истинно олиготрофных штамма *Rhizobium alarii* и *Pedobacter roseus*. По результатам данной работы, было отмечено, что большинство штаммов, произрастающих на бедной среде, принадлежат к организмам с широкой нормой реакции по шкале концентрации углерода, и представляют собой достаточно известные и распространенные бактерии. Олиготрофия является физиологической, а не таксономической особенностью, что сильно затрудняет работу с почвенными олиготрофами. Однако, на основании результатов последних исследований олиготрофов водной среды, можно полагать, что олиготрофы могут отличаться от копиотрофов типами и количеством генов, отвечающих за синтез транспортных белков, что оставляет надежду на разработку количественного метода определения олиготрофов почв.

В главах 3-4 для оценки влияния различных удобрений на изменение супрессивности почв были проведены биологические тесты с использованием *R. solani* и сахарной свеклы, *F. oxysporum* и льна. Также анализировалась взаимосвязь развития патогенов со многими микробиологическими и химическими параметрами почв, включая отношение олиготрофов к копиотрофам (подсчитывались колонии на бедной и богатой углеродом среде) и биоразнообразии сообществ грибов и бактерий. Для этого образцы ДНК из образцов почвы были амплифицированы с использованием специфических праймеров и полученные амплификаты разделены с использованием денатурирующего градиентного электрофореза. Для определения переменных, связанных с супрессивностью, проводился многофакторный анализ.

Было установлено, что в результате удобрения почвы совокупно компостом, жидким и твердым навозом (SCD) симптомы *Fusarium* на льне были минимальны, и усиливались в результате добавления компоста (С) в одном из полей (глава 3). Прочие параметры подверглись малозначительным изменениям, за исключением рН (достоверно выше при обработке SCD). Значения площади под кривой прогрессии заболевания (AUDPC) отрицательно коррелировали только с величинами рН, но не с другими параметрами, включая соотношение копиотрофов к олиготрофам. Последующий дискриминантный анализ выявил, что различия в AUDPC также обусловлены влиянием количества углерода в почве и индексом разнообразия аммонийоокисляющих бета-протеобактерий (АОВ). Разнообразие видов АОВ сообщества, в свою

очередь, было связано с большим уровнем азота и меньшими величинами AUDPC.

Результаты анализа данных, полученных в экспериментах с *R. solani*, сильно отличались от результатов с *F. oxysporum*: влияния удобрений на развитие патогена обнаружено не было, также не было обнаружено достоверного влияния на прочие измеряемые характеристики (глава 4). Это во многом обусловлено фактом совокупного исследования образцов почв с двух различных полей; где зачастую наблюдались прямо противоположные эффекты: например, использование удобрения SCD привело к увеличению скорости распространения *R. solani* в одном из полей, тогда как на другом поле значения AUDPC были наименьшими. Данные наблюдения указывают на высокую сложность технологии управления развитием патогенных заболеваний почв с помощью внесения органических удобрений, т.к. их влияние на устойчивость растений к воздействию патогенов может сильно отличаться в зависимости от типа ранее засеваемых культур, истории вносимых ранее удобрений и особенностей сельскохозяйственного возделывания почв. В ходе исследования также было обнаружено влияние некоторых химических характеристик (рН, содержание органики, количество водорастворимого углерода) на состояние сообществ бактерий, грибов, *Pseudomonas* и АОВ.

Одним из наиболее интересных результатов данной работы является обнаружение олиготрофного штамма, принадлежащего к виду *Collimonas*, представители которого обладают способностью к хитинолизису. Поэтому дальнейшая работа была сосредоточена на исследовании нового штамма IS343, что представляло особый интерес в аспекте исследования прямой взаимосвязи между олиготрофией и суппресивностью (глава 5). Было установлено, что *Collimonas sp.* IS343 способен разлагать хитин и подавлять рост мицелия *R. solani* как в богатой углеродом среде, так и в среде с минимальным содержанием углерода. В отличие от хорошо изученного штамма *Collimonas fungivorans* Ter331, штамм IS343 не реагировал увеличением скорости роста на повышение концентрации углерода в жидкой среде, обладал большей скоростью роста в низкоуглеродной среде и более высоким уровнем выживаемости в почве. Таким образом, мы показали, что штамм IS343 в лучшей степени приспособлен к условиям малой доступности углерода, чем штамм Ter331. Также, штамм IS343 был способен более эффективно подавлять распространение *R. solani* в тестах *in vitro*. Добавление *Collimonas sp.* IS343 в почву способствовало замедлению распространения симптомов *R. solani* на сахарной свекле, что говорит о возможном подавлении мицелия *R. solani* и в почве. Наблюдаемые эффекты могут быть связаны как с эффективной конкуренцией за доступные питательные вещества между штаммом IS343 и *R. solani*, так и с присутствием хитиназной активности у *Collimonas sp.* IS343.

В связи с обнаруженной ролью бактерий, участвующих в цикле азота, в подавлении обоих патогенов (*R. solani* и *F. oxysporum*), мы исследовали взаимосвязь содержания генов,

обеспечивающих функционирование азотного цикла в почве, с количеством доступного азота и показателями супрессивности (глава 6). Содержание кодирующих генов аммоний-монооксигеназы (*amoA*) и нитрит-редуктаз (*nirK*, *nirS*) определялись методом real-time PCR. Мы не обнаружили взаимосвязи количества данных генов с величинами AUDPC при заражении сахарной свеклы и льна *R. solani* и *F. oxysporum* f. sp. *Lini* соответственно. В то же время, между содержанием *nirS*, *nirK* и общей фракцией азота была установлена статистически достоверная нелинейная связь. На основании полученных данных мы предположили, что взаимосвязь между количеством *amoA*, *nirS*, *nirK* и AUDPC является косвенной, и она обусловлена содержанием общего и доступного азота почвы.

Данное исследование привнесло новые сведения о потенциальных индикаторах здоровых почв. Однако, требуется приложить еще немало усилий на пути к пониманию процессов, определяющих состояние здоровых почв и подобрать соответствующие универсальные индикаторы. Нами были раскрыты отдельные аспекты взаимосвязи между здоровьем почв и олиготрофными бактериями, малоизученной группой почвенных микроорганизмов, обладающих высокой функциональной значимостью.

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Curriculum vitae

Ilya Senechkin was born on 22.07.1982 in Puschino (Moscow Region) - a town of biological sciences. At school (1989-1999) he was attending classes with a special interest in biochemistry. Ilya did his BSc study (1999-2003) at the Biotechnology Department of Moscow State Academy of Fine Chemical Technology, and carried out research on the organic synthesis of betulinic acid based drug compounds possessing improved anti-melanoma activity. Ilya's MSc study (2003-2005) was performed at The Institute of Molecular Genetics (Russian Academy of Sciences, Moscow); the research was about the effect of His6-tagging of glutamylendopeptidase on its folding mechanism, which led to an MSc degree with specialization in Molecular and Cellular Biotechnology. Ilya also completed a summer practice (2004) at The Institute of Molecular Physiology, Max-Planck-International (Dortmund, Germany) studying vesicular transport proteins in the laboratory. He continued with a PhD program in microbiology and soil health that took 4 years (2005-2009) in Wageningen, The Netherlands; 4 additional years (2009-2013) were needed in Russia to complete analysis and write-up of the research data, resulting in the thesis titled "Oligotrophic bacteria and root disease suppression in organically managed soils". Starting from 2010 Ilya has been working in the area of in-vitro fertilization (IVF) treatment in Moscow and continues his international activities as a representative of the Swedish company Vit.

List of publications

Senechkin, I.V., Speksnijder, A.G.C.L., Semenov, A.M., Van Bruggen, A.H.C., Van Overbeek, L.S. (2010) Isolation and partial characterization of bacterial strains on low organic carbon medium from soils fertilized with different organic amendments. *Microbial Ecology* 60:829-839.

Van Overbeek, L.S., Senechkin, I. and Van Bruggen, A.H.C. (2012) Variation in microbial responsiveness and *Rhizoctonia solani* AG2-2IIIB growth in soil under different organic amendment regimes. *Canadian Journal of Plant Pathology* 34:268-276.

Senechkin, I.V., Van Overbeek, L.S., Er, H.L., De Vos, O.J. and Van Bruggen, A.H.C. (2013) Interaction of *Collimonas* Strain IS343 with *Rhizoctonia solani* at low carbon availability in vitro and in soil. *European Journal of Plant Pathology* 136:789-802.

Senechkin, I.V., Van Overbeek, L.S. and Van Bruggen, A.H.C. (2014) Greater *Fusarium* wilt suppression after complex than after simple organic amendments as affected by soil pH, total carbon and ammonia-oxidizing bacteria. *Applied Soil Ecology*. In press.

PE&RC PhD Training Certificate

With the educational activities listed below the PhD candidate has complied with the educational 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 (4.5 ECTS)

- Multiplex detection and quantification of microbial genes to report soil health (2005)

Writing of project proposal (4.5 ECTS)

- Multiplex detection and quantification of microbial genes to report on soil health

Post-graduate courses (3 ECTS)

- Advanced statistics; PE&RC (2006)
- Interactions between soil and plant communities; PE&RC/FE/SENSE (2006)
- Interactions between soil and plant communities; PE&RC/FE/SENSE (2007)

Deficiency, refresh, brush-up courses (2 ECTS)

- Academic writing I and II; CENTA (2006)

Competence strengthening / skills courses (3ECTS)

- Soil quality; Prof. Brussaard (2007)

PE&RC Annual meetings, seminars and the PE&RC weekend (1.2 ECTS)

- PE&RC Day (2006)
- PE&RC Day (2007)
- Symposium; PE&RC (2007)

Discussion groups / local seminars / other scientific meetings (7.5 ECTS)

- Agricultural production systems in temperate systems (2006-2009)
- Plant Research International groups: Molecular Phytopathology cluster and Biointeractions and Plant Health group (2006-2009)

International symposia, workshops and conferences (5 ECTS)

- 11th International Symposium on Microbial Ecology (ISME); Vienna, Austria (2006)
- 2nd Annual Meeting of the Ecogenomics research Program (2006)
- 3rd Annual Meeting of the Ecogenomics research Program (2007)
- 4th Annual Meeting of the Ecogenomics research Program (2008)
-

Supervision of 2 MSc students (6 ECTS)

- N-Cycling genes quantification in organic soils (Robert Bara)
- Characterization of newly isolated *Collimonas* str. And its interaction with *R. solani* (Er Hong Ling)

