Pseudomonas spp. producing antimicrobial metabolites in agricultural soils: links between abundance, soil factors and soil disease resistance

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Pseudomonas spp. producing antimicrobial metabolites in agricultural soils: links between abundance, soil factors and soil disease resistance

A thesis submitted to attain the degree of DOCTOR of SCIENCES of ETH ZURICH (Dr. sc. ETH Zurich)

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«...ha dell’incredibile!»
(D.L.)
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SUMMARY

*Pseudomonas* spp. producing antimicrobial metabolites inhibit the growth of soilborne plant pathogenic fungi and are used as biocontrol agents to control plant diseases. They are abundant in soils defined “suppressive”, where pathogens are present but plants show no or reduced disease symptoms. *Pseudomonas* spp. are suggested to contribute to the disease suppressiveness of these specific soils. However, it is not known, if these bacteria can generally be associated with, or contribute to soil disease resistance in common agricultural soils. The main aim of this thesis was therefore to investigate, with focus on wheat production, if the abundance of *Pseudomonas* producing antimicrobial metabolites can be linked to soil resistance to two important soilborne pathogens, *Pythium ultimum* and *Gaeumannomyces tritici*. This thesis was part of the National Research Program 68 “Soil as a Resource”, aiming at increasing the sustainability of soil use in Switzerland. Therefore, this thesis focused on representative agricultural soils in Switzerland.

In the first part of this thesis, the abundance of pseudomonads producing antimicrobial metabolites was determined on the roots of wheat grown in ten representative Swiss agricultural soils. To this end, the following biosynthesis genes of three antimicrobial metabolites, which have been demonstrated to be involved in disease suppression were quantified using real-time quantitative PCR: *phlD* (biosynthesis of 2,4-diacetylphloroglucinol), *phzF* (biosynthesis of phenazines) and *prnD* (biosynthesis of pyrrolnitrin). In addition, disease resistance to *Pythium ultimum* and *Gaeumannomyces tritici* was determined in these soils in pot experiments where plants were grown in presence of increasing inoculum quantities of these pathogens. Among the investigated soils, there were large differences in both disease resistance levels and abundance of antimicrobial genes. However, no significant correlations were found between gene abundances and soil disease resistance, but both were significantly linked to certain soil characteristics. For example, the abundance of *phlD* and *Pythium* resistance were both significantly positively linked to nitrate concentration in soil.

The influence of different cropping systems, such as conservation tillage and organic agriculture, on antimicrobial metabolite producing *Pseudomonas*, is not well known, but this knowledge is needed to implement conservation biocontrol strategies involving these bacteria. Thus, in the second part of this thesis, the abundance of *phlD*,
and *phzF* harbouring *Pseudomonas* and *prnD* harbouring bacteria was determined in two long-term trials (the FAST trial of Agroscope and the DOK trial of the Research Institute for Organic Agriculture) both comparing organic with conventional farming, the first additionally comparing intensive with conservation tillage. The obtained results did not indicate that particular cropping systems can promote antimicrobial metabolite producing *Pseudomonas* in general, but specific groups of these bacteria can be favoured by cropping systems. For instance, in the treatments of the FAST trial, *phlD*+ *Pseudomonas* were significantly more abundant in conventional, conservation tillage plots, compared to organically managed conservation tillage plots. In contrast, in the DOK trial, populations of *Pseudomonas* spp. harbouring biosynthesis genes of antimicrobial metabolites were not significantly different in conventional and organic treatments. However, similarly to the results obtained for the 10 field sites, there was no clear link between soil resistance to pathogens and abundance of antimicrobial metabolite producing *Pseudomonas*. These results suggest that the cropping system can affect the abundance of antimicrobial metabolite producing *Pseudomonas*, but that these bacteria cannot be used as indicators of soil disease resistance. The development of conservation biocontrol strategies based on promoting naturally present biocontrol pseudomonads will be very challenging, since soil disease resistance is probably shaped by a consortium of different microorganisms, which might be different for each pathogen, soil type and crop plant.

Results from the first and second part of this thesis indicate that *Pseudomonas* play a minor role in soil disease resistance, and other microorganism might be the key players. Therefore, in the last part of this thesis, bacterial and fungal communities in the same soils studied in the first part of the thesis were characterised by high-throughput amplicon sequencing of the 16S rRNA and ITS genes. The interactions in the microbial communities, in particular, the links between taxa containing biocontrol bacteria, taxa containing pathogenic fungi, and soil disease resistance against pathogens were investigated with network and correlation analysis. No significant links were found between the relative abundance of taxa at the genus level and soil disease resistance. However, specific interactions were found at the operational taxonomic unit (OTU) level. Specific OTUs were associated with soil resistance against specific pathogens. These results indicate that soil disease resistance is based on very specific microbial interactions, which likely involve different bacterial taxa for each
pathogen. Since the functions of single OTUs cannot be obtained from amplicon sequencing data, the mechanisms behind these interactions remain elusive. In the future, microbial processes involved in soil disease resistance should be studied additionally with functional approaches, e.g. using metatranscriptomics.

In summary, this thesis contributes to understanding the ecology of *Pseudomonas* producing antimicrobial metabolites in agricultural soils, their interaction with plant pathogenic fungi and their role in soil disease resistance.
**ZUSAMMENFASSUNG**


Häufigkeit von \textit{phlD} und die Resistenz gegen \textit{Pythium} signifikant positiv mit der Nitratkonzentration im Boden korreliert.


Die Resultate der ersten zwei Teile dieser Dissertation deuten darauf hin, dass \textit{Pseudomonas} eine untergeordnete Rolle in der Resistenz der Böden gegenüber

Zusammenfassend trägt diese Dissertation zum Verständnis der Ökologie von Pseudomonas, welche antimikrobielle Metaboliten produzieren, in landwirtschaftlichen Böden bei, sowie zum Verständnis der Interaktionen dieser Bakterien mit pflanzenpathogenen Pilzen und ihrer Rolle in der Resistenz der Böden gegen Krankheiten.
CHAPTER 1

General Introduction
1.1. Soilborne plant pathogens: ecology, importance and control strategies
Certain fungal, oomycete and plasmodiophoromycete pathogens attack primarily plant roots and lower parts of the stem. These pathogens are often called soilborne pathogens, and their attack leads to reduced water and nutrient uptake, which in turn causes stunting, reduced yield and senescence in upper plant parts (Agrios, 2005). Soilborne pathogens survive on plant residues and organic matter in soils, where they form long-term survival structures such as sclerotia (De Coninck et al., 2015). Many soilborne fungal pathogens have a hemibiotrophic lifestyle, consisting of an initial biotrophic phase followed by a necrotrophic phase, while typical soil-borne pathogenic oomycetes have an only necrotrophic lifestyle (De Coninck et al., 2015). Major soil-borne pathogens include different oomycete species, such as *Pythium* spp., and *Phytophthora* spp., as well as fungi, such as different species of the ascomycete genera *Fusarium*, *Verticillium*, *Thielaviopsis*, *Gaeumannomyces*, *Colletotrichum*, and the basidiomycete *Rhizoctonia* (Agrios, 2005; De Coninck et al., 2015). Some root and soil borne pathogens have narrow host ranges, with one pathogen species or strain parasitizing only one host species, such as for example *Gaeumannomyces tritici*. Other pathogens have a wide host range spanning many crop species, for example *Rhizoctonia solani* and *Pythium ultimum* (Agrios, 2005). The damage caused by soilborne pathogens is often underestimated (De Coninck et al., 2015), because signs of infections, such as typical brown lesions on roots caused by many pathogens (Agrios, 2005) are not directly visible on the above ground parts of plants. Especially in the case of ubiquitous pathogens such as *Pythium* spp., the damage is not detected by growers unless the plants can be compared with plants from a fungicide-treated healthy control plot in the same field (Paulitz, 2006). In fact, the damage caused by *Pythium* in wheat cultivations was not recognized, until it was shown that treatments with metalaxyl significantly increased yields (Cook et al., 1980). Soilborne pathogens are difficult to control with fungicides, because of the difficult accessibility of roots for treatments, and because the application of fungicides in the soil is controversial from an ecological point of view (Agrios, 2005; Haas and Defago, 2005; Paulitz, 2006; De Coninck et al., 2015). Therefore, soilborne pathogens have mainly been controlled through agricultural practices, such as crop rotation, removal of plant residues after harvest, and tillage (Agrios, 2005). However, these cultural practices are not effective control methods for all pathogens; for instance pathogens such as *Fusarium* spp., or
Plasmodiophora brassicae that form long-term survival structures in the soil, cannot be controlled by crop rotation (Agrios, 2005). Crop rotation is also not effective to control pathogens such as Pythium and Rhizoctonia, which have a broad host spectrum and can survive from one cropping cycle to the next on the roots of weeds (Paulitz, 2006). Seed treatments with fungicides are used against certain soil- and seedborne pathogens, for example against Fusarium in wheat (Moya-Elizondo and Jacobsen, 2016), Rhizoctonia in soybean (Dorrance et al., 2003), Alternaria in vegetables (Mancini and Romanazzi, 2014) and Phytophtora capsici in pumpkin (Babadoost and Islam, 2003). However, often there is only a partial reduction of symptoms after seed treatments with fungicide (Babadoost and Islam, 2003; Moya-Elizondo and Jacobsen, 2016). In contrast to foliar plant pathogens, soilborne plant pathogens can, in most cases, not be controlled with resistant varieties, since there simply are none against many of these pathogens (Paulitz, 2006). In this context, new methods to control soilborne pathogens are needed to increase agricultural production. Biological control of soilborne pathogens with antagonistic fungi or bacteria is an alternative to synthetic fungicides used for seed treatments. While biological control, for example with antifungal bacteria, is less effective against some soilborne pathogens compared to synthetic fungicides (Moya-Elizondo and Jacobsen, 2016), they offer other advantages, such as less ecological concerns (Berg, 2009) and growth promoting effects for plants, since many biocontrol bacteria also solubilize nutrients (Berg, 2009; Berendsen et al., 2012).

1.2. Suppressive soils: history and link to fluorescent pseudomonads producing antimicrobial metabolites

The study of soil bacteria able to inhibit the growth of soilborne plant pathogens started with the discovery of particular soils, where plant pathogens were present, but plants showed no or reduced disease symptoms (Weller et al., 2002). These soils are defined as “disease suppressive” or “pathogen suppressive” soils (Weller et al., 2002). While suppressive soils, for example Fusarium suppressive soils (Atkinson, 1892), were observed already in the nineteenth century, the underlying mechanisms were identified almost one century later. In particular, with experiments where soils were pasteurised and then inoculated with pathogens, the microbial nature of soil
suppressiveness was discovered (Ahl et al., 1986; Stutz et al., 1986; Weller et al., 2002; Lemanceau et al., 2006). Comparing the disease incidence in sterilized soils to natural soils, it was noted that all soils can inhibit pathogens to a certain degree (Weller et al., 2002). This effect was defined “general suppression” and inhibition is observed against all pathogens, in contrast to suppressive soils, where only a specific pathogen is usually strongly inhibited (Weller et al., 2002).

To understand the microbial basis of suppressiveness, bacterial strains were isolated from these soils and characterised (Lemanceau et al., 2006). The strains isolated worldwide from suppressive soils that showed effects against pathogens, were mainly of the genera *Pseudomonas*, *Bacillus* and *Serratia* (Haas and Defago, 2005; Berg, 2009). Among the first studies characterising the soil bacteria involved in suppressiveness, were the studies in the take-all decline soils of the Pacific Northwest of the USA (Weller and Cook, 1983). In these soils, the incidence of take-all of wheat, caused by *G. tritici*, declined after several years of wheat monoculture, eventually leading to take-all suppressive soils (Weller et al., 2002). The decline of take all incidence on wheat has been linked to an increased rhizosphere population of bacteria belonging to the *Pseudomonas fluorescens* group with antifungal characteristics in these soils (Weller et al., 2002; Cook, 2007). In particular, model strain *P. synxantha* 2-79, with various antifungal properties, was isolated from take all decline soils in this region (Weller and Cook, 1983). Similarly, also in the tobacco black root rot (caused by *T. basicola*) suppressive soils of the Broye region in Switzerland, the suppression of the pathogen was attributed to fluorescent pseudomonads, and model strain *P. protegens* CHA0 was isolated from these soils (Stutz et al., 1986). Other early studies on soil suppressiveness were carried out in the *Fusarium* suppressive soils of the Châteaurenard region in France, where the competition between pathogenic and apathogenic *Fusarium* strains was found to be important for suppressiveness (Alabouvette, 1986).

Already in these early studies, it emerged that bacteria could inhibit pathogenic fungi with different mechanisms (Figure 1), namely competition for resources on the root surface (Lockwood, 1988), induction of systemic resistance in the plant against pathogens (Kloepper et al., 1992; Maurhofer et al., 1998; Kloepper et al., 2004; Bakker et al., 2007), and production of secondary metabolites with antifungal effects (Keel et al., 1992; Défago, 1993; Haas and Defago, 2005; Lemanceau et al., 2006). These
mechanisms were studied in particular in the genus *Pseudomonas* (Haas and Keel, 2003; Haas and Defago, 2005). Competition for nutrients is thought to play a minor role in the inhibition of pathogens by *Pseudomonas* spp. (Haas and Defago, 2005), except for the competition for iron (Kloepper et al., 1980; Ahl et al., 1986; Lemanceau et al., 1992; Raaijmakers et al., 2008), which can also regulate induced systemic resistance through siderophores produced by the bacteria (Bakker et al., 2007). In contrast, induced systemic resistance has been shown to be a mechanism by which many biocontrol microorganisms protect plants against pathogens, also pseudomonads (Bakker et al., 2013). For example, fluorescent pseudomonads induce systemic resistance in *Arabidopsis* against *Pseudomonas syringae* (Weller et al., 2012), against *Peronospora* (Iavicoli et al., 2003), and against tobacco necrosis virus (Maurhofer et al., 1998). In *Pseudomonas*, induced systemic resistance is triggered by flagellins, cell surface components such as lipopolysaccharides (Bakker et al., 2007), siderophores (Maurhofer et al., 1994a), and antimicrobial secondary metabolites such as 2,4-diacetylphloroglucinol (Iavicoli et al., 2003; Bakker et al., 2007).

Secondary metabolites with broad antimicrobial effects were identified as being a central mechanism in the antagonism of biocontrol bacteria and pathogenic fungi. Phenazine compounds were shown to be essential for the inhibition of *Gaeumannomyces tritici*, by using a mutant lacking the phenazine biosynthesis operon (Thomashow and Weller, 1988). Similarly, 2,4-diacetylphloroglucinol (DAPG) has been shown to be an important mechanisms of fluorescent pseudomonads to inhibit pathogens, such as *T. basicola* and *G. tritici* (Keel et al., 1992). Other metabolites produced by fluorescent pseudomonads that have antifungal effects include pyrrolnitrin (Cartwright et al., 1995), pyoluteorin (Maurhofer et al., 1994b), hydrogen cyanide (Voisard et al., 1989), and various cyclic lipopeptides (Bender et al., 1999; Raaijmakers et al., 2006).
Figure 1: Summary of mechanisms of plant protection against pathogens by biocontrol
*Pseudomonas* spp. These bacteria can protect plants against pathogens with three main mechanisms: i) Competition for nutrients and plant exudates in the rhizosphere; ii) Induced systemic resistance, where *Pseudomonas* spp. prime plants against pathogen attacks with various signalling molecules; iii) Antibiosis, where *Pseudomonas* spp. produce antimicrobial metabolites that have deleterious effects for fungal and oomycete cells. The main antimicrobial metabolites of biocontrol *Pseudomonas* spp. involved in antibiosis are 2,4-diacetylphloroglucinol (DAPG), phenazines (PHZ), pyrrolnitrin (PRN), pyoluteorin (PLT), cyclic lipopeptides (CLP) and hydrogen cyanide (HCN).

Soil suppressiveness has been linked to a higher abundance of *Pseudomonas* spp. producing antimicrobial metabolites, compared to conducive soils. DAPG producing pseudomonads were found to be more abundant in take-all suppressive soils compared to conducive soils (Raaijmakers et al., 1997; de Souza et al., 2003c), *T. basicola* suppressive soils (Ramette et al., 2003) and in soils suppressive to *Fusarium* wilt of pea (Landa et al., 2002), while phenazine producing pseudomonads were found to be more abundant in *Fusarium* suppressive soils compared to conducive soils (Mazurier et al., 2009). In particular, for DAPG producers it was found that an abundance greater than $10^5$ cells per gram of root is needed to inhibit pathogen growth in take-all decline soils (Raaijmakers and Weller, 1998). However, in other studies, DAPG producing pseudomonads were similarly abundant in suppressive and
conducive soils (Mazurier et al., 2009; Almario et al., 2013a; Kyselkova et al., 2014). In another study, the abundance of DAPG producing pseudomonads was similar in conducive and suppressive soils, but different genotypes were detected in the two soils (Frapolli et al., 2010). Therefore, the role of antimicrobial metabolite producing *Pseudomonas* spp. in disease suppressiveness is unclear, and it remains to be elucidated if there is a quantitative link between their abundance and the degree of soil disease resistance.

### 1.3. *Pseudomonas* metabolites linked to biological control

Antimicrobial metabolites with antifungal effects are produced by *Pseudomonas* in the soil and on roots primarily to compete and communicate with other microorganisms (Venturi and Keel, 2016). In fact, these metabolites tend to have a broad spectrum activity against many fungal and bacteria taxa (Keel et al., 1992). 2,4-diacetylphloroglucinol (DAPG) is one of the most studied *Pseudomonas* antimicrobial metabolites. DAPG is a phenolic polyketide metabolite encoded by the operon *phlACBD* (Bangera and Thomashow, 1999). Its importance for fungal pathogen suppression was recognized testing the effect of mutants lacking biosynthesis genes on fungi, and by directly adding the metabolite to fungal cultures (Keel et al., 1992). DAPG inhibited the growth of a variety of plant pathogens, including different *Fusarium* species, different *Pythium* species, *Rhizoctonia solani*, *Thielaviopsis basicola* and *Gaeumannomyces tritici* (Keel et al., 1992). DAPG is produced by *P. protegens* strains and by some strains of the *P. fluorescens* group sub-clade 2 (Flury et al., 2016), such as the model strains *P. protegens* CHA0 (Stutz et al., 1986), *P. protegens* Pf-5 (Howell and Stipanovic, 1980) and *Pseudomonas sp.* F113 (Shanahan et al., 1992). Fluorescent pseudomonads were the only group known to produce DAPG, but recently the DAPG biosynthesis operon was detected also in certain Betaproteobacteria (Almario et al., 2017). In the oomycete *Pythium*, DAPG causes the disruption of hyphae and disintegration of the cell content (de Souza et al., 2003a). The biochemical mode of action of DAPG was studied in the model fungus *Neurospora crassa* (Troppens et al., 2013), where it leads to changes in mitochondrial membrane morphology and cellular calcium homeostasis. The effect of DAPG on fundamental cellular processes might be the reason for its broad spectrum antimicrobial activity (Troppens et al., 2013).
Phenazines are a second class of important antimicrobial metabolites produced by *Pseudomonas* (Mavrodi et al., 2006). They are a class of numerous different heterocyclic nitrogen containing compounds, of which approximately 100 are naturally produced by bacteria (Mavrodi et al., 2006). In *Pseudomonas*, phenazines are encoded by the operon *phzABCDEFG* (Mavrodi et al., 1998). Similarly to DAPG, phenazines have a broad spectrum antimicrobial activity and inhibit the growth of different pathogenic fungi, such as *Gaeumannomyces tritici* (Thomashow and Weller, 1988), *Rhizoctonia solani* (Mavrodi et al., 2012; Mohd Jaaffar et al., 2017), *Fusarium* (Anjaiah et al., 1998) and *Pythium* (Anjaiah et al., 1998; Perneel et al., 2007). Among soil bacteria, phenazines are produced by different genera of Gram-positive and Gram-negative Proteobacteria, such as *Pseudomonas*, *Burkholderia*, *Streptomyces* and *Brevibacterium* (Mavrodi et al., 2006). In the genus *Pseudomonas*, phenazines are produced by different strains of *Pseudomonas aeruginosa*, *Pseudomonas chlororaphis*, *Pseudomonas syxantha* (Mavrodi et al., 2006), *Pseudomonas orientalis*, *Pseudomonas aridus* and *Pseudomonas cerealis* (Parejko et al., 2013). The mode of action of phenazines is not fully understood (Mavrodi et al., 2013), but one mechanism that causes their broad spectrum activity is the formation of reactive oxygen compounds (ROS) after the uptake of phenazines by fungal cells (Morales et al., 2010).

A third well-studied antimicrobial metabolite produced by *Pseudomonas* is pyrrolnitrin. This tryptophan-derived, organohalogenic metabolite is produced by a restricted number of taxa of the Gram-negative Proteobacteria (Costa et al., 2009). It is encoded by the four-gene operon *prnABCD* (Costa et al., 2009). It has been shown to inhibit the growth of different plant pathogens, such as the bacterial pathogen *Streptomyces* (El Banna and Winkelmann, 1998), *Rhizoctonia solani* (Hwang et al., 2002), and *Fusarium* (Costa et al., 2009). Pyrrolnitrin is produced by some strains of the genera *Pseudomonas*, *Serratia* and *Burkholderia* (Costa et al., 2009). Among *Pseudomonas*, pyrrolnitrin is produced by a number of *P. protegens* and *P. chlororaphis* strains (Flury et al., 2016). Its broad spectrum antimicrobial activity is explained by its inhibitory effect on the respiratory chain in mitochondria, where it inhibits the terminal electron transport system (Tripathi and Gottlieb, 1969).

Pyoluteorin is a halogenated pyrrole (Takeda, 1958), that has been found to play an important role in the inhibition of *Pythium ultimum* (Howell and Stipanovic, 1980; Maurhofer et al., 1994b). It is produced by *Pseudomonas aeruginosa* (Gross and Loper,
and by some strains of the *Pseudomonas fluorescens* group Sub-clade 1, mainly *P. protegens* strains that also produce DAPG (Flury et al., 2016). It is encoded by the gene locus *plt*, consisting of 17 genes (Gross and Loper, 2009). Pyoluteorin production is influenced by phloroglucinol, an intermediate of the DAPG biosynthesis pathway, where a high quantity of produced phloroglucinol causes a decrease in the pyoluteorin production in nearby cells (Schnider-Keel et al., 2000; Clifford et al., 2016). Hydrogen cyanide (HCN) is a volatile antimicrobial metabolite produced by many strains of the *Pseudomonas fluorescens* group sub-clades 1 and 2 (Flury et al., 2016). Its importance in pathogen inhibition on plant roots was recently questioned, since its concentration in natural environments was not high enough to affect plant pathogens (Rijavec and Lapanje, 2016). In contrast, HCN is involved in soil chemical processes, indirectly increasing the availability of phosphate (Rijavec and Lapanje, 2016). Further, antimicrobial metabolites with antifungal effects are the cyclic lipopeptides, such as orfamide and sessilins (Raaijmakers et al., 2006; Olorunleke et al., 2015; Ma et al., 2016). In various studies, it was found that between approximately 1% and 60% of *Pseudomonas* strains isolated from soil and root samples produce cyclic lipopeptides, but also various strains of *Bacillus* and *Paenibacillus* (McSpadden Gardener, 2004; Raaijmakers et al., 2010). Cyclic lipopeptides have a lytic and growth inhibitory effect against a broad range of microorganisms, including bacteria, fungi, oomycetes and viruses (Nybroe and Sørensen, 2004). Cyclic lipopeptides from *Pseudomonas* have been found to have strong inhibitory effects on *Pythium* and *Phytophthora*, because of their ability to lyse zoospores (de Souza et al., 2003b; van de Mortel et al., 2009). Lipopeptides from *Bacillus* were found to be the main antifungal metabolite involved in the inhibition of *Cladosporium*, *Botrytis*, *Fusarium* and *Pythium* (Cawoy et al., 2015). Cyclic lipopeptides further influence bacterial motility and biofilm formation, and are elicitors of induced systemic resistance (Ongena et al., 2007; Raaijmakers et al., 2010).

Recently, it was found that some *Pseudomonas* strains additionally produce metabolites with insecticidal properties, such as the Fit toxin, a homologue of the Mcf toxin produced by the entomopathogenic bacterium *Photorabdus* (Péchy-Tarr et al., 2008; Ruffner et al., 2013; Ruffner et al., 2015). Interestingly also some antimicrobial metabolites produced by *Pseudomonas*, such as cyclic lipopeptides and hydrogen cyanide, have been shown to be involved in insecticidal activity in fluorescent
pseudomonads of subclade 1 (Flury et al., 2017).

1.4. Ecology of antimicrobial metabolite producing pseudomonads

*Pseudomonas* producing antimicrobial metabolites have been found in soils worldwide (Raaijmakers et al., 1997; Wang et al., 2001; de Souza et al., 2003c; Mazurier et al., 2009; Kim, 2013). In particular, their abundance was associated with soil suppressiveness in some studies (Raaijmakers et al., 1997; Mazurier et al., 2009), but *Pseudomonas* producing antimicrobial metabolites were also found to be similarly abundant in conducive soils (Almario et al., 2013a; Kyselkova et al., 2014). These studies have mostly been conducted on a small number of suppressive and adjacent conducive soils, therefore it is still not clear if the abundance of antimicrobial producing *Pseudomonas* is an indicator of soil suppressiveness or reduced disease incidence when looking at a broader range of agricultural soils.

Environmental factors influence the presence and abundance of antimicrobial producing *Pseudomonas*. For instance, (Mavrodi et al., 2012) showed that irrigation influences the abundance of DAPG and phenazine producing pseudomonads. DAPG producers were more abundant in irrigated soils compared to phenazine producers while the opposite was the case in dryland soils (Mavrodi et al., 2012). This results could be explained by the role that phenazine producing pseudomonads play in biofilm formation, which could be an advantage for root colonization under arid conditions (Mavrodi et al., 2013).

Soil physical and chemical properties influence the production of antimicrobial metabolites in different ways. An interesting example is the suppression of tobacco black root rot by *P. protegens* CHA0 in suppressive soils of Morens, Switzerland (Stutz et al., 1986). It was found that the cause of the suppressiveness was the higher abundance of iron-rich vermiculite clay in the suppressive soil compared to the conducive soil (Keel et al., 1989). Iron-rich vermiculite favours the transcription of DAPG biosynthesis genes, thus enhancing plant protection from tobacco black root rot (Almario et al., 2013b).

Macro- and micronutrient concentrations in soils have been shown to influence the concentration of antimicrobial metabolites produced by *Pseudomonas* (Duffy and Défago, 1999; Ownley et al., 2003). An increased zinc concentration for example
increased the production of various antimicrobial metabolites by \textit{P. protegens} CHA0 in an artificial medium (Duffy and Défago, 1999), while ammonium, pH, sand, sodium, sulfur and zinc concentrations were positively correlated with the biocontrol activity of phenazine producing \textit{Pseudomonas} (Ownley et al., 2003).

Also biotic factors have been found to influence the expression of antimicrobial metabolite biosynthesis genes, for example the host plant at the species and cultivar level (Notz et al., 2001; Rochat et al., 2010), host plant age (Notz et al., 2001) and pathogen infection (Notz et al., 2001; de Werra et al., 2008).

However, these studies have mostly been carried out in artificial soils or in growth media (Duffy and Défago, 1999; Notz et al., 2001; Rochat et al., 2010), or with a reduced number of \textit{Pseudomonas} strains (Notz et al., 2001; Ownley et al., 2003), therefore, the role these biotic and abiotic factors play in relation to abundance and expression of antimicrobial metabolite biosynthesis genes in natural \textit{Pseudomonas} populations requires further clarification.

1.5. Methods for quantification of antimicrobial metabolite producing \textit{Pseudomonas} spp. in environmental samples

Pseudomonads with the ability to produce antimicrobial metabolites can be quantified within natural bacterial communities by amplifying the biosynthesis genes of these metabolites (Raaijmakers et al., 1997; Garbeva et al., 2004; Mazurier et al., 2009; Mavrodi et al., 2012; Almario et al., 2013a). Two different types of methods have been commonly used in the last years: i) cultivation-dependent methods, where \textit{Pseudomonas} colonies are isolated from environmental samples using selective growth media, and subsequently the proportion of cells harbouring antimicrobial metabolite biosynthesis genes is determined by PCR (Meyer et al., 2010; Mavrodi et al., 2012), and ii) cultivation-independent methods, such as quantitative real-time PCR (qPCR) targeting biosynthesis genes of antimicrobial metabolites (Garbeva et al., 2004; Mavrodi et al., 2007; Almario et al., 2013a).

Cultivation-dependent methods are based on dilution series performed with the environmental sample in growth media, allowing to obtain a quantitative measure of the number of \textit{Pseudomonas} cells per quantity of soil or root harbouring a certain metabolite biosynthesis gene (Landa et al., 2006; Meyer et al., 2010; Mavrodi et al.,
These methods are defined as dilution-endpoint assays (Mavrodi et al., 2012), or most probable number (MPN) assays (Meyer et al., 2010). The advantage of these methods is that only viable cells are quantified, i.e. the DNA from dead cells that is still present in the sample is not quantified. However, there are also disadvantages, such as the failure to detect the non-culturable fraction of the Pseudomonas population, and the selection for certain strains or species through the growth medium (Li et al., 2013). Therefore, increasingly, methods are used that amplify biosynthesis genes for antimicrobial metabolites directly in environmental samples. For example, qPCR allows to obtain the number of target genes, or cells harbouring the target gene, per unit of environmental sample (Garbeva et al., 2004; Mavrodi et al., 2007; Almario et al., 2013a). However, qPCR accuracy and replicability is dependent on the experimental procedure, in particular the construction of an appropriate standard curve and selection of primers and probes. Therefore, guidelines for the minimal information needed to interpret data obtained with qPCR assays have been published (Bustin et al., 2009). Moreover, the amplification of DNA directly extracted from environmental samples can lead to biases due to inhibition of co-extracted substances, such as humic acids in soils (Kreader, 1996). Steps have to be taken to minimize or control inhibition of PCR amplification, such as the addition of substances that bind to the inhibitors (Kreader, 1996), or the use of internal standards to normalise the data and control for the effect of the inhibition (Von Felten et al., 2010; DeCoste et al., 2011).

1.6. Other microorganisms involved in soil disease resistance

Pseudomonas spp. have been linked to the suppressiveness of soils in different studies (Weller et al., 2002; de Souza et al., 2003c; Weller, 2007; Mazurier et al., 2009). Pseudomonas model strains isolated from suppressive soils have been studied in depth for the mechanisms of pathogen inhibition (Haas and Keel, 2003; Haas and Defago, 2005), and some strains have also been commercialised as active ingredients of biocontrol products (Berg, 2009). In addition to Pseudomonas spp., some other soil and root microorganisms have been associated with soil suppressiveness or have been found to inhibit plant pathogens. One of the first studied examples are the Fusarium suppressive soils of the Châteaurenard region in France, where aphaathogenic Fusarium stains were found to be linked with suppressiveness (Alabouvette, 1986).
Other bacterial taxa linked to pathogen inhibition include *Bacillus* and *Paenibacillus* (McSpadden Gardener, 2004; Raaijmakers et al., 2010; Cawoy et al., 2015), which produce different lipopeptides that have been shown to inhibit pathogens such as *Rhizoctonia solani* and different oomycetes (McSpadden Gardener, 2004; Cawoy et al., 2015). Bacteria of these two genera have been commercialised as biocontrol products against *Rhizoctonia*, *Fusarium*, *Pythium* and *Phytophthora* (Berg, 2009). In particular, *Bacillus* and *Paenibacillus* strains offer some advantages for commercialisation compared to *Pseudomonas*, because of their ability to form spores, and therefore have a longer shelf-life (Kloepper et al., 2004). Certain strains of the genera *Serratia*, *Stenotrophomonas* and *Burkholderia* have also been found to inhibit plant pathogens (Berg et al., 2006; Vleesschauwer, 2007; Bach et al., 2016). Some of these strains share certain antimicrobial metabolites with *Pseudomonas*, for example pyrrolnitrin, which is also produced by *Burkholderia* and *Serratia* (Garbeva et al., 2004), cyclic lipopeptides, which are also produced by *Bacillus* and *Paenibacillus* (Raaijmakers et al., 2010), and phenazines, which are also produced by *Burkholderia* and many other soil bacteria (Mavrodi et al., 2010). The fact that different taxa of soil bacteria and fungi have disease suppressive capacities indicates that pathogen inhibition in soils is likely a complex process orchestrated by the interactions between different plant beneficial and plant pathogenic microorganisms (Lemanceau et al., 2006).

**1.7. Microbiome studies and soil disease resistance**

Disease suppressiveness and pathogen inhibition in soils is probably regulated by complex interactions within the microbiome (Lemanceau et al., 2006; Raaijmakers et al., 2008). Moreover, the non-culturable fraction of soil and microbial communities could play a role in suppressive soils as well, but this cannot be studied with traditional microbiological techniques (Nesme et al., 2016). The advent of new sequencing techniques, such as high-throughput amplicon sequencing of environmental DNA, has allowed to study the entire soil and plant associated microbial communities, leading to the discovery of many new taxa (Massart et al., 2015; Mauchline and Malone, 2017). To unravel the taxa which are associated with soil suppressiveness, the microbial communities in suppressive soils can be compared with those of adjacent conducive soils to identify the taxa enriched in the suppressive soils, which therefore are
considered putative indicator taxa for suppressive soils (Mendes et al., 2011). This information can then be used to select new bacterial strains for the development of biocontrol products, or for the assembly of beneficial microbial consortia targeted to specific soils (Massart et al., 2015; Schlaeppi and Bulgarelli, 2015). These approaches require an combination of “omics” techniques with cultivation dependent techniques (Schlaeppi and Bulgarelli, 2015). Other approaches to exploit beneficial microorganisms for disease control, which do not depend on the isolation of strains, are conservation biocontrol or breeding-based approaches (Berg et al., 2017). For the first, the aim is to adapt or improve agricultural cultivation techniques in a way which favours the abundance and activity of naturally occurring beneficial microorganisms. For the latter, plants are bred based on selection for genotypes which accumulate and benefit from beneficial microbes.

The taxa which have been associated with suppressive soils in recent microbiome studies include gamma- and beta-Proteobacteria (Pseudomonadaceae, Burkholderiaceae, Xanthomonadales) and the Firmicutes (Lactobacillaceae) in a *Rhizoctonia solani* suppressive soil (Mendes et al., 2011). Further, the fungal genus *Mortierella* has been associated with *Fusarium* suppressive soils (Xiong et al., 2017) and Actinobacteria, Firmicutes and Acidobacteria with *Fusarium* suppressive soils in Australia at a continental scale (Trivedi et al., 2017). In a study focusing on Gammaproteobacteria in *Fusarium*-infested banana cultivations (Köberl et al., 2017), *Pseudomonas* and *Stenotrophomonas* were associated with healthy plants, while Enterobacteriaceae were associated with diseased plants. All these studies were mostly carried out comparing single suppressive soils that were adjacent to conducive soils. Therefore, it is not clear if the microbial taxa found to be associated with suppressiveness in the studies mentioned above, are generally linked to soil disease resistance in agricultural soils.

Moreover, microbial community studies that aim to identify taxa associated with soil suppressiveness have several limitations. Methodological limitations include biases in DNA extraction, where not all types of cells can be lysed equally well, resulting in biased relative abundances (Nesme et al., 2016), the selection of appropriate primers (Beckers et al., 2016), and limited reference databases (Choi et al., 2016). Microbial community studies are frequently carried out by amplifying a fragment of the 16S rRNA gene, which allows to distinguish taxa at the order, family and at the genus level,
but not at the species or below species level (Ranjan et al., 2016). Therefore, the role or function of taxa in soil is difficult to assign with currently available microbiome sequencing methods, and has to be considered with care. The genus *Fusarium*, for example, comprises pathogenic, aphathogenic, and disease suppressive strains, which can be present in the same soil (Alabouvette, 1986), while the genus *Pseudomonas* contains plant beneficial, plant pathogenic and human pathogenic strains (Rumbaugh, 2014).

### 1.8. Plant-beneficial pseudomonads in soils managed organically or with reduced tillage

Soil conservation systems, such as no tillage or reduced tillage systems, are increasingly adopted by farmers worldwide to minimise soil erosion, increase water retention and preserve soil structure (Pittelkow et al., 2014). Several studies showed that soil bacterial communities are influenced by tillage intensity, with specific taxa being more frequent under no tillage compared to conventional tillage (Navarro-Noya et al., 2013; Chávez-Romero et al., 2016).

Similarly, organic agriculture is increasingly common because it requires less external inputs and increases soil fertility (Mäder et al., 2002; Fließbach et al., 2007). Organically managed soils were found to harbor an overall greater microbial diversity than conventionally managed soils (Mäder et al., 2002; Li et al., 2012; Hartmann et al., 2015), and certain taxa, such as Proteobacteria, were found to be enriched in organically managed soils, while other taxa, such as Actinobacteria and Chloroflexi, were enriched in conventionally managed soils (Li et al., 2012; Hartmann et al., 2015; Pershina et al., 2015).

While the beneficial effect of conservation tillage and organic agriculture for soil structure and fertility is well studied, the effect of these cropping systems on specific, plant beneficial and pathogenic microorganisms is not well known. For instance, only a small number of studies quantified antimicrobial metabolite producing pseudomonads in reduced tillage soils or in organically managed soils. Rotenberg et al. (2007), for example, found that DAPG producing *Pseudomonas* were more abundant in the rhizosphere of maize grown in no tillage plots compared to moderately tilled plots, and Hiddink et al. (2005), detected more DAPG producing *Pseudomonas* in
conventionally managed fields compared to organically managed fields. These studies just provide a first insight into the interplay of cropping systems and plant-beneficial microorganisms. In order to successfully apply conservation biocontrol strategies, further studies are needed.

1.9. The National Research Program 68 in the context of the Swiss pesticide reduction plan and soil conservation strategies

In Switzerland, the majority of agricultural soils are subject to crop rotation, because this is a requirement to obtain governmental subsidies for farms with more than three ha (regulation on agricultural subsidies, Direktzahlungsverordnung, available online: www.admin.ch). This, among other advantages, limits the damage caused by soilborne pathogens. Seed coatings with synthetic fungicides are allowed against seed- and soilborne pathogens in conventional agriculture (Regulation on agricultural subsidies, Direktzahlungsverordnung, available online: www.admin.ch), while in organic agriculture, seeds are treated with preventative measures, such as hot water treatments to reduce pathogen pressure (FiBL, 1999). However, concerns about the ecological impact of synthetic fungicides and pesticides are increasing among the public. Therefore, a pesticide reduction plan is being discussed by the Swiss parliament (Eidgenössisches Departement für Wirtschaft, 2014). The promotors of the pesticide reduction plan aim to reduce the use of pesticides in Swiss agriculture by increasing the costs of pesticides, by increasing the monitoring of pesticides in the environment, with a stronger control of pesticide use, and by favouring alternative pest control strategies (Bosshard, 2016). In this context, the development of new biocontrol strategies based on soil bacteria is crucial for a more sustainable agriculture, where biocontrol methods will be widely adopted also in conventional crop production.

This thesis was carried out within the frame of the National research program 68: “Soil as a resource” of the Swiss national science foundation. This program aims to improve the sustainability of soil use in Switzerland from an ecological and economical point of view. The present thesis was part of the “soil biology cluster”, containing five projects linked to sustainable soil use in agriculture (http://www.nfp68.ch/en/projects/key-aspect-2-soil-biology, accessed on 19.06.2017). The project “Soil bacteria: healthy
soils thanks to soil bacteria” aimed to investigate how plant beneficial *Pseudomonas* are linked to soil disease resistance against pathogens, and how this knowledge can be used to develop new biocontrol methods. In particular, the objectives of the project were the following:

1) To develop new tools to measure the abundance and activity of plant beneficial *Pseudomonas*, and to characterise Swiss agricultural soils for their abundance and activity, as well as for the ability of the soils to sustain natural biocontrol activity;

2) To investigate the influence of agricultural practices on the abundance and activity of plant beneficial *Pseudomonas* and soil disease resistance;

3) To develop new approaches for the biocontrol of insect pests and plant diseases based on *Pseudomonas* bacteria;

4) To test new biocontrol strategies based on field applications of beneficial pseudomonads in combination with entomopathogenic nematodes.

The project was a collaboration between different research groups; this thesis focused on objectives 1 and 2, as detailed in the following section.

Figure 2: Samples from the ten Swiss agricultural soils analyzed in this thesis (Chapter 2 and Chapter 5).
1.10. Objectives of the thesis

The aim of this thesis is to gain a deeper understanding of the ecology of fluorescent pseudomonads with antimicrobial activity and disease suppressing capacity, in agricultural soils with different physical and chemical characteristics. The focus of the thesis is on the link between these bacteria and the resistance of soils against pathogens. A major objective was to test the hypothesis that the abundance of antimicrobial metabolite producing pseudomonads is positively correlated with the degree of soil resistance against pathogens. This thesis, should, however, not only focus on *Pseudomonas* spp. with antimicrobial properties, but also include the genus *Pseudomonas* in general, and also assess the relations of other bacterial taxa to disease resistance. In contrast to most studies performed on this topic so far, this thesis should not only compare a suppressive with a conducive soil, but include a set of agricultural soils with a wider range of susceptibility/resistance to soilborne diseases. A further aim was to investigate the effect of different cropping systems, such as conservation tillage and organic agriculture, on the abundance of antimicrobial metabolite producing pseudomonads. In detail, the objectives of the present thesis were the following:

I) Development of quantitative, real time PCR (qPCR) assays to quantify pseudomonads producing antimicrobial compounds on roots and in soil. Cultivation-independent methods to quantify antimicrobial metabolite producing *Pseudomonas* are needed to investigate natural populations. Therefore, at the start of the thesis, qPCR methods to quantify *Pseudomonas* producing 2,4-diacetylphloroglucinol (DAPG), and *Pseudomonas* producing phenazines from soil and roots, targeting the genes *phlD* and *phzF* respectively, were developed. An already existing qPCR method for pyrrolnitrin (Garbeva et al., 2004) was adapted.

II) Investigation of the link between abundance of pseudomonads producing antimicrobial metabolites known to be involved in biocontrol and soil disease resistance. The abundance of antimicrobial metabolite producing pseudomonads has often been compared in suppressive and adjacent conducive soils, and in some studies their abundance has been found to be linked to soil suppressiveness.
(Raaijmakers et al., 1997; Mazurier et al., 2009). However, it is not clear if and how the abundance of antimicrobial metabolite producing *Pseudomonas* is linked to disease resistance in a broader range of soils. Therefore, the first major aim of the thesis was to quantify i) groups of known biocontrol *Pseudomonas* spp. harbouring the antimicrobial metabolite biosynthesis genes *phlD* (DAPG biosynthesis), *phzF* (phenazines biosynthesis) and *prnD* (pyrrolnitrin biosynthesis) by qPCR in ten representative agricultural soils of Switzerland (Figure 2); ii) to assess the resistance of these soils to soilborne diseases caused by pathogens such as *Pythium ultimum* and *Gaeumannomyces tritici*; and iii) to investigate links between disease resistance and abundance of these *Pseudomonas* groups.

III) **Investigation of the impact of agricultural methods on soil disease resistance and *Pseudomonas* abundance.** Still very little is known on how the abundance of beneficial soil bacteria is influenced by different agricultural methods. Therefore, a further objective of this thesis was to investigate the impact of different agricultural cropping systems, such as conservation tillage and organic agriculture, on population sizes of the above mentioned *Pseudomonas* groups and on soil disease resistance in two long-term field trials comparing conservation tillage with conventional tillage, and organic agriculture with conventional agriculture. In addition, the abundance of the genus *Pseudomonas* in soils managed with different cropping systems was investigated using a 16S rRNA gene amplicon sequencing.

IV) **Unravelling the interactions between plant beneficial bacteria and pathogenic fungi in soil microbial communities.** Many microorganisms other than *Pseudomonas* spp. are involved in pathogen inhibition in soils (Berg, 2009). However, it is still not well known which microbial taxa are determinants for pathogen inhibition on roots and in soils, since studies typically compare the microbial communities in one suppressive soil and an adjacent conducive soil (Mendes et al., 2011; Kyselkova et al., 2014; Xiong et al., 2017). Therefore, the last objective of this thesis was to unravel the interactions between plant beneficial bacteria and plant pathogenic fungi in soil microbial communities by analysing the links between these
two groups in a range of soils with different levels of disease resistance/susceptibility. To this end, a high-throughput amplicon sequencing of partial 16S rRNA and ITS gene fragments was performed in nine representative agricultural soils. Subsequently the link between the relative abundances of bacterial taxa containing strains known to be involved in pathogen inhibition, and fungal taxa containing soil borne plant pathogens was analysed. Moreover, taxa linked to *P. ultimum* and *G. tritici* resistance in soil and root microbial communities were identified.

1.11. References


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CHAPTER 1


CHAPTER 2

Relationships between root pathogen resistance, abundance and expression of *Pseudomonas* antimicrobial genes, and soil properties in representative Swiss agricultural soils


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FD performed the greenhouse disease resistance experiments, developed the qPCR assays and collected the qPCR data, analysed the disease resistance and qPCR data, performed the correlation analyses, and wrote the manuscript together with MM, CK and NI. NI performed gene expression experiments and data analysis, as well as development of reporter strains.
2.1. Abstract

Strains of *Pseudomonas* that produce antimicrobial metabolites and control soilborne plant diseases have often been isolated from soils defined as disease-suppressive, i.e., soils, in which specific plant pathogens are present, but plants show no or reduced disease symptoms. Moreover, it is assumed that pseudomonads producing antimicrobial compounds such as 2,4-diacetylphloroglucinol (DAPG) or phenazines (PHZ) contribute to the specific disease resistance of suppressive soils. However, pseudomonads producing antimicrobial metabolites are also present in soils that are conducive to disease. Currently, it is still unknown whether and to which extent the abundance of antimicrobials-producing pseudomonads is related to the general disease resistance of common agricultural soils. Moreover, virtually nothing is known about the conditions under which pseudomonads express antimicrobial genes in agricultural field soils. We present here results of the first side-by-side comparison of 10 representative Swiss agricultural soils with a cereal-oriented cropping history for (i) the resistance against two soilborne pathogens; (ii) the abundance of *Pseudomonas* bacteria harboring genes involved in the biosynthesis of the antimicrobials DAPG, PHZ, and pyrrolnitrin on roots of wheat, and (iii) the ability to support the expression of these genes on the roots. Our study revealed that the level of soil disease resistance strongly depends on the type of pathogen, e.g., soils that are highly resistant to *Gaeumannomyces tritici* often are highly susceptible to *Pythium ultimum* and vice versa. There was no significant correlation between the disease resistance of the soils, the abundance of *Pseudomonas* bacteria carrying DAPG, PHZ, and pyrrolnitrin biosynthetic genes, and the ability of the soils to support the expression of the antimicrobial genes. Correlation analyses indicated that certain soil factors such as silt, clay, and some macro- and micronutrients influence both the abundance and the expression of the antimicrobial genes. Taken together, the results of this study suggests that pseudomonads producing DAPG, PHZ, or pyrrolnitrin are present and abundant in Swiss agricultural soils and that the soils support the expression of the respective biosynthetic genes in these bacteria to various degrees. The precise role that these pseudomonads play in the general disease resistance of the investigated agricultural soils remains elusive.
2.2. Introduction

The ability of soilborne plant pathogens to attack and damage host plants is influenced by biotic and abiotic soil factors (Weller et al., 2002; Haas and Defago, 2005; Lemanceau et al., 2006; Almario et al., 2014). In some soils, even susceptible crop plants suffer only a little or not at all from specific diseases although soilborne pathogens are present (Weller et al., 2002). In general, two different types of natural pathogen suppression are thought to occur in agricultural soils. First, the general disease suppression, where different soilborne pathogens are controlled to a certain degree depending on the total microbial activity in the soil and/or on abiotic soil factors (Weller et al., 2002; Lemanceau et al., 2006). Second, the specific disease suppression, where the soil restricts the activity of a distinct species of plant pathogen based on its interactions with a specific group of microorganisms (Weller et al., 2002; Haas and Defago, 2005; Lemanceau et al., 2006; Berendsen et al., 2012; Raaijmakers and Mazzola, 2016).

Soils with specific disease suppression have been described worldwide (Cook and Rovira, 1976; Stutz et al., 1986; Weller et al., 2002; Lemanceau et al., 2006; Chng et al., 2015) and for diverse soilborne plant pathogens. They include soils suppressive to *Gaeumannomyces graminis var. tritici* (recently renamed *G. tritici* (Hernández-Restrepo et al., 2016)) causing take-all of wheat (Weller et al., 2002), *Thielaviopsis basicola* causing black root rot of tobacco (Stutz et al., 1986; Almario et al., 2014), *Fusarium oxysporum* causing wilt on tomatoes (Alabouvette, 1986; Tamietti et al., 1993), *Pythium* spp. causing seedling damping-off (Martin and Hancock, 1986), and *Rhizoctonia solani* causing damping-off and root rot on various crop species (Mendes et al., 2011). Such soils are commonly referred-to as suppressive soils. By contrast, conducive soils do not restrict the development of soilborne diseases (Haas and Defago, 2005).

Suppressive soils have been found to host distinct microbial communities that are thought to be responsible for the natural disease control effect (Weller et al., 2002; Haas and Defago, 2005; Mendes et al., 2011; Klein et al., 2013; Mendes et al., 2013; Kyselkova et al., 2014; Cha et al., 2016). In particular, bacteria of the *Pseudomonas fluorescens* group have been isolated from suppressive soils and used as plant or soil inoculants. Several strains proved to be very efficient at colonizing roots, protecting plants from different diseases, and increasing plant productivity (Mercado-Blanco and
Bakker, 2007; Lugtenberg and Kamilova, 2009; Höfte and Altier, 2010). Thus, it has been suggested that such pseudomonads contribute to soil suppressiveness (Weller et al., 2002; Haas and Defago, 2005; Garbeva et al., 2006; Lemanceau et al., 2006; Weller, 2007; Mazurier et al., 2009). The capacity of many root-associated pseudomonads to release antimicrobial compounds placed them in the focus of research on the nature of soil disease suppressiveness. Many P. fluorescens group strains produce an array of potent antimicrobials, among which 2,4-diacetylphloroglucinol (DAPG), phenazines (PHZ), pyrrolnitrin (PRN), and hydrogen cyanide (HCN) are most prominent (Blumer and Haas, 2000; Haas and Defago, 2005; Raaijmakers and Mazzola, 2012; Mavrodi et al., 2013). All these antimicrobials were shown, mostly in pot and gnotobiotic assays, to play indeed an important role in the Pseudomonas-mediated protection of plants from soilborne pathogenic fungi and oomycetes (Thomashow and Weller, 1988; Voisard et al., 1989; Keel et al., 1992; Maurhofer et al., 1992; Pierson and Thomashow, 1992; Hwang et al., 2002; Chin-A-Woeng et al., 2003; Weller, 2007; Mavrodi et al., 2013).

The role of these antimicrobial compounds in disease-suppressive soils is still not fully understood. They are indeed produced in some field soils, as demonstrated for DAPG in the Quincy take-all decline soil (Raaijmakers et al., 1999) and for PHZ in wheat fields of the Columbia Plateau, USA (Mavrodi et al., 2012a). Several studies performed during the last 15 years aimed at investigating whether disease-suppressive soils are specifically enriched for Pseudomonas genotypes producing antimicrobials compared to conducive soils. In fact, in the Pacific Northwest of the USA, DAPG-producing pseudomonads were found to be more abundant in take-all suppressive soils than in adjacent conducive soils (Raaijmakers et al., 1997). However, DAPG producers were not more abundant in Fusarium suppressive soils of Châteaurenard (France) than in adjacent conducive soils, in contrast to PHZ-producing pseudomonads, which were more abundant in the suppressive soils (Mazurier et al., 2009). In some studies, the total abundance of DAPG-producing pseudomonads was found to be similar in suppressive and conducive soils (Ramette et al., 2006; Almario et al., 2013a), but suppressive soils harbored distinct genotypes of DAPG producers (Frapolli et al., 2010). Moreover, abundances of plant-beneficial pseudomonads with antimicrobial activity were mostly investigated in specific disease-suppressive soils, and very little is known about the occurrence of these bacteria in common agricultural soils and on
how soil factors might impact these bacteria. In wheat fields of the Pacific Northwest of the USA, it was found that the abundance of DAPG- and PHZ-producing pseudomonads on wheat roots is influenced by irrigation (Mavrodi et al., 2012b). Based on such observations, the abundance and genotypic diversity of antimicrobials-producing *Pseudomonas* bacteria in soil seems not a sufficient argument to explain the disease suppressiveness of some soils. It has been suggested that (i) other bacterial species contribute importantly to the disease suppressiveness (Mendes et al., 2011; Kyselkova et al., 2014), and (ii) somehow the expression of antimicrobial genes in *Pseudomonas* bacteria is favored in suppressive soils and hampered in conducive soils (Ramette et al., 2006; Almario et al., 2014). Indeed, studies on the abundance of antimicrobial metabolite-producing pseudomonads do not consider the complex interactions in the rhizosphere that ultimately modulate the production of the antimicrobials in the rhizosphere (Rochat et al., 2010; de Werra et al., 2011). To date, little is known about the biotic and abiotic factors affecting the expression of biosynthetic genes for these metabolites in soil. Studies conducted under gnotobiotic conditions indicate that the expression of DAPG, HCN, and PRN biosynthesis genes is influenced by the crop species and variety (Rochat et al., 2010; de Werra et al., 2011; Latz et al., 2015) and for DAPG also by the interaction with other microorganisms and the iron availability in the rhizosphere (Notz et al., 2001; Notz et al., 2002; Maurhofer et al., 2004; Jousset et al., 2010; Jousset et al., 2011; Almario et al., 2013b). How biotic and abiotic soil factors affect antimicrobial gene expression under natural conditions in agricultural soils remains, however, unexplored.

There is a clear lack of studies investigating the link between natural disease resistance and abundance and expression of antimicrobial *Pseudomonas* genes in common agricultural soils. To address this gap, in the present study 10 representative Swiss agricultural soils with a cereal-oriented cropping history and differing in their physical and chemical characteristics were compared for their resistance to two soilborne pathogens of wheat, i.e., *G. tritici* (Gt) and *Pythium ultimum* (Pu). In parallel, the 10 soils were planted with wheat and pseudomonads harboring the biosynthetic genes required for the production of the antimicrobial compounds DAPG, PHZ, and PRN were quantified on roots using qPCR. In addition, the expression of these genes and the HCN biosynthetic genes was monitored by flow cytometry using fluorescent reporter strains of the representative model pseudomonads *P. protegens* CHA0 and *P.
chlororaphis PCL1391. To our best knowledge, this is the first side-by-side comparison using root-associated pseudomonads as bio-indicators to explore relationships between abundance and expression of antimicrobial genes, soil disease resistance and soil physicochemical characteristics in a range of common agricultural soils.

2.3. Material and Methods

2.3.1 Sampling and physicochemical analysis of field soils

Soil samples were collected in 10 farmer’s fields across Switzerland (Figure 1) in May 2013. The main characteristics of the 10 field soils are listed in Table 1. Field sites had a history of multi-year cereal-oriented crop rotation and were chosen to represent predominant Swiss agricultural soil types and climate conditions. All fields were cropped with winter wheat in the year of sampling. For sampling, soil cores of 15–20 cm depth were extracted with disinfected soil recovery augers between the rows of wheat plants at 20 random locations in each field in order to obtain a representative sample. Then, extracted soil samples were sieved (mesh size, 10 by 10 mm) in order to remove stones, plant residues or other larger material, pooled and thoroughly mixed. For each site, approximately 120 kg of sieved soil were collected and stored in barrels for 3 months at 15°C before the start of the experiments in order to equilibrate the soils, i.e., to minimize effects of different environmental conditions (e.g., temperature, soil moisture) prevailing at the different sampling sites at the time of sampling. The storage temperature was chosen because it can be considered as the average temperature in Switzerland during the growing season of wheat from April to September (according to long-term monthly temperature averages recorded by the Swiss Federal Office MeteoSwiss, http://www.meteoswiss.admin.ch/home/climate/past/homogenous-monthly-data.html). Soil parameter analyses were carried out by the Labor für Boden- und Umweltanalytik (Eric Schweizer AG, Steffisburg, Switzerland) following standard protocols used in Swiss agriculture (Agroscope, 2006). Concentrations of soluble, readily plant-available macronutrients were determined following H₂O extraction, for which soil samples were suspended in distilled water at a ratio of 1:10 (g mL⁻¹). Reserve macronutrients and micronutrients were extracted with ammonium acetate EDTA, for which soil samples dried at 65°C were suspended at a ratio of 1:10 (g mL⁻¹) in a solution consisting of acetic acid (0.5 mol L⁻¹), ammonium acetate and EDTA (0.02 mol L⁻¹) adjusted to a pH of 4.65. Soil
suspensions were then vigorously shaken for 1 h and filtered prior to analysis by mass spectrometry. The soil parameter analyses were carried out (i) directly after sampling and (ii) after the end of the experiments. Except for nitrate, the soil parameters did not change significantly between the two analyses (Table 1).

**Figure 1: Location of the 10 field sites in Switzerland used for soil sampling.** Cd, Cadenazzo; Cx, Courtedoux; Cz, Cazis; De, Delley; Es, Eschikon; Gr, Grangeneuve; Ta, Taenikon; Ut, Utzenstorf; Vo, Vouvry; Wi, Witzwil. Empty topographical map of Switzerland courtesy of Federal Office of Topography swisstopo.

**2.3.2 Assessment of field soil resistance against root pathogens**

In order to test the natural resistance of the 10 Swiss agricultural soils toward soilborne pathogens, pot experiments were carried out in the greenhouse with cucumber and Pu and wheat and Gt. The Pu inoculum was prepared by inoculating 25 g of autoclaved millet seeds moistened with 10 mL of sterile water with three plugs of a culture of Pu strain ETH-2 (isolated from a Swiss agricultural soil) grown on Oxoid malt agar (Thermo Fisher Scientific, Reinach, Switzerland) for 7 days. Pu millet seed cultures were grown for 7 days at 18°C and then cut into small fragments for soil inoculation. The Gt inoculum was prepared by inoculating 100 g of autoclaved oat seeds without spelts and moistened with 100 mL of sterile, distilled water with 20 plugs of cultures of Gt strain I-17 (Lebreton et al., 2007) grown on Oxoid potato dextrose agar for 14 days. Gt oat cultures were grown for 4 weeks at 24°C in the dark and then dried in a sterile cabinet on sterile filter paper for 3 days. Seeds of cucumber (*Cucumis sativus* cv. Chinese Snake) and spring wheat (*Triticum aestivum* cv. Rubli) were surface-sterilized for 30 min in 1.5% (v/v) NaOCl, rinsed with sterile distilled
water and pre-germinated on sterile moist filter paper for 2 days in the dark at 24°C. For the plant experiments, 250-mL plastic pots of were filled with a 4:1 mixture (wt/wt) of the respective field soil and quartz sand (grain size of 0.5–2.2 mm diameter). Pathogen inoculum at different concentrations was thoroughly mixed into soil. Pu inoculum was added at 0.125, 0.25, 0.5, or 1.0 g inoculum per pot, whereas Gt inoculum was added at 0.2, 0.6, 2.0, or 6.0 g per pot. Pots in control treatments contained field soil without pathogen addition. Three seedlings of cucumber or wheat, respectively, were then planted per pot. For each treatment, six replicate pots were prepared. Plants were grown in the greenhouse at 70% relative humidity with light (210 mmol m⁻² sec⁻¹) for 16 h at 22°C (cucumber) or 18°C (wheat), followed by an 8-h dark period at 18°C (cucumber) or 15°C (wheat). Plants were watered routinely to keep the soil at constant moisture. The position of the pots was changed at random every other day to avoid position effects. After incubation for 10 days (cucumber) or 21 days (wheat), total shoot fresh weights per pot were assessed.
Table 1: Characteristics of agricultural soils sampled at different Swiss farmers’ fields cropped with wheat.

<table>
<thead>
<tr>
<th>Coordinates</th>
<th>Cademazzo (Cd)</th>
<th>Courtedoux (Cs)</th>
<th>Cazis (Ca)</th>
<th>Delley (De)</th>
<th>Eschikon (Es)</th>
<th>Grandeneuve (Gr)</th>
<th>Tannikon (Ta)</th>
<th>Uttendorf (Ut)</th>
<th>Veovy (Vo)</th>
<th>Witzwil (Wi)</th>
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<td>47.482525</td>
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<td>46.330365</td>
<td>46.983912</td>
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<tr>
<td>Organic matter (%)</td>
<td>1.7</td>
<td>2.6</td>
<td>2.2</td>
<td>1.7</td>
<td>2.4</td>
<td>1.7</td>
<td>3.5</td>
<td>4.7</td>
<td>1.5</td>
<td>11.6</td>
</tr>
<tr>
<td>Clay (%)</td>
<td>8.4</td>
<td>25.7</td>
<td>7.8</td>
<td>16.8</td>
<td>24.1</td>
<td>19.2</td>
<td>27.0</td>
<td>20.4</td>
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<td>31.0</td>
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<tr>
<td>Silt (%)</td>
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<td>61.4</td>
<td>32.9</td>
<td>26.7</td>
<td>27.9</td>
<td>16.0</td>
<td>24.0</td>
<td>32.7</td>
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<td>30.5</td>
</tr>
<tr>
<td>Sand (%)</td>
<td>47.8</td>
<td>10.5</td>
<td>58.4</td>
<td>55.7</td>
<td>46.7</td>
<td>64.3</td>
<td>47.1</td>
<td>44.3</td>
<td>59.0</td>
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<td>pH</td>
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<td>7.7</td>
<td>7.3</td>
<td>7.0</td>
<td>6.6</td>
<td>6.9</td>
<td>7.0</td>
<td>7.1</td>
<td>7.3</td>
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<tr>
<td>Soluble macronutrients (H₂O, 1:10)³</td>
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<td></td>
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<td></td>
<td></td>
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<tr>
<td>Nitrate (analysis 1) (mg kg⁻¹)</td>
<td>8.2</td>
<td>12.6</td>
<td>26.7</td>
<td>23.1</td>
<td>34.6</td>
<td>29.4</td>
<td>34.3</td>
<td>30.3</td>
<td>10.0</td>
<td>52.0</td>
</tr>
<tr>
<td>Nitrate (analysis 2) (mg kg⁻¹)</td>
<td>80.9</td>
<td>10.1</td>
<td>153.9</td>
<td>21.8</td>
<td>192.8</td>
<td>9.1</td>
<td>148.0</td>
<td>80.1</td>
<td>77.7</td>
<td>91.9</td>
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<tr>
<td>P (mg kg⁻¹)</td>
<td>4.3</td>
<td>2.2</td>
<td>1.9</td>
<td>6.0</td>
<td>1.5</td>
<td>2.5</td>
<td>3.6</td>
<td>7.0</td>
<td>4.2</td>
<td>1.1</td>
</tr>
<tr>
<td>K (mg kg⁻¹)</td>
<td>3.7</td>
<td>18.3</td>
<td>22.2</td>
<td>30.7</td>
<td>22.3</td>
<td>14.8</td>
<td>43.4</td>
<td>56.8</td>
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<td>Ca (mg kg⁻¹)</td>
<td>36.4</td>
<td>164.8</td>
<td>155.7</td>
<td>144.6</td>
<td>117.4</td>
<td>76.9</td>
<td>118.1</td>
<td>139.6</td>
<td>103.5</td>
<td>332.2</td>
</tr>
<tr>
<td>Mg (mg kg⁻¹)</td>
<td>5.2</td>
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<td>11.5</td>
<td>21.8</td>
<td>15.9</td>
<td>7.3</td>
<td>14.2</td>
</tr>
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<td>Reserve macronutrients (NH₄·Ac + EDTA, 1:10)³</td>
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</tr>
<tr>
<td>P (mg kg⁻¹)</td>
<td>41.5</td>
<td>45.9</td>
<td>77.9</td>
<td>211.5</td>
<td>29.5</td>
<td>38.4</td>
<td>206.6</td>
<td>291.7</td>
<td>137.3</td>
<td>33.7</td>
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<tr>
<td>K (mg kg⁻¹)</td>
<td>46.6</td>
<td>182.2</td>
<td>50.5</td>
<td>140.8</td>
<td>110.9</td>
<td>98.9</td>
<td>261.1</td>
<td>249.9</td>
<td>50.5</td>
<td>81.6</td>
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<tr>
<td>Ca (mg kg⁻¹)</td>
<td>870.1</td>
<td>4608.0</td>
<td>21880.0</td>
<td>3386.0</td>
<td>2611.0</td>
<td>1746.0</td>
<td>4122.0</td>
<td>3927.0</td>
<td>27170.0</td>
<td>69900.0</td>
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<tr>
<td>Mg (mg kg⁻¹)</td>
<td>79.7</td>
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<td>116.6</td>
<td>392.5</td>
<td>191.8</td>
<td>256.1</td>
<td>537.3</td>
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<tr>
<td>Micronutrients (NH₄·Ac + EDTA, 1:0)³</td>
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<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Fe (mg kg⁻¹)</td>
<td>248.2</td>
<td>336.5</td>
<td>552.1</td>
<td>836</td>
<td>381.4</td>
<td>372.8</td>
<td>1385.0</td>
<td>1275.0</td>
<td>785.0</td>
<td>1500.0</td>
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</tr>
<tr>
<td>B (mg kg⁻¹)</td>
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<td>0.4</td>
<td>0.8</td>
<td>0.1</td>
<td>0.0</td>
<td>0.6</td>
<td>0.8</td>
<td>1.0</td>
<td>0.9</td>
</tr>
<tr>
<td>Mn (mg kg⁻¹)</td>
<td>148.8</td>
<td>790.8</td>
<td>754.9</td>
<td>528.3</td>
<td>686.7</td>
<td>601.2</td>
<td>911.1</td>
<td>682.4</td>
<td>184.9</td>
<td>207.5</td>
</tr>
<tr>
<td>Zn (mg kg⁻¹)</td>
<td>1.9</td>
<td>6.1</td>
<td>9.25</td>
<td>4.51</td>
<td>7.0</td>
<td>3.3</td>
<td>11.6</td>
<td>14.3</td>
<td>4.3</td>
<td>14.7</td>
</tr>
</tbody>
</table>

¹Soils from farmers’ fields were sampled in May 2013. All fields were planted with winter wheat at the time of sampling. Soil parameters were analysed by the Labor für Boden- und Umweltanalytik, Enis Schweizer AG, Steffisburg, Switzerland after sampling in 2013.
³Geographical coordinates of each field site were measured by GPS. The field site localization was described by the south–north orientation (SN) and the east–west (EW) orientation codes.
³Soluble macronutrients were extracted with water. Reserve macronutrients and micronutrients were extracted with ammonium acetate EDTA. See Materials and Methods section for details.
³Soil parameters were analysed twice, once after sampling and once at the end of the experiments, i.e. after prolonged storage. Only nitrate levels (shown in the table) were significantly different between the first and the second analysis.
2.3.3 Development of qPCR methods for quantification of pseudomonads harboring DAPG, PHZ, and PRN biosynthetic genes

To quantify the abundance of DAPG and PHZ producing bacteria in soil, we developed quantitative real-time polymerase chain reaction (qPCR) assays targeting \textit{phlD} and \textit{phzF} genes. These genes encode, respectively, a polyketide synthase involved in the synthesis of phloroglucinols from malonyl-CoA (Bangera and Thomashow, 1996; Achkar et al., 2005) and an isomerase involved in the synthesis of phenazine-1-carboxylic acid (Mavrodi et al., 1998; Blankenfeldt et al., 2004). Alignments were created with publicly available \textit{phlD} and \textit{phzF} sequences from GenBank and conserved regions were chosen for the design of primers and probes (Table 2), which was carried out with the Primer 3 Plus software (Untergasser et al., 2007). The parameters were amplicon length between 100 and 200 bp, melting temperature (TM) between 50 and 70 °C, TM of probe 5 °C higher than TM of primers, and the default setting of the program for self-complementarity and 3-end stability. Partial sequences of \textit{phlD} (GenBank accession CP003190.1:6563260-6563937) of strain \textit{P. protegens} CHA0 (Jousset et al., 2014) and \textit{phzF} (locus tag, PFLU3_RS28075) of \textit{P. synxantha} 2–79 (Nesemann et al., 2015) were used for primer design. The specificity of the primers was tested \textit{in silico} with Primer-Blast (Ye et al., 2012) and \textit{in vitro} with genomic DNA from 28 DAPG-producing strains and 38 PHZ-producing strains of the \textit{P. fluorescens} group and nine additional PHZ-producing strains (Supplementary Table S1). Results of these tests revealed that our qPCR assays amplify \textit{phlD} and \textit{phzF} genes exclusively from DAPG and PHZ producing species of the \textit{P. fluorescens} lineage. The PRN biosynthetic genes were quantified on wheat roots by the qPCR method of Garbeva et al. (2004). That assay targets a gene for the class IA oxygenase PrnD that is involved in the final step of PRN biosynthesis (Kirner et al., 1998). In contrast to our \textit{phlD} and \textit{phzF} primers, the primers of Garbeva et al. (2004) have broader specificity and, in addition to \textit{Pseudomonas}, amplify \textit{prnD} from PRN-producing strains of \textit{Burkholderia} and \textit{Serratia}. The efficiency of \textit{phlD} and \textit{phzF} primers at low gene copy numbers was evaluated using \textit{in vitro} standard curves prepared by serially diluting genomic DNA of \textit{P. protegens} CHA0 and \textit{P. synxantha} 2–79. The genomic DNA was prepared by growing both strains in lysogeny broth (LB) (Bertani, 1951) overnight at 24°C on a rotary shaker at 180 rpm and extracting DNA with the Wizard Genomic DNA Purification Kit (Promega AG, Dübendorf, Switzerland). The concentration of purified DNA was quantified by
fluorimetry with Qbit (Thermo Fisher Scientific). We also generated an in vivo standard curve for each qPCR assay to quantify the corresponding target genes on wheat roots. To this end, aliquots of 1 g of 21-days-old roots of spring wheat cv. Rubli grown in autoclaved soil were inoculated with decreasing concentrations of a mixture of bacterial cells belonging to different strains carrying the respective target gene. Strains used for in vivo standard curves are listed in Supplementary Table S1. Bacterial cells were harvested from overnight cultures in LB, washed and suspended in sterile 0.9% NaCl solution. Cell suspensions from each strain were set to the same optical density at 600 nm (OD$_{600}$) and then mixed together at equal proportions. The mixed suspensions were adjusted to an OD$_{600}$ of 0.125, corresponding to approximately $10^8$ CFU mL$^{-1}$, serially diluted and inoculated at $10^1$, $10^2$, $10^3$, $10^4$, $10^5$, $10^6$, $10^7$ and $10^8$ CFU g$^{-1}$ roots for the preparation of the standard curve. For each concentration and for the control without bacteria, three replicates were performed. The inoculated root samples used for standard curves were processed with the same method as the samples from pot experiments with the different soils (see following chapter). In vivo standard curves were prepared as described above for the phlD, phzF and prnD qPCR, using strains listed in Supplementary Table S1. Since all in vivo standard curves were prepared with bacterial cells recovered from wheat roots, the CT values can be directly converted to numbers of bacteria harboring phlD, phzF, or prnD per g root. Our qPCR data also directly reflect the abundance of the antimicrobial biosynthesis genes because phlD, phzF, or prnD are present in single copy in genomes of the P. fluorescens group (Flury et al., 2016). A survey of published bacterial genomes revealed that phzF and prnD are also found as a single copy in other bacterial species such as Burkholderia (phzF and prnD), Pectobacterium (phzF) or Serratia (prnD).
Table 2: Primers and probes used to quantify antimicrobial genes with qPCR.

<table>
<thead>
<tr>
<th>Metabolite, target gene</th>
<th>Primers and probes&lt;sup&gt;1&lt;/sup&gt;</th>
<th>Sequence (5'-3')</th>
<th>Annealing temperature (°C)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>DAPG&lt;sup&gt;2&lt;/sup&gt;, <em>phiID</em></td>
<td>PhilD_65F_DEG</td>
<td>GGT RTG GAA GAT GAA RAA RTC</td>
<td>50</td>
<td>This study; (Flury et al., 2017)</td>
</tr>
<tr>
<td></td>
<td>PhilD_153P_DEG</td>
<td>FAM-ATG GAG TTC ATS ACV GCY TCG TC-BHQ1</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>PhilD_236R_DEG</td>
<td>GCC YRA BAG YGA GCA YTA C</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>PhzF_2Fm</td>
<td>ACC GGC TGT ATC TGG AAA CC</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>PhzF_2Pm</td>
<td>FAM-GCC GCC AGC ATG GAC CAG CCG AT-BHQ1</td>
<td>62</td>
<td>This study</td>
</tr>
<tr>
<td></td>
<td>PhzF_2Rm</td>
<td>TGA TAG ATC TCG ATG GGA AAG GTC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phenazine, <em>phzF</em></td>
<td>PrnD_F</td>
<td>TGC ACT TCG CGT TCG AGA C</td>
<td>60</td>
<td>Garbeva et al. (2004)</td>
</tr>
<tr>
<td></td>
<td>PrnD_P</td>
<td>FAM-CGA CGG CCG TCT TGC GGA TC-BHQ1</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>PrnD_R</td>
<td>GTT GCG CGT CGT AGA AGT TCT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pyrrolnitrin, <em>prnD</em></td>
<td>CMV_1F</td>
<td>TCA TCA TTT CCA CTC CAG GCT C</td>
<td>62</td>
<td>Von Felten et al. (2010)</td>
</tr>
<tr>
<td>Internal control, APA9 plasmid&lt;sup&gt;3&lt;/sup&gt;</td>
<td>CMV_1R</td>
<td>TCA TCC CTC TGC TCA TAC GAC TG</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<sup>1</sup>TaqMan probes were labelled with fluorescein (FAM) at the 5' end and with the black hole quencher 1 (BHQ-1) at the 3' end.

<sup>2</sup>DAPG, 2,4-diacetylphloroglucinol.

<sup>3</sup>Plasmid from cassava mosaic virus.

### 2.3.4 qPCR-based quantification of antimicrobial genes on roots of wheat grown in field soils

To standardize the root material for qPCR quantification of DAPG, PHZ and PRN biosynthetic genes, soil samples from the 10 Swiss field sites were planted with spring wheat cv. Rubli in a greenhouse pot experiment. Plastic pots of 8 cm diameter and 30 cm height were part-filled with field soil and three wheat seedlings, prepared as described above, were planted per pot. Six pots per field soil were prepared. Wheat plants were grown for 2.5 months under the conditions described above for the Gt resistance assays. Root samples were collected, rinsed with tap water, incubated overnight in a sterile 0.9% NaCl solution at 3°C, and then vigorously agitated at 350 rpm for 30 min. Roots were separated from the root-wash suspensions and kept for
dry weight assessment. Root-wash suspensions were centrifuged at 3500 rpm for 20
min. The supernatant was discarded and aliquots of 0.5 ml of the resulting root-wash
pellet were used for DNA extraction. To each sample, $10^9$ copies of the APA9 plasmid
from a cassava mosaic virus were added as an internal standard (Von Felten et al.,
2010). DNA extraction was performed with the MPBio soil kit (MP Biomedicals,
Illkirch, France) following the protocol of the manufacturer. The concentration of
extracted DNA was measured with Qbit. qPCR reactions consisted of 10 mL TaqMan
Gene Expression Master Mix (Applied Biosystems, Foster City, CA, USA), 2 mL of the
respective forward and reverse primer solutions (10 mM), 2 mL of the respective
probe solution (2.5 mM), 0.5 mL of bovine serum albumin solution (20 mg mL$^{-1}$), and
2 mL of template DNA in a total reaction volume of 20 mL. Primer and probe sequences
are indicated in Table 2. Cycling conditions consisted of 2 min at 50°C (to permit
uracil-DNA glycosylase activity), an initial denaturation step of 10 min at 95°C, and 40 cycles
of 15 s at 95°C, 30 s at annealing temperature (see Table 2) and 30 s at 72°C. In all
samples, the added APA9 plasmid was quantified with the primers listed in Table 2,
following the method of Von Felten et al. (2010). The results from the APA9 plasmid
quantification were used to normalize DNA extraction.

To compare the abundance of $\textit{phlD}$ and $\textit{phzF}$ measured with qPCR with the abundance
measured using a cultivation-dependent terminal endpoint dilution assay, also called
most probable number PCR (MPN-PCR) method (Ramette et al., 2006), a greenhouse
experiment was carried out with soil samples from the Cazis and Taenikon field sites
and spring wheat cv. Rubli cultivated under the same conditions as described above.
After 3 weeks, $\textit{phzF}$ and $\textit{phlD}$ qPCR assays were performed on one fraction of each
harvested root-wash pellet as described above, while the other fractions were serially
diluted into microtiter plate wells (200 ml volume) containing King’s medium B (King
et al., 1954) broth amended with 100 mg L$^{-1}$ cycloheximide, 13 mg L$^{-1}$ chloramphenicol
and 40 mg L$^{-1}$ ampicillin. The microtiter plates were incubated at 24°C for 3 days and
MPN-PCR was performed as described by Ramette et al. (2006), except for adapting
annealing temperatures to the primers used in the present study (Table 2).

### 2.3.5 Quantification of resident $\textit{Pu}$ and $\textit{Gt}$ by qPCR

$\textit{Pythium ultimum}$ and $\textit{Gaeumannomyces tritici}$ populations naturally present on roots
of wheat were quantified using the qPCR methods developed by Cullen et al. (2007)
and Bithell et al. (2012), respectively. The reaction mix was prepared as described above for antimicrobial genes, and cycling conditions were set as described previously (Cullen et al., 2007; Bithell et al., 2012). In vitro standard curves were performed with genomic DNA of Pu isolate ETH-2 (concentration range from 0.1 ng to 200 ag per reaction) and of Gt isolate I-17 (10 ng to 10^-4 ng per reaction). Genomic DNA of the two pathogens was extracted with the DNeasy plant mini kit (Qiagen, Hombrechtikon, Switzerland) from lyophilized mycelia prepared from cultures grown in potato dextrose broth (Difco, Becton, Dickinson and Company, Franklin Lakes, USA) for 7 days at 24°C with agitation at 180 rpm.

2.3.6 Construction and culture of Pseudomonas reporter strains

Bacterial strains and plasmids used for generation of Pseudomonas reporter strains for monitoring antimicrobial gene expression are listed in Table 3. Pseudomonas and Escherichia coli strains were routinely cultured at 30 and 37°C, respectively, on nutrient agar plates, in LB and in nutrient yeast broth (Stanisich and Holloway, 1972). When appropriate, selective antibiotics were added to the media at the following concentrations: ampicillin, 100 mg mL^-1; chloramphenicol, 50 mg mL^-1; gentamicin, 10 mg mL^-1; kanamycin, 25 mg mL^-1; and tetracycline, 125 mg mL^-1. Genomic DNA from P. protegens strain CHA0 and P. chlororaphis strain PCL1391 was isolated as previously described (Schnider-Keel et al., 2000). Plasmids were extracted and purified using the QIAprep Spin Miniprep kit (Qiagen) or the JETStar Plasmid Purification Midi kit (Genomed, Basel, Switzerland). PCRs were done using the PrimeSTAR HS DNA polymerase kit (Takara Bio Inc., Shiga, Japan) as described elsewhere (Péchy-Tarr et al., 2005). All DNA digestion and ligation reactions were done using standard techniques (Sambrook and Russell, 2001; Schnider-Keel et al., 2001). DNA extractions from agarose gels were carried out with the QIAquick Gel Extraction kit (Qiagen). Transformations of electro-competent cells with plasmid or purified ligation products were performed by electroporation (Schnider-Keel et al., 2000). To amplify genomic DNA or to detect the presence of recombinant DNA in E. coli colonies by screening, 100–200 ng of DNA were amplified using the GoTaq DNA polymerase kit (Promega, Dübendorf, Switzerland). All PCR constructs intended for transformation were verified by sequence analysis. DNA sequencing was carried out
by GATC Biotech AG (Konstanz, Germany). Sequences were analyzed using the DNASTAR Lasergene software package version 11.0.

In order to tag \textit{P. protegens} CHA0 and \textit{P. chlororaphis} PCL1391 with green fluorescent protein (GFP), a single copy of a \textit{gfp} variant gene constitutively expressed from the \textit{P}_{\text{tac}} promoter, was inserted into the chromosome using the pBK-miniTn7-\textit{gfp2} delivery plasmid (Koch et al., 2001) and the Tn7 transposition helper plasmid pUX-BF13 (Bao et al., 1991) as described previously (Pechy-Tarr et al., 2013). For use as reporters to monitor the expression of HCN, DAPG, PRN, and PHZ biosynthetic genes, strain CHA0-\textit{gfp} was transformed with pME9011 (\textit{hcnA-mcherry}), pME9012 (\textit{phlA-mcherry}) or pME11011 (\textit{prnA-mcherry}), and strain PCL1391-\textit{gfp} with pME11017 (\textit{phzA-mcherry}) (Table 3). To construct the \textit{prnA-mcherry} reporter plasmid pME11011, a 629-bp fragment containing the CHA0 \textit{prnA} promoter was amplified from pME7116 (Baehler et al., 2005) using primers P5 BamHI new and P6 (Table 3). The obtained fragment was digested with BamHI and EcoRI and ligated into the \textit{mcherry}-based promoter-probe vector pME9010 (Rochat et al., 2010) opened with the same enzymes. Similarly, for the construction of the \textit{phzA-mcherry} reporter plasmid pME11017, a 961-bp fragment containing the \textit{phzA} promoter (Chin-A-Woeng et al., 2001) was amplified from genomic DNA of PCL1391 (Flury et al., 2016) using primers phzAF and phzAR (Table 3). The PCR product was digested with BamHI-SacI and the resulting fragment was first cloned into pUK21 and, from there, into pME9010, both opened with the same restriction enzymes.

\subsection*{2.3.7 Assay to monitor antimicrobial gene expression in field soils}

Antimicrobial gene expression and colonization levels of GFP-marked \textit{P. protegens} CHA0 and \textit{P. chlororaphis} PCL1391 harboring mCherry-based reporter plasmids were monitored on roots of spring wheat grown in the soils sampled at the 10 different Swiss field sites. Untreated seeds of spring wheat cv. Rubli were surface-sterilized for 12 min in 4\% NaClO (vol/vol) and then washed with sterile distilled water. Seeds were germinated on soft agar (Agar Agar Serva at 9 g L^{-1}; Serva, Heidelberg, Germany) for 48 h at room temperature in the dark. The wheat seedlings were then transferred to 200-mL Erlenmeyer flasks (5-cm opening; Simax, Czech Republic) containing 60 g of soil. In each flask, three seedlings were placed into the soil and inoculated with 1 mL of a suspension of washed cells of the respective \textit{Pseudomonas} reporter strain adjusted
within a range from $3.6 \times 10^7$ to $7.8 \times 10^7$ cells mL$^{-1}$. Washed cells were prepared from LB cultures grown without antibiotic addition under the conditions described above to an OD$_{600}$ of 0.8 to 1.5, depending on the reporter strain used, whereby choosing a growth stage at which no significant expression of the antimicrobial genes occurred. This was done by determining, with a fluorimetry assay (Baehler et al., 2005), the time point at which the relative red fluorescence emitted by the *Pseudomonas* strains carrying mCherry-based reporter plasmids was not yet significantly different from the red background fluorescence emitted by control strains carrying the empty vector pME9010. Wild-type and GFP-tagged *P. protegens* CHA0 and *P. chlororaphis* PCL1391 (with and without pME9010) were included as control treatments for properly setting green and red fluorescence backgrounds for the FACS-based flow cytometry analysis described below. Flasks were sealed with cotton wool plugs and incubated in a growth chamber set to 60% relative humidity for 16 h with light (176 mE m$^{-2}$ s$^{-1}$) at 25°C, followed by an 8-h dark period at 20°C. After 5 days of incubation, wheat roots from each flask were removed, washed using distilled water to remove loosely adhering soil particles from roots and pooled in 10 mL of autoclaved, ultrapure water contained in a sterile 50-mL Falcon tube. Tubes were agitated for 20 min at 300 rpm in order to remove the majority of bacteria from the roots. The resulting suspensions were filtered using a 5.0-mm sterile syringe single-use filter (Sartorius Stedim Biotech GmbH, Goettingen, Germany), transferred on ice and immediately analyzed by FACS as described below. Dry weights of wheat roots were recorded and the number of GFP-marked *Pseudomonas* cells present in the root washes were determined by FACS and recorded as cells g$^{-1}$ of root.
Table 3: Bacterial strains, plasmids and primers used for construction of reporter strains.

<table>
<thead>
<tr>
<th>Strain, plasmid, or oligonucleotide</th>
<th>Relevant characteristics(^1) or sequences (5’→ 3’)</th>
<th>Reference or source</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Pseudomonas protegens</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CHA0</td>
<td>Wild type; biocontrol agent; DAPG(^{+}), PRN(^{+}), HCN(^{+})</td>
<td>Stutz et al. (1986)</td>
</tr>
<tr>
<td>CHA0-gfp</td>
<td>CHA0::attTn7-gfp; Gm(^{r})</td>
<td>Péchy-Tarr et al. (2013)</td>
</tr>
<tr>
<td><em>Pseudomonas chlororaphis</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PCL1391</td>
<td>Wild type; biocontrol agent; HCN(^{+}) PHZ(^{+})</td>
<td>Chin-A-Woeng et al. (1998)</td>
</tr>
<tr>
<td>PCL1391-gfp</td>
<td>PCL1391::attTn7-gfp; Gm(^{r})</td>
<td>This study</td>
</tr>
<tr>
<td><em>Escherichia coli</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DH5(\alpha)</td>
<td>Laboratory strain</td>
<td>Sambrook and Russel (2001)</td>
</tr>
<tr>
<td><strong>Plasmids</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pBK-miniTn7-gfp(^{2})</td>
<td>pUC19-based delivery plasmid for miniTn7-gfp(^{2}); mob(^{+}) Gm(^{r})</td>
<td>(Koch et al., 2001)</td>
</tr>
<tr>
<td>pME7116</td>
<td>prnA-gfp transcriptional fusion; reporter of PRN biosynthetic gene expression in CHA0; Tc(^{r})</td>
<td>Baehler et al. (2005)</td>
</tr>
<tr>
<td>pME9010</td>
<td>mCherry-based promoter-probe vector derived from pPROBE'-gfp (AAV); Km(^{r})</td>
<td>Rochat et al. (2010)</td>
</tr>
<tr>
<td>pME9011</td>
<td>hcnA-mcherry transcriptional fusion; reporter of HCN biosynthetic gene expression in CHA0; Km(^{r})</td>
<td>Rochat et al. (2010)</td>
</tr>
<tr>
<td>pME9012</td>
<td>phlA-mcherry transcriptional fusion; reporter of DAPG biosynthetic gene expression in CHA0; Km(^{r})</td>
<td>Rochat et al. (2010)</td>
</tr>
<tr>
<td>pME11011</td>
<td>prnA-mcherry transcriptional fusion; reporter of PRN biosynthetic gene expression in CHA0; Km(^{r})</td>
<td>This study</td>
</tr>
<tr>
<td>pME11017</td>
<td>phzA-mcherry transcriptional fusion; reporter of PHZ biosynthetic gene expression in PCL1391; Km(^{r})</td>
<td>This study</td>
</tr>
<tr>
<td>pUK21</td>
<td>Cloning vector; Km(^{r})</td>
<td>Vieira and Messing (1991)</td>
</tr>
<tr>
<td>pUX-BF13</td>
<td>Helper plasmid encoding Tn7 transposition functions; R6K-replicon; Ap(^{r})</td>
<td>Bao et al. (1991)</td>
</tr>
<tr>
<td><strong>Oligonucleotides(^{2})</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>P5 BamHI new</td>
<td>CCGGATCCCCGGCTCAAGGACAGTTGGTTC, BamHI</td>
<td>This study</td>
</tr>
<tr>
<td>P6</td>
<td>GGAAATCCCGAGGTAACGGAAGCCGCCATC, EcoRI</td>
<td>Baehler et al. (2005)</td>
</tr>
<tr>
<td>phzAF</td>
<td>CGGGATCCCTAATCCATTTTGAAGCACC, BamHI</td>
<td>This study</td>
</tr>
<tr>
<td>phzAR</td>
<td>CGAGCTCGCTCAATCTCCATATGAATAAGGGGGGCT, SacI</td>
<td>This study</td>
</tr>
</tbody>
</table>

\(^{1}\) Abbreviations: Ap\(^{r}\), ampicillin; Cm\(^{r}\), chloramphenicol; Gm\(^{r}\), gentamicin; Km\(^{r}\), kanamycin; and Tc\(^{r}\), tetracycline resistance, respectively.

\(^{2}\) Restriction sites are underlined.
2.3.8 FACS analysis

Green fluorescent protein and mCherry expression levels in *Pseudomonas* reporter cells in natural soils were quantified with a Becton–Dickinson LSRFortessa flow cytometer. Size and granularity of *Pseudomonas* cells and particles were determined by measuring the forward scatter (FSC-A) and side scatter (SSC-A) signals, respectively. FSC-A signals were collected with a photodiode detector (set to 350 V), in the range of 483 to 493 nm (488/10 BP filter), with a threshold set to 200. SSC-A signals were detected with a photomultiplier tube (PMT) (detector G, set to 300 V) in the range of 483 to 493 nm (488/10 BP filter). Green fluorescence signals were collected with the PMT detector E (set at the voltage of 676 V), in the range of 515 to 545 nm (530/30 BP filter, 505 LP mirror). Red fluorescence signals were detected using the PMT detector C (set to 700 V), between 600 and 620 nm (610/20 BP filter, 600 LP mirror). For FACS analysis, aliquots of 300 mL of filtered root-washes were placed into Nunc MaxiSorp flat-bottom 96-well plates (Sigma–Aldrich, Buchs, Switzerland), and each sample was mixed three times. The analyzed volume was standardized to 200 mL, allowing the detection of 500–10,000 GFP events, depending on the analyzed soil. Gating of GFP-marked bacteria was done by delimiting on the FSC-A/FITC-A density plot particles with green fluorescence values above the background fluorescence noise (i.e., autofluorescence emitted by root and soil particles, cell fragments or bacterial cells not expressing GFP). Control samples obtained from soils amended with pure water, wild-type *P. protegens* CHA0 and *P. chlororaphis* PCL1391, and GFP-tagged strains CHA0-*gfp* and PCL1391-*gfp* were used to identify the GFP background fluorescence in soil extraction samples. The red fluorescence emitted by the gated GFP-tagged *Pseudomonas* cells was then analyzed on the PE-Texas Red-A histogram and on the FSC-A/PE-Texas Red-A density plot, allowing to detect and analyze all GFP-marked cells actively expressing their mCherry-based reporter fusion. Control samples including CHA0-*gfp* and PCL1391-*gfp* without mCherry-based vector, or carrying the empty pME9010 vector were used to define the mCherry background fluorescence among the GFP-marked *Pseudomonas* population. The median of red fluorescence emitted by the *Pseudomonas* cells was calculated using the BD FACSDiva software version 8.0 (Becton–Dickinson). To determine root colonization levels of *Pseudomonas* reporter strains, the GFP tag was used to count by
FACS the number of reporter cells present in the analyzed 200 mL of filtered root-wash and then to calculate their concentration per gram of dry roots.

2.3.9 Data analysis

Statistical data analysis was carried out with the open source software R version 3.2.3 (RCoreTeam, 2015). Shoot fresh weights of cucumber and wheat plants obtained from the Pu and Gt infection assays, respectively, were analyzed as a proxy for soil disease resistance. Shoot weights from samples where pathogen inoculum was added were normalized against the shoot weights from non-infested control plants grown in the same soil, to minimize variation due to different nutrient contents in the different soils. Data were checked for normal distribution with the Shapiro–Wilk test and by plotting QQ-Plots. Equality of variance was verified with Bartlett’s test. Analysis of variance was carried out with a non-parametric test (Kruskal–Wallis test, significance level \( p < 0.05 \)), followed by a post hoc test (kruskalmc, R package ‘Pgirmess’).

The abundance of \( \text{phlD, phzF, and prnD} \) harboring bacterial cells on wheat roots was calculated with the \textit{in vivo} standard curves described above. Efficiencies and detection limits of qPCR assays determined by \textit{in vivo} standard curves are indicated in Supplementary Table S2. Cycle threshold values obtained from the \textit{in vivo} standard curves and from the samples were normalized for differences in DNA extractions as described by (Von Felten et al., 2010). Normalized values were used for further analysis. The average values obtained from the three technical replicates of each qPCR assay were used for statistical analysis, which was performed as described above for shoot weights.

Data on the expression of reporters of antimicrobial genes in the different soils represent the medians of three independent repetitions of the same experiment, with nine replicates per treatment in each experiment. Significant differences between treatments were calculated with a non-parametric Kruskal–Wallis test (significance level \( p < 0.05 \)), followed by Dunn’s test for post hoc comparisons.

Data used for the heat map showing rankings of pathogen suppression, abundance of antimicrobial genes, and expression of antimicrobial genes by reporter strains in the different soils were normalized using the function ‘scale’ (R package ‘stats’). Correlations between pathogen suppression, gene abundance, gene expression and abiotic soil parameters were inferred with Spearman’s rho rank correlation...
(significance level \( p < 0.05 \)). Data were displayed in a heat map with the functions ‘levelplot’ (R package ‘lattice’) or ‘corrplot’ (R package ‘corrplot’).

2.4. Results

2.4.1 Resistance of Swiss agricultural soils to soilborne pathogens

The general resistance of 10 Swiss agricultural soils (Figure 1 and Table 1) to soilborne pathogens was tested in a greenhouse assay in which increasing quantities of Gt or Pu inoculum were added to soil samples planted with spring wheat or cucumber, respectively. Resistance to both pathogens varied between soils. In the Gt-infested soils at 0.6 g inoculum per pot, shoot fresh weights of wheat plants ranged from 31% in Grangeneuve soil to 107% in Taenikon soil of the weights of control plants grown in not artificially infested soils (Figure 2A). The shoot fresh weights of wheat grown in infested Taenikon soil were significantly higher (1.3–3.5-fold) than those of plants grown in infested soils from Cazis, Eschikon, Grangeneuve, Vouvry, and Witzwil. Similar trends were observed at other inoculum quantities (Supplementary Figure S1). Plants grown in Taenikon soil had the highest shoot fresh weights at all Gt concentrations used except the highest (6 g of inoculum per pot), at which the fungal pathogen heavily affected plant growth in all the soils and reduced shoot weights by 60–90% (Supplementary Figure S1D).

When the soils were artificially infested with Pu inoculum at 0.125 g per pot, median shoot fresh weights of cucumber plants ranged from 0% in Vouvry soil to 106% in Cazis soil of those of control plants grown in non-infested soils (Figure 2B). The shoot fresh weights of cucumber grown in Pu-infested Cazis soil were significantly higher (three–fivefold) than those of plants grown in infested soils from Cadenazzo, Courtedoux, Delley, Taenikon, and Vouvry (Figure 2B) and similar trends were observed for other inoculum densities (Supplementary Figure S2). Plants grown in soil from the Cazis field site had the highest shoot weights at all Pu concentrations, while plants grown in soil from the Vouvry field had the lowest shoot weights at all levels of the pathogenic oomycete except at 0.25 g per pot.
Individual soils did not display equal resistance levels to the two soilborne pathogens. While the soils from the Taenikon and Delley field sites were the most resistant against Gt, they were among the least resistant against Pu (Figure 2 and Supplementary Figures S1, S2). Likewise, the soil from Cazis was the most resistant against Pu, but was only moderately resistant against Gt.

To account for potential effects of resident Gt and Pu populations on the outcome of the soil resistance experiments, a qPCR method targeting the ITS rRNA gene region was used to detect and quantify the two pathogens in the rhizoplane of spring wheat plants grown in the not artificially infested control treatments of all soils. Gt could not
be detected in any of the samples. By contrast, Pu was detected on the plant roots in all ten soils, but there was no significant difference in the abundance of the oomycete pathogen among the individual soils (Supplementary Figure S3).

In summary, the 10 agricultural soils strongly varied in their resistance against soilborne pathogens. However, the soil resistance levels observed for the two investigated pathogens in general were different; i.e., some soils displaying high resistance to Pu were highly susceptible to Gt and vice versa, pointing to specificity in the buffering capacity of individual soils toward specific soilborne pathogens.

2.4.2 Abundance of phlD+ pseudomonads, phzF+ pseudomonads, and prnD+ bacteria on roots of wheat grown in Swiss agricultural soils

The abundance of bacterial cells harboring phlD, phzF, and prnD required for the biosynthesis of the antimicrobials DAPG, PHZ, and PRN, respectively, was quantified by qPCR on roots of spring wheat grown in the 10 Swiss agricultural soils. As detailed in section “Materials and Methods,” we assume that phlD+ and phzF+ cells quantified in our assays correspond to cell numbers of DAPG and PHZ producing pseudomonads, whereas prnD+ cells correspond to cell numbers of PRN producing bacteria. Since the investigated genes are present as one copy per bacterial cell, we also refer to the abundance of cells harboring an antimicrobial gene as gene abundance.

The abundance of the phlD+ pseudomonads in all studied soils in general was higher than the abundance of the phzF+ and prnD+ bacteria, and in some soils reached 10^7 cells per gram of root dry weight, whereas the abundance of the latter remained below 10^6 gene copies per gram of root dry weight (Figure 3). For individual genes, pronounced differences in abundance between soils were observed. The biggest differences were found for phlD+ cells with approximately 10^4-fold higher numbers on roots of wheat grown in soil from Taenikon compared to those grown in soil from Vouvry (Figure 3A). The abundance of phlD+ pseudomonads was significantly higher in soils from Courtedoux, Delley, Eschikon, Grangeneuve, Taenikon, Utzenstorf, and Witzwil than in soils from Cadenazzo, Cazis, and Vouvry. The abundances of phzF+ pseudomonads and prnD+ bacteria varied at maximum 100-fold between the different soils. The number of pseudomonads harboring the phzF gene was significantly higher on roots samples from Courtedoux and Delley soils compared to samples extracted from Vouvry soil (Figure 3B). The abundance of prnD+ bacteria was significantly higher
in soil samples from Cadenazzo, compared to those from Courtedoux, Cazis, and Vouvry (Figure 3C). Taken together, for all three investigated antimicrobial genes pronounced differences in the abundances were found between the individual agricultural soils, indicating that the different soils may sustain to different extents specific populations of pseudomonads producing DAPG, PRN, and/or PHZ.

**Figure 3**: Abundance of bacterial cells harboring genes required for the biosynthesis of the antimicrobial compounds (A) 2,4-diacetylphloroglucinol (phlD), (B) phenazine (phzF) and (C) pyrrolnitrin (prnD) on roots of spring wheat in 10 Swiss agricultural soils. Cells harboring the antimicrobial genes were quantified by qPCR. The dotted line indicates 10⁵ cells per gram of root dry weight. For each soil, six replicates were used. Letters indicate significant differences (Kruskal-Wallis test, p<0.05). Sampling sites: Cd, Cadenazzo; Cx, Courtedoux; Cz, Cazis; De, Delley; Es, Eschikon; Gr, Grangeneuve; Ta, Taenikon; Ut, Utzenstorf; Vo, Vouvry; Wi, Witzwil.

### 2.4.3 Expression of antimicrobial genes in Swiss agricultural soils

The relative capacity of the 10 different Swiss agricultural soils to sustain the expression of biosynthetic genes for the antimicrobial compounds DAPG, HCN, PRN, and PHZ on roots was followed using GFP-tagged *Pseudomonas* strains carrying
mCherry-based reporter plasmids inoculated into soil microcosms planted with spring wheat. *P. protegens* CHA0-gfp carrying plasmid pME9012 (*phlA*-mcherry), pME9011 (*hcnA*-mcherry), or pME11011 (*prnA*-mcherry), or *P. chlororaphis* PCL1391-gfp carrying pME11017 (*phzA*-mcherry) served as reporter strains. GFP fluorescence (identifying the tagged reporter strains) and relative mCherry fluorescence intensities (reporting expression levels of respective antimicrobial genes) of cells in root washes extracted from the different soils were recorded with FACS-based flow cytometry. Data are presented as total antimicrobial gene expression by all cells of the respective reporter strain per gram dry weight of roots, and they were calculated by multiplying the median gene expression per cell with the total number of reporter cells per gram dry weight of roots (Supplementary Table S3). The levels of total expression of all investigated antimicrobial genes varied significantly among the 10 field soils, with the highest variations observed for DAPG and HCN biosynthetic genes (Figure 4). The soil from Cadenazzo supported the highest levels of total *phlA* expression on the roots, which were approximately 16-fold higher than those measured on roots growing in soil from Taenikon, which yielded the lowest expression levels (Figure 4A). The Cadenazzo soil also supported highest levels of total *hcnA* expression among all 10 soils, and these levels were approximately 20-fold higher than *hcnA* expression levels recorded in the soil from the Grangeneuve field site, which was the least favorable to the expression of this antimicrobial gene (Figure 4B). Levels of total expression of PRN and PHZ biosynthetic genes appeared to be less variable among the 10 soils. In particular, *prnA* expression levels were eightfold higher in the soil from Utzenstorf that supported the highest expression compared to the soil from Eschikon that supported the lowest expression (Figure 4C). Levels of overall *phzA* expression varied only about fivefold between the most contrasting soils from the Cadenazzo and Taenikon field sites, respectively (Figure 4D). Several individual soils sustained total expression levels for all investigated antimicrobial genes to a similar extent. This was evident notably for soils from Cadenazzo and Vouvry in which all four antimicrobial genes attained high total expression levels, while soils from Eschikon, Grangeneuve and Taenikon supported significantly lower overall expression levels of these genes (Figure 4).
Figure 4: Relative expression of genes required for the biosynthesis of the antimicrobial compounds (A) 2,4-diacetylphloroglucinol (*phlA*), (B) hydrogen cyanide (*hcnA*), (C) pyrrolnitrin (*prnA*), and (D) phenazines (*phzA*) on roots of spring wheat in 10 Swiss agricultural soils. Expression was monitored by fluorescence-activated cell-sorting-based flow cytometry using GFP-tagged strains of *Pseudomonas protegens* (CHA0-*gfp*) carrying reporter plasmids pME9012 (*phlA-mcherry*), pME9011 (*hcnA-mcherry*), or pME11011 (*prnA-mcherry*) or of *Pseudomonas chlororaphis* (PCL1391-*gfp*) carrying reporter plasmid pME11017 (*phzA-mcherry*). Seedlings inoculated with the reporter strains were grown in soil microcosms for five days prior to analysis of bacterial cells in root washes. Data are shown as relative fluorescence units (RFU) per gram of root dry weight, and were calculated as the median mCherry expression per GFP-tagged *Pseudomonas* cell multiplied with the total number of GFP-tagged *Pseudomonas* cells per gram of root. Results from three independent experiments with nine replicates each are presented. Since Kruskal-Wallis analyses did not reveal significant experiment x treatment interactions, data of the three experiments were pooled for statistical analysis. Letters indicate significant differences (Dunn test, p<0.05). Sampling sites: Cd, Cadenazzo; Cx, Courtedoux; Cz, Cazis; De, Delley; Es, Eschikon; Gr, Grangeneuve; Ta, Taenikon; Ut, Utzenstorf; Vo, Vouvry; Wi, Witzwil.
The Cadenazzo and Vouvry soils appeared to favor also highest levels of root colonization \((1.9 \times 10^7\) to \(2.4 \times 10^7\) cells g\(^{-1}\) of dry root weight) by \(P.\ protegens\) and \(P.\ chlororaphis\) among the 10 soils tested, while the soils from Eschikon, Grangeneuve, and Taenikon were among those supporting lower levels of root colonization \((2.9 \times 10^6\) to \(9.7 \times 10^6\) cells g\(^{-1}\) of dry root weight) (Figure 5 and Supplementary Table S3). However, whereas the soil from Cadenazzo was indeed also the field soil supporting highest \(phlA\), \(hcnA\), \(prnA\), and \(phzA\) expression levels in individual reporter cells, the median single cell expression of these genes was significantly lower in the soil from Vouvry (Supplementary Table S3). For the Vouvry soil, it thus seems that the high total expression in the reporter population on the roots was mainly due to the higher colonization levels attained in this soil. Likewise, the relatively high single cell expression but low colonization levels in the Eschikon and Taenikon soils (Figure 5 and Supplementary Table S3) resulted in the significantly lower overall expression as compared to the Cadenazzo and Vouvry soils (Figure 5). By contrast, in the soil from Grangeneuve, single cell expression levels of antimicrobial genes as well as colonization levels were consistently relatively low (Supplementary Table S3). Similar overall gene expression levels may therefore reflect contrasting levels of single cell gene expression and colonization in individual soils.

In summary, the expression of the investigated antimicrobial genes strongly varied between the studied agricultural soils. Some soils seem to favor higher levels of overall antimicrobial gene expression whereas others apparently support the expression of these genes much less. The findings suggest that the expression of different antimicrobial genes may be induced by the same soil factors (or similar combinations thereof).

**2.4.4 Relationships between pathogen resistance and abundance and expression of antimicrobial genes in Swiss agricultural soils**

Results obtained from experiments on pathogen resistance of soils, abundance and expression of antimicrobial genes were displayed in a gradient map (Figure 6). Soils were grouped in three clusters. The first cluster consisted of soils supporting high abundances but low expression levels of antimicrobial genes (i.e., soils from Eschikon and Taenikon field sites). The second cluster consisted of soils supporting high
antimicrobial gene expression levels but low abundances of bacteria harboring antimicrobial genes (i.e., soils from Cadenazzo and Vouvry field sites). The third cluster consisted of soils ranging in between the two other clusters. Two soils displayed high resistance against soilborne plant pathogens, i.e., the soil from Taenikon against Gt and the soil from Cazis against Pu. Although the soil from Taenikon was the one supporting the highest abundance of pseudomonads harboring the DAPG biosynthetic gene \textit{phlD}, resistance to the soilborne pathogens did not generally cluster with high abundances or expression levels of antimicrobial genes (Figure 6).

Correlation analysis revealed that the expression of the four studied antimicrobial genes was positively correlated (Figure 7B). A similar trend could be observed for the
abundance of the antimicrobial genes (Figure 7A). Resistance to soilborne pathogens was not significantly correlated to the abundance of antimicrobial genes, although a weak positive correlation could be observed between the abundance of bacteria harboring individual antimicrobial genes and resistance to pathogens (Figure 7A). These results suggest that the expression of the investigated antimicrobial genes is similarly influenced by biotic and abiotic factors prevailing in the respective soils. The same could be true for the abundance of bacteria harboring these antimicrobial genes. Our results further indicate that the abundance of such bacteria probably plays only a limited role in the resistance of the investigated cereal crop-oriented agricultural soils to the soilborne pathogens Pu and Gt.

Figure 6: Heatmap showing normalized-values of disease resistance, antimicrobial gene abundance and antimicrobial gene expression measured in 10 representative Swiss agricultural soils with a cereal-oriented cropping history. The color scale depicts highest (fuchsia) via intermediate (white) to lowest (blue) values for each variable. Sampling sites: Cd, Cadenazzo; Cx, Courtedoux; Cz, Cazis; De, Delley; Es, Eschikon; Gr, Grangeneuve; Ta, Taenikon; Ut, Utzenstorf; Vo, Vouvry; Wi, Witzwil.
2.4.5 Relationships between soil parameters, pathogen resistance, abundance and expression of antimicrobial genes

The physical and chemical properties of the 10 Swiss agricultural soils investigated in this study were analyzed (Figure 1 and Table 1) and correlated with their resistance to pathogens, the abundance of antimicrobial genes and the expression of antimicrobial genes (Figure 8). Macronutrients were extracted from soils with the water or ammonium acetate-EDTA (AA-EDTA) soil extraction procedures routinely used in Swiss agriculture to account for, respectively, soluble, i.e., readily plant-available macronutrients and bound, reserve macronutrients that become available to plants at mid or long term (Agroscope, 2006; Stünzi, 2007). Plant micronutrients were extracted with AA-EDTA.

![Heatmap showing Spearman’s rank correlations for disease resistance and abundance of antimicrobial genes (A) and for expression levels of antimicrobial genes (B) in 10 representative Swiss agricultural soils with a cereal-oriented cropping history.](image)

Organic carbon and clay, and silt and sand inversely influenced antimicrobial gene abundance and expression. Abundance of DAPG biosynthesis genes (recorded for \textit{phlD}) was significantly positively correlated with clay and significantly negatively correlated with silt, while expression of these genes (recorded for \textit{phlA}) was significantly negatively correlated with organic carbon and clay and significantly positively correlated with silt (Figure 8). Similar trends were also observed for the pyrrolnitrin and phenazine biosynthetic genes \textit{prnA} and \textit{phzF}, respectively. No clear positive or negative correlation was found between pH and antimicrobial gene

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**Figure 7:** Heatmap showing Spearman’s rank correlations for disease resistance and abundance of antimicrobial genes (A) and for expression levels of antimicrobial genes (B) in 10 representative Swiss agricultural soils with a cereal-oriented cropping history. The abundance of antimicrobial genes is defined as the number of bacterial cells harboring the indicated gene. Significant correlations (p<0.05) are highlighted with asterisks. The color scale to the right of the matrix indicates rho correlation coefficients.
abundance or expression (Figure 8). Nitrate concentration in soil was positively correlated with the abundance of bacteria harboring the investigated antimicrobial genes; in particular, it was significantly positively correlated with the abundance of *phlD*<sup>+</sup> pseudomonads (Figure 8). Antimicrobial gene expression was neither clearly positively nor clearly negatively correlated with nitrate. Of the other macronutrients, potassium inversely influenced abundance and expression of antimicrobial genes. Reserve potassium extracted with AA-EDTA was significantly positively correlated with the abundance of *phlD*<sup>+</sup> and *phzF*<sup>+</sup> pseudomonads and significantly negatively with *phlA* and *phzA* expression (Figure 8). A similar trend was also observed for water-extracted, i.e., readily plant available magnesium. Among the measured micronutrients, a significant effect was only observed for manganese, which was significantly negatively correlated with *phzA* and *hcnA* expression (Figure 8).

Resistance to soilborne pathogens was not significantly correlated to any soil factor in the case of Gt, and significantly positively correlated to nitrate and magnesium extracted with water in the case of Pu (Figure 8). The resistance to both pathogens was positively, but not significantly correlated to organic carbon, clay, potassium, manganese, and zinc.

Taken together, these analyses indicate that soil physical and chemical properties have contrasting and subtle effects on the abundance and expression of antimicrobial genes. No pronounced correlations between soil properties and general disease resistance of soils could be observed for the 10 agricultural field sites investigated. Remarkably, all soil factors that were positively correlated with the abundance of *phlD*<sup>+</sup> and *phzF*<sup>+</sup> pseudomonads were also positively correlated with Pu resistance.

### 4.5. Discussion

Resistance to soilborne diseases and beneficial microbial populations involved have been studied extensively in soils specifically suppressive to one particular pathogen species (Weller et al., 2002; Mendes et al., 2011; Raaijmakers and Mazzola, 2016). However, virtually nothing is known about interactions between soilborne pathogens and beneficial microorganisms in common agricultural soils, i.e., in soils that lack specific disease suppressiveness. Similarly, it is not clear whether a particular soil may simultaneously exhibit suppressiveness toward multiple soilborne pathogens. To our best knowledge, the present study is the first to compare side-by-side a range of
common agricultural soils for their resistance toward two soilborne pathogens, *P. ultimum* (Pu) and *G. tritici* (Gt), as well as for the abundance and expression of biosynthetic genes required for the production of antimicrobial compounds by plant-beneficial pseudomonads.

![Heatmap showing Spearman's rank correlations between soil parameters, disease resistance, and abundance and expression of antimicrobial genes in 10 representative Swiss agricultural soils with a cereal-oriented cropping history.](image)

Figure 8: Heatmap showing Spearman's rank correlations between soil parameters, disease resistance, and abundance and expression of antimicrobial genes in 10 representative Swiss agricultural soils with a cereal-oriented cropping history. Significant correlations (p<0.05) are highlighted with asterisks. The color scale to the right of the matrix indicates rho correlation coefficients.

First, we investigated the capacity of different Swiss agricultural soils to buffer the attack of two soilborne pathogens by testing the growth of crop plants in these soils after amendment with increasing quantities of pathogens up to very high concentrations. Individual soils differed markedly in their respective resistance to the two pathogens. Several soils that showed comparatively high suppressiveness toward Pu were poorly or only moderately resistant to Gt and vice versa. These results indicate that conventional agricultural soils do not necessarily exhibit a general level of resistance toward a range of soilborne pathogens, but rather display variable resistance levels toward specific pathogens, which are likely modulated by different microbial and abiotic soil factors. Nevertheless, soils suppressive to more than one pathogen have been reported, notably the *Fusarium* wilt of pea suppressive soils in Mt. Vernon, WA, USA (Landa et al., 2002; Weller, 2007), which are also suppressive to
take-all of wheat (Allende Molar (2006); Allende-Molar and Weller, personal communication).

Soil resistance to soilborne pathogens has often been linked to the abundance of pseudomonads producing antimicrobial compounds (Stutz et al., 1986; Raaijmakers et al., 1997; Weller et al., 2002; Haas and Defago, 2005; Weller, 2007; Raaijmakers et al., 2008; Mazurier et al., 2009; Almario et al., 2014). For this reason, we have used a qPCR approach targeting biosynthetic genes for DAPG, PHZ, and PRN in bacterial cells present on roots of wheat grown in the agricultural soils that we tested for disease resistance. While the qPCR assays used in this study to quantify \( \text{phlD}^+ \) and \( \text{phzF}^+ \) bacteria are specific for fluorescent pseudomonads (see Supplementary Table S1), the assay used to quantify \( \text{prnD}^+ \) cells additionally detects \textit{Burkholderia} and \textit{Serratia} (Garbeva et al., 2004). However, 16S rRNA amplicon sequencing performed on root samples from wheat grown in the same soils (Chapter 5) showed that the relative abundance of \textit{Pseudomonas} (4.4–25.2%) was markedly higher than the relative abundances of \textit{Burkholderia} (0.003–0.53%) and \textit{Serratia} (0.003–1.21%). Therefore, we assume that the \( \text{prnD} \) genes detected in the present study predominantly derive from pseudomonads. Most studies that quantified pseudomonads harboring antimicrobial genes so far were carried out using cultivation-dependent approaches, e.g., colony plating/colony hybridization assays or endpoint dilution assays followed by PCR (Raaijmakers et al., 1997; Meyer et al., 2010; Mavrodi et al., 2012a; Mavrodi et al., 2012b). An examination of genome sequences published by Flury et al. (2016) and other investigators indicated that fluorescent pseudomonads harbor only one copy of \( \text{phlD} \), \( \text{phzF} \), or \( \text{prnD} \) per cell. Accordingly, we did not find any significant difference between the abundances of \( \text{phlD} \) and \( \text{phzF} \) quantified by qPCR or a cultivation-dependent endpoint dilution assay in samples from Taenikon (Supplementary Figure S4). Still, qPCR assays can potentially detect viable but non-culturale or even dead cells, thus caution is required when comparing our findings with results of cultivation-dependent experiments.

Studies simultaneously investigating the abundance of several antimicrobial genes in soil are rare. Raaijmakers et al. (1997) used a colony-hybridization assay to quantify pseudomonads harboring DAPG or PHZ genes in different take-all suppressive and conducive US soils. They found pseudomonads harboring DAPG genes to be enriched in suppressive soils, but much less abundant or below the detection limit in conducive
soils and they did not detect pseudomonads harboring PHZ genes in any of the tested soils. In our study, we found \textit{phzF}\textsuperscript{+} pseudomonads to be present in all investigated agricultural soils. However, in general their abundance was quite low, ranging from 2.5 to 5.3 log cells/g root, compared to another US study on soils of the Columbia Plateau of the Pacific Northwest, in which the abundance of PHZ-producing pseudomonads detected by endpoint-dilution assays coupled with \textit{phzF}-specific PCR was up to 100-fold higher in certain soils (Mavrodi et al., 2012a; Mavrodi et al., 2012b). It has been suggested that PHZ producing pseudomonads are more abundant in dryland fields without irrigation, where yearly rainfall ranges from 150 to 300 mm, compared to irrigated fields (Mavrodi et al., 2012b). At the sampling sites of the present study, average annual rainfall was high, ranging from 610 mm per year to 1780 mm per year, which could be a possible reason for the rather low abundance of \textit{phzF}\textsuperscript{+} pseudomonads detected here.

The abundance of DAPG producing pseudomonads in the investigated soils varied strongly, from 2.2 to 8.0 log cells/g root. We detected higher but also lower numbers compared to previous studies, which may be explained by the very different types of soils we investigated (Table 1). Two studies using terminal endpoint dilution assays followed by \textit{phlD}-specific PCR, one by Meyer et al. (2010) on two Swiss agricultural soils and one by Mavrodi et al. (2012b) on irrigated fields of the Pacific Northwest, detected \textit{phlD}\textsuperscript{+} pseudomonads at levels ranging from 4.5 to 6.5 log CFU/g on roots of wheat. The population levels of pseudomonads on roots of wheat grown in the Delley soil reported by Meyer et al. (2010) were similar to the numbers of \textit{phlD}\textsuperscript{+} pseudomonads detected by qPCR in our study. Bacteria harboring \textit{prnD} were detected in the 10 Swiss soils investigated here at abundances comparable to those found in different types of agricultural soils in a previous study (Garbeva et al., 2004).

The abundance of antimicrobial genes, respectively, of the bacteria harboring these genes, does only reflect the bacterial population that potentially could produce a particular antimicrobial compound but is not useful to identify the conditions that favor the proliferation and the consequent niche domination for a given bacterial species, as well as the production of specific antimicrobials \textit{in situ}. The present study is the first to monitor side-by-side the expression of several biosynthetic genes for antimicrobial compounds in different agricultural soils, i.e., in particular \textit{phlA}, \textit{hcnA}, \textit{prnA}, and \textit{phzA}, reflecting the biosynthesis of DAPG, HCN, pyrrolnitrin, and phenazines.
(Baehler et al., 2005; Chin-A-Woeng et al., 2005; Rochat et al., 2010). This was done by FACS-based monitoring of dual-labeled Pseudomonas reporter strains that carry a GFP cell tag and a mCherry-based reporter allowing to record the relative expression of a specific antimicrobial gene in individual cells of a Pseudomonas population. A similar technique was used by de Werra et al. (2008) and Rochat et al. (2010) for measuring the expression of phlA, hcnA, and prnA genes by Pseudomonas reporter strains on roots of different plant varieties in soilless systems. However, this is the first time that the combination of FACS and fluorescent reporter strains was applied to measure antimicrobial gene expression on plant roots grown in different natural soils. Moreover, to our best knowledge, there were no reports so far on phenazine gene expression in soil or on plant roots. To date, only a handful of studies attempted to determine the expression of antimicrobial genes of pseudomonads in soil (DeCoste et al., 2011; Novinscak and Filion, 2011; Almario et al., 2013b), mainly because of practical challenges. In our study, our initial attempts of tracking fluorescence of reporter strains extracted from roots and soil with microscopy revealed impracticable because of the strong autofluorescence of the investigated soil and root material. To address this problem, we modified the FACS approach of Rochat et al. (2010) and used GFP as a constitutively expressed cell marker and mCherry-based reporters for monitoring antimicrobial gene expression. This approach allowed to specifically track the Pseudomonas reporter cells and their expression of select antimicrobial genes in natural soil. However, due to the lack of suitable fluorescent proteins allowing reliable visualization of bacteria cells and gene expression in natural soil, a major limitation of this method is that it is impossible to monitor the expression of two or more antimicrobial genes in the same bacterial cell simultaneously. An alternative method of measuring gene expression in soils involves the extraction of total RNA followed by quantitative reverse transcription PCR of bacterial transcripts of interest. This method was used to quantify the expression of DAPG and HCN biosynthetic genes in natural and artificial soils (DeCoste et al., 2011; Novinscak and Filion, 2011). However, the main problem with this approach is the inefficiency of RNA extraction from a complex material such as soil along with the limited abundance of transcripts for antimicrobial genes.
We observed an interesting trend where in soils from Cadenazzo and Vouvry the expression of the four studied genes was markedly enhanced, while lower levels of gene expression occurred in soils from Eschikon, Grangeneuve, and Taenikon. The observation that a specific group of soils is capable of promoting or hampering the expression of four distinct antimicrobial genes in pseudomonads suggests that something in the abiotic or biotic composition of these soils can modulate the production of corresponding plant-beneficial compounds in the *P. protegens* and *P. chlororaphis* reporter strains. Furthermore, we observed that the soil type also influenced the capacity of both pseudomonads to colonize wheat, e.g., plants grown in soils from Cadenazzo and Vouvry supported higher populations than those grown in the Eschikon soil (Figure 5). However, the differences in root colonization by the two pseudomonads do not explain entirely the difference in gene expression observed in the different soils (Figures 4, 5 and Supplementary Table S3).

The relative importance of the abundance of *Pseudomonas* spp. for suppression of soilborne pathogens was questioned recently (Kyselkova et al., 2014). Indeed, DAPG and HCN producing pseudomonads could be isolated not only from suppressive but also from conducive soils, leading to the hypothesis that differential environmental factors prevailing in the two types of soils may shape the expression of relevant biocontrol genes in *Pseudomonas* bacteria and thus disease suppression (Ramette et al., 2006). Moreover, the abundance of the DAPG biosynthesis gene *phlD* was not indicative of the resistance of soils to black root rot of tobacco (Almario et al., 2013b; Kyselkova et al., 2014). Our results support the hypothesis that the abundance of pseudomonads producing antimicrobial metabolites may, in fact, be less important for disease suppression in the rhizosphere than previously hypothesized, at least for certain soils. We failed to detect any significant positive correlation between the resistances to the two soilborne pathogens *Pu* or *Gt* and the abundance of *Pseudomonas* harboring antimicrobial genes in the investigated agricultural soils (Figure 7). The lack of a significant positive correlation between disease resistance and abundance of antimicrobial pseudomonads could also be because only the *phlD*+ bacteria were detected at more than 10^5 cells/g root, which is close to the threshold considered to be relevant for biocontrol activity (Raaijmakers et al., 1999; Haas and Defago, 2005) in many soils. By analogy, the populations of PHZ and PRN producing pseudomonads might therefore also be too low to contribute to efficient pathogen
suppression in the soils of our study. Although correlations between gene abundances and disease resistance were never significant, it is still worth to note that for both pathogens they were mostly positive (Figure 7A). The expression of antimicrobial genes by reporter strains cannot be directly correlated to pathogen suppression data in the present study. Nevertheless, our findings suggest that high expression levels of antimicrobial genes in a particular soil may not necessarily be indicative of high levels of pathogen suppression (Figure 6). Furthermore, soils supporting the high expression of antimicrobial genes mostly harbored lower numbers of antimicrobials-producing pseudomonads (Figure 6). We thus speculate that the specific biotic and abiotic factors operating in the distinct agricultural soils might differently influence the abundance and expression of antimicrobial genes. At the present stage, it remains therefore elusive to which extent and how exactly pseudomonads producing DAPG, PHZ, and PRN contribute to the disease resistance of the investigated agricultural soils. Moreover, only two soilborne pathogens were investigated here and individual soils strongly differed in their response to Pu and Gt. It is likely that different relations to gene abundances and expression would be found for other pathogen and plant species. Soil nutrients are known to be important factors influencing resistance to pathogens (Martin and Hancock, 1986; Löbmann et al., 2016) and the abundance of pseudomonads producing antimicrobial compounds (Meyer et al., 2010; Kim, 2013). Recently, Löbmann et al. (2016) found that soils can influence the resistance to Pu in two ways: through abiotic effects that inhibit pathogen growth, which was the case in soils with a high pH, high calcium, and high clay content; or through balanced nutrient contents, which were hypothesized to stimulate the proliferation of plant-beneficial microorganisms. Löbmann et al. (2016) postulated this effect in soils with high P, K, Mg, sand and organic matter contents, although the involved plant-beneficial microorganisms were not identified in their study. In our study, we found significant positive correlations between Pu resistance and the contents of nitrate and Mg in soil. However, the same two nutrients were also positively correlated with the abundance of the DAPG biosynthesis gene phlD, so we cannot conclude on how exactly these nutrients have a positive impact on soil resistance. They could have a negative effect on the pathogen, have a positive effect on the growth of the plants and increase their pathogen resistance, or have an indirect positive effect on soil resistance by promoting beneficial bacteria.
We found no significant correlation between pH and abundance of *Pseudomonas* harboring antimicrobial genes, probably because the soils of this study all had a pH close to neutral and ranged from 6.0 (Cadenazzo) to 7.7 (Cazis) (Table 1). The influence of pH of agricultural soils on the population sizes of pseudomonads producing antimicrobial compounds is poorly understood. Previously, indigenous pseudomonads were found to be more abundant in soils with a neutral pH than in soils with an acidic pH (Kim, 2013). Likewise, *P. protegens* CHA0 inoculated into soils with an acidic pH reached lower population sizes compared to when it was inoculated into soils with neutral or basic pH (Mascher et al., 2013).

We obtained contrasting results for clay and silt, where clay was positively and silt negatively correlated with gene abundance (Figure 8). For gene expression, the opposite was the case. The precise relation between *Pseudomonas* abundance and clay content is not known. However, high clay content in soil was previously shown to reduce the expression of the HCN biosynthetic gene *hcnC* (Novinscak and Filion, 2011) and the biocontrol activity of phenazine-producing strains (Ownley et al., 2003). The pronounced effect of clay on biocontrol activity of pseudomonads was also observed in studies carried out in artificial soils (Keel et al., 1989; Almario et al., 2013b; Almario et al., 2014). In particular, vermiculitic clay supported a higher level of biocontrol activity and HCN production than illitic clay (Keel et al., 1989), and the expression of *phlA* was greater in the presence of vermiculite than in the presence of illite (Almario et al., 2013b).

The plant macronutrients nitrate, potassium and magnesium also inversely influenced abundance and expression of antimicrobial genes. To the best of our knowledge, it has not been investigated if high macronutrient contents in the soil directly stimulate the growth of antimicrobial pseudomonads. We hypothesize that the positive correlation between abundance of antimicrobial pseudomonads and certain macronutrients such as nitrate could also be due to an indirect effect, e.g., via stimulation of plant growth and increased root exudation. Support for this hypothesis comes from a recent study on maize roots, where nitrogen concentration in soil was found to be positively correlated with plant root exudation and abundance of rhizosphere bacteria (Zhu et al., 2016). For the micronutrients, a significant, though negative, correlation was found only for manganese and the expression of the HCN and PHZ biosynthetic genes (Figure
However, in previous studies, several micronutrients were described as factors that affect abundance, gene expression, metabolite production and biocontrol activity in pseudomonads. For instance, copper negatively influenced the abundance of *Pseudomonas* spp. in agricultural soils (Brandt et al., 2006), while the bioavailability of iron affected the expression of *phlA* and the production of HCN in artificial soils (Keel et al., 1989; Almario et al., 2013b). The biocontrol activity of phenazine-producing strains was positively correlated to zinc and negatively to iron and manganese levels in soil (Ownley et al., 2003). Moreover, the production of several antimicrobial metabolites, notably DAPG, pyoluteorin and PRN by strain *P. protegens* CHA0 was stimulated by zinc, but these experiments were performed in culture media (Duffy and Défago, 1997; 1999). The lack of significant correlations between micronutrient contents in soil and abundance and expression of antimicrobial genes in our study could be due to the relatively high micronutrient content in the sampled soils. In fact, all studied soils had sufficient amounts of micronutrients according to the classification for farmers approved by the Swiss government (Flisch et al., 2009). Boron concentration was an exception, as three out of 10 sampled soils tested as poor (Table 1).

### 4.6. Conclusion

Results of this study suggest that resistance of soils to pathogens, and abundance and expression of antimicrobial genes are not generally positively or negatively correlated in a wide range of diverse agricultural soils. Complex interactions depending on the host–pathogen system and the soil composition determine pathogen resistance of soils and the abundance and expression of antimicrobial genes. The abundance of antimicrobial metabolites producing pseudomonads in the investigated agricultural soils and the expression of biosynthetic genes for these compounds as studied here using reporter strains seem to be differentially shaped by multiple soil factors. This could explain, at least in part, why soils that sustain high numbers of these bacteria, often support only low levels of antimicrobial gene expression and vice versa. Therefore, to better understand the links between soil characteristics and abundance and expression of antimicrobial genes in pseudomonads, future studies should include extreme soils (i.e., highly acidic or alkaline soils or soils enriched in or depleted of specific nutrients). Pseudomonads are probably only one among many microbial...
groups determining the natural pathogen tolerance or resistance of agricultural soils (Mendes et al., 2011; Kyselkova et al., 2014; Cha et al., 2016; Raaijmakers and Mazzola, 2016). In future work, the potential of *Pseudomonas* bacteria as bio-indicators of soil resistance has to be re-evaluated with care. It will probably be difficult to identify specific groups of microorganisms as general indicators of soil health and disease resistance, since natural disease suppression likely requires individual compositions of the beneficial microbiota depending on the soil type, the crop species, the soilborne disease and maybe even the cropping system.

### 4.7. Acknowledgements

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### 4.8. References


Rochat, L., Péchy-Tarr, M., Baehler, E., Maurhofer, M., and Keel, C. (2010). Combination of fluorescent reporters for simultaneous monitoring of root colonization and antifungal gene expression by a biocontrol pseudomonad on cereals with flow...


*Pseudomonas fluorescens* helps suppress black root rot of tobacco under 
gnotobiotic conditions. *EMBO J* 8(2), 351.

fluorescens* strains F113, CHA0 and Pf153 in the rhizosphere of maize by strain-
specific real-time PCR unaffected by the variability of DNA extraction efficiency. 


populations responsible for specific soil suppressiveness to plant pathogens. 

Primer-BLAST: A tool to design target-specific primers for polymerase chain 

exudation, the rhizosphere microbiome and nitrogen-use-efficiency of maize. 
Table S1: Strains used for specificity testing of qPCR assays and for in-vivo standard curves.

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<th>Amplification with PhILD_65F/PhILD_236R_DEG</th>
<th>In vivo standard curve[^2]</th>
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[^1]: Produced by the strain.
[^2]: Refers to the in-vivo standard curve.
[^3]: Not determined.

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**Pseudomonas aeruginosa lineage**

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**Pseudomonas fluorescens lineage**

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<td>+</td>
<td>Cucumber rhizosphere, Switzerland</td>
<td>Wang et al. (2001)</td>
</tr>
<tr>
<td><em>Pseudomonas fluorescens</em> Q37-87</td>
<td>DAPG</td>
<td>nd</td>
<td>+</td>
<td>Wheat rhizosphere, WA, USA</td>
<td>Keel et al. (1996)</td>
</tr>
<tr>
<td><em>Pseudomonas fluorescens</em> Q65e-80</td>
<td>DAPG</td>
<td>nd</td>
<td>+</td>
<td>Wheat rhizosphere, WA, USA</td>
<td>Harrison et al. (1993)</td>
</tr>
<tr>
<td><em>Pseudomonas fluorescens</em> Q86-87</td>
<td>DAPG</td>
<td>nd</td>
<td>+</td>
<td>Wheat rhizosphere, WA, USA</td>
<td>Keel et al. (1996)</td>
</tr>
<tr>
<td><em>Pseudomonas kilonensis</em> DSM 13647</td>
<td>DAPG</td>
<td>nd</td>
<td>+</td>
<td>Agricultural soil, Germany</td>
<td>Sikorsky et al. (2001)</td>
</tr>
<tr>
<td><em>Pseudomonas kilonensis</em> P12</td>
<td>DAPG</td>
<td>nd</td>
<td>+</td>
<td>Tobacco rhizosphere, Switzerland</td>
<td>Keel et al. (1996)</td>
</tr>
<tr>
<td><em>Pseudomonas orientalis</em> L1-3-08 rif</td>
<td>PHZ</td>
<td>+</td>
<td>nd</td>
<td>Wheat rhizosphere, WA, USA</td>
<td>Parejko et al. (2013)</td>
</tr>
</tbody>
</table>
| Table S1 continued
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Pseudomonas protegens PF</td>
<td>DAPG, PRN</td>
<td>nd</td>
<td>+</td>
</tr>
<tr>
<td>Pseudomonas protegens BRIP</td>
<td>DAPG, PRN</td>
<td>nd</td>
<td>+</td>
</tr>
<tr>
<td>Pseudomonas protegens CHA0</td>
<td>DAPG, PRN</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Pseudomonas protegens PT-5</td>
<td>DAPG, PRN</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Pseudomonas protegens PGNR1</td>
<td>DAPG, PRN</td>
<td>nd</td>
<td>+</td>
</tr>
<tr>
<td>Pseudomonas sp. R11-45-07</td>
<td>PHZ</td>
<td>+</td>
<td>nd</td>
</tr>
<tr>
<td>Pseudomonas sp. R2-7-07</td>
<td>PHZ</td>
<td>+</td>
<td>nd</td>
</tr>
<tr>
<td>Pseudomonas sp R4-34-07</td>
<td>PHZ</td>
<td>+</td>
<td>nd</td>
</tr>
<tr>
<td>Pseudomonas sp. R4-35-07</td>
<td>PHZ</td>
<td>+</td>
<td>nd</td>
</tr>
<tr>
<td>Pseudomonas sp. CIPhz19</td>
<td>PHZ</td>
<td>+</td>
<td>nd</td>
</tr>
<tr>
<td>Pseudomonas sp. CMR12a</td>
<td>PHZ, DAPG</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Pseudomonas sp. CMR5c</td>
<td>PHZ, DAPG</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Pseudomonas sp. L1-11-07</td>
<td>PHZ</td>
<td>+</td>
<td>nd</td>
</tr>
<tr>
<td>Pseudomonas sp. PILH1</td>
<td>DAPG</td>
<td>nd</td>
<td>+</td>
</tr>
<tr>
<td>Pseudomonas sp. R11-23-07</td>
<td>PHZ</td>
<td>+</td>
<td>nd</td>
</tr>
<tr>
<td>Pseudomonas sp. R5-89-07</td>
<td>PHZ</td>
<td>+</td>
<td>nd</td>
</tr>
<tr>
<td>Pseudomonas sp. R5-90-07</td>
<td>PHZ</td>
<td>+</td>
<td>nd</td>
</tr>
<tr>
<td>Pseudomonas sp. SLPH 10</td>
<td>PHZ</td>
<td>+</td>
<td>nd</td>
</tr>
<tr>
<td>Strain</td>
<td>Metabolite</td>
<td>Activity</td>
<td>Source</td>
</tr>
<tr>
<td>---------------------------------------------</td>
<td>------------</td>
<td>----------</td>
<td>-----------------------------</td>
</tr>
<tr>
<td>Pseudomonas sp. R14-24-07</td>
<td>PHZ</td>
<td>+</td>
<td>nd</td>
</tr>
<tr>
<td>Pseudomonas thiirvalensis DSM 13194</td>
<td>DAPG</td>
<td>nd</td>
<td>+</td>
</tr>
<tr>
<td>Pseudomonas thiirvalensis PITR2</td>
<td>DAPG</td>
<td>nd</td>
<td>+</td>
</tr>
</tbody>
</table>

1 Only the metabolites of interest for this study, i.e. phenazines (PHZ), 2,4-diacetylphloroglucinol (DAPG), and pyrrolnitrin (PRN) are indicated. Many of these strains produce additional antimicrobial metabolites. For more detailed information, see the reference of each strain and Flury et al. (2016).

2 Indicated strains were selected to generate in-vivo standard curves for phlD, phzF and prnD qPCR used for the quantification of DAPG, PHZ and PRN biosynthesis genes.

3 Legend: ++ = amplification, - = no amplification, nd = not determined.

4 American Type Culture Collection, 10801 University Blvd, Manassas, VA 20110, USA.

5 Phylogeny according to Mulet et al., (2010).

6 Pseudomonas Genetic Stock Centre, East Carolina University, Greenville, NC 27858-4353, USA.

7 All-Russian Collection of Microorganisms, Russia, 142290, Moscow Region, Pushchino, pr. Nauki, 5, IBPM.

8 Kindly provided by Jos M. Raaijmakers, Netherlands Institute of Ecology, Wageningen, The Netherlands.
TABLE S2: Efficiencies and detection limits of qPCR assays using *in vivo* standard curves for quantification

<table>
<thead>
<tr>
<th>Target</th>
<th>Efficiency (%)</th>
<th>Slope</th>
<th>$R^2$</th>
<th>Detection limit (gene copies/g root dry weight)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>phlD</em></td>
<td>92.19</td>
<td>-3.525</td>
<td>0.992</td>
<td>2,000</td>
</tr>
<tr>
<td><em>phzF</em></td>
<td>110.56</td>
<td>-3.090</td>
<td>0.987</td>
<td>200</td>
</tr>
<tr>
<td><em>prnD</em></td>
<td>86.07</td>
<td>-3.708</td>
<td>0.900</td>
<td>2,000</td>
</tr>
</tbody>
</table>

1 Mixtures of strains containing the target genes were used. The strains used for each *in vivo* standard curve are indicated in Table S1. The target concentrations ranged from $10^8$ cells per gram of root to 0 cells per gram of root (noninoculated control) in tenfold dilutions (see Materials and Methods). The quantity of template used per reaction was 2 µl of undiluted DNA extracted from rhizosphere wash.

2 Efficiency = $10^{(-1/slope)} - 1$

3 Three biological replicates were used for each concentration.
Table S3: Antimicrobial gene expression at the single cell level and root colonization by GFP-marked reporters of *Pseudomonas protegens* (CHA0-gfp) or *Pseudomonas chlororaphis* (PCL1391-gfp) carrying mcherry-based fusions to *phIA*, *hcnA*, *prnA* and *phzA*, respectively, in soils from 10 Swiss fields planted with wheat\(^1\).

<table>
<thead>
<tr>
<th>Soil</th>
<th><em>P. protegens</em> CHA0-gfp</th>
<th></th>
<th></th>
<th><em>P. chlororaphis</em> PCL1391-gfp</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><em>phIA</em> expression (RFU per cell) (\times 10^5)</td>
<td><em>hcnA</em> expression (RFU per cell) (\times 10^5)</td>
<td><em>prnA</em> expression (RFU per cell) (\times 10^5)</td>
<td>Number of cells g(^{-1}) dry root (\times 10^5)</td>
<td><em>phzA</em> expression (RFU per cell) (\times 10^5)</td>
</tr>
<tr>
<td>Cd</td>
<td>49.9 a</td>
<td>3.18 a</td>
<td>1.77 a</td>
<td>1.88 ab</td>
<td>7.04 a</td>
</tr>
<tr>
<td>Cx</td>
<td>38.4 ab</td>
<td>1.72 cde</td>
<td>0.86 c</td>
<td>0.40 def</td>
<td>4.91 ab</td>
</tr>
<tr>
<td>Cz</td>
<td>28.4 cde</td>
<td>1.26 def</td>
<td>0.95 c</td>
<td>1.93 ab</td>
<td>4.50 abc</td>
</tr>
<tr>
<td>De</td>
<td>36.2 abc</td>
<td>4.82 a</td>
<td>1.65 ab</td>
<td>1.39 cde</td>
<td>2.59 d</td>
</tr>
<tr>
<td>Es</td>
<td>32.6 bcd</td>
<td>3.00 ab</td>
<td>1.73 ab</td>
<td>0.29 g</td>
<td>4.04 abc</td>
</tr>
<tr>
<td>Gr</td>
<td>15.9 fg</td>
<td>1.75 cde</td>
<td>1.48 b</td>
<td>0.35 fg</td>
<td>3.23 c</td>
</tr>
<tr>
<td>Ta</td>
<td>29.1 de</td>
<td>1.96 bc</td>
<td>1.65 ab</td>
<td>0.58 efg</td>
<td>2.59 d</td>
</tr>
<tr>
<td>Ut</td>
<td>12.7 g</td>
<td>1.26 g</td>
<td>1.86 a</td>
<td>0.85 bcd</td>
<td>2.66 d</td>
</tr>
<tr>
<td>Vo</td>
<td>25.3 ef</td>
<td>1.75 ef</td>
<td>1.06 c</td>
<td>2.41 a</td>
<td>3.51 bc</td>
</tr>
<tr>
<td>Wi</td>
<td>15.6 g</td>
<td>1.32 g</td>
<td>0.99 c</td>
<td>1.09 bc</td>
<td>3.44 bc</td>
</tr>
</tbody>
</table>

\(^1\)Expression and root colonization by reporter strains was monitored by fluorescence-activated cell-sorting-based flow cytometry using CHA0-gfp carrying plasmids pME9012 (phIA-mcherry), pME9011 (hcnA-mcherry), or pME11011 (prnA-mcherry) or PCL1391-gfp carrying pME11017 (phzA-mcherry). Seedlings inoculated with the reporter strains were grown in soil microcosms for five days prior to analysis of bacterial cells in root washes. Data on expression are shown as relative fluorescence units (RFU) and represent the median mCherry expression per GFP-tagged *Pseudomonas* cell. Root colonization levels were recorded as number of GFP-tagged cells per gram of dry root. Results from three independent experiments with nine replicates each are presented. Letters indicate significant differences (Dunn test, \(p<0.05\)).

\(^2\)Soils were sampled at the following field sites: Cd, Cadenazzo; Cx, Courtedoux; Cz, Cazis; De, Delley; Es, Eschikon; Gr, Grangeneuve; Ta, Taenikon; Ut, Utzenstorf; Vo, Vouvry; Wi, Witzwil.
4.9.2 Supplementary Figures

Figure S1: Relative resistance of 10 representative Swiss agricultural soils planted with spring wheat to increasing inoculum concentrations of *Gaeumannomyces tritici*. Inoculum concentrations per pot were (A) 0.2 g, (B) 0.6 g, (C) 2.0 g, and (D) 6.0 g. Each pathogen concentration and soil was tested in six replicate pots. Soil resistance is shown as fresh shoot weight of plants in artificially pathogen-infested soil compared to shoot weight of control plants grown in non-infested soil. The dotted line indicates 50% of shoot weight compared to the control. Letters indicate significant differences (Kruskal-Wallis test, p<0.05). Sampling sites: Cd, Cadenazzo; Cx, Courtedoux; Cz, Cazis; De, Delley; Es, Eschikon; Gr, Grangeneuve; Ta, Taenikon; Ut, Utzenstorf; Vo, Vouvry; Wi, Witzwil.
Figure S2: Relative resistance of 10 representative Swiss agricultural soils planted with cucumber to increasing inoculum concentrations of *Pythium ultimum*. Inoculum concentrations per pot were (A) 0.125 g, (B) 0.25 g, (C) 0.5 g, and (D) 1.0 g. Each pathogen concentration and soil was tested in six replicate pots. Soil resistance is shown as fresh shoot weight of plants in artificially pathogen-infested soil compared to shoot weight of control plants grown in non-infested soil. The dotted line indicates 50% of shoot weight compared to the control. Letters indicate significant differences (Kruskal-Wallis test, p<0.05). Sampling sites: Cd, Cadenazzo; Cx, Courtedoux; Cz, Cazis; De, Delley; Es, Eschikon; Gr, Grangeneuve; Ta, Taenikon; Ut, Utzenstorf; Vo, Vouvry; Wi, Witzwil.
Figure S3: Abundance of resident *Pythium ultimum* on roots of spring wheat grown in 10 Swiss agricultural soils. Abundance was determined with a qPCR assay targeting the internal transcribed spacer (ITS) rRNA gene region. For each soil, six replicates were used. The quantity of *P. ultimum* DNA per gram of roots is shown (ag, attogram). No significant differences between samples from different soils were found (Kruskal-Wallis test, p<0.05). Sampling sites: Cd, Cadenazzo; Cx, Courtedoux; Cz, Cazis; De, Delley; Es, Eschikon; Gr, Grangeneuve; Ta, Taenikon; Ut, Utzenstorf; Vo, Vouvry; Wi, Witzwil.

Figure S4: Comparison between most probable number PCR (MPN-PCR) and qPCR for the determination of the abundance of *phlD*+ and *phzF*+ cells on roots of spring wheat. Six replicates of roots of wheat grown in Taenikon soil were used. The same primers (see Table 2) were used for MPN-PCR and qPCR. No significant difference between the two methods was detected.
4.9.3 Supplementary References


CHAPTER 3

Conservation tillage and organic farming induce minor variations in *Pseudomonas* abundance, their antimicrobial function and soil disease resistance

A version of this chapter is in preparation for *FEMS Microbiology Ecology*, by

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FD coordinated the sampling, performed the greenhouse disease resistance experiments, collected the qPCR data, performed the amplicon sequencing library preparation, analysed the data with the help of KS, and wrote the manuscript (with revisions and comments from all co-authors). Gene expression data were provided by NI.
3.1. Abstract
Conservation tillage and organic farming are strategies used worldwide to preserve the stability and fertility of soils. While positive effects on soil structure have been extensively shown, the effects on specific root- and soil-associated microorganisms are less known. The aim of this study was to investigate how conservation tillage and organic farming influence the frequency and activity of plant-beneficial pseudomonads. Amplicon sequencing using the 16S rRNA gene revealed that Pseudomonas is among the most abundant bacterial taxa in the root microbiome of field-grown wheat, independent of agronomical treatments. However, pseudomonads carrying genes required for the biosynthesis of specific antimicrobial compounds were enriched in samples from conventionally farmed plots without tillage. In contrast, disease resistance tests indicated that soil from conventional no tillage plots is less resistant to the soilborne pathogen Pythium ultimum compared to soil from organic reduced tillage plots, which showed the highest resistance of all compared cropping systems. Reporter strain-based gene expression assays did not reveal any differences in Pseudomonas antimicrobial gene expression between soils from different cropping systems. Our results suggest that plant-beneficial pseudomonads can be favored by certain soil cropping systems; but soil resistance against plant diseases is likely determined by a multitude of biotic factors in addition to Pseudomonas.

3.2. Introduction
Sustainable cropping systems, such as conservation tillage and organic agriculture, are increasingly adopted by farmers worldwide to prevent soil erosion and nutrient losses as well as to increase soil organic matter and water retention in the soil (Mäder et al., 2002; Pittelkow et al., 2014; Giller et al., 2015). Soil conservation cropping systems, where crops are sown directly in the field with no or minimal ploughing, have been found to be advantageous particularly in non-irrigated cultivation systems in dry climates (Pittelkow et al., 2014). While in temperate climates with high rainfall, no tillage systems slightly decreases yields (Anken et al., 2004; Pittelkow et al., 2014), it nevertheless has a positive effect on soil structure and soil biota (Anken et al., 2004; Karlen et al., 2013; Verzeaux et al., 2016). No tillage leads to more stable soil aggregates and a higher soil organic matter content in the upper soil layers (Peigné et
al., 2007). It has often be hypothesized that no tillage has positive effects on soil macrobiota and microbiota (Peigné et al., 2007; Navarro-Noya et al., 2013), however, results from field studies are so far not consistent. The abundance and diversity of individual taxonomical groups can be differentially influenced by tillage. Soil bacterial communities have been found to be different in tillage versus no tillage systems, with certain taxa being more frequent under no tillage compared to conventional tillage (Navarro-Noya et al., 2013; Carbonetto et al., 2014; Chávez-Romero et al., 2016; Degrune et al., 2016; Guo et al., 2016; Wang et al., 2016).

Organic agriculture becomes more and more common because it requires less external inputs and increases soil fertility (Mäder et al., 2002; Fließbach et al., 2007). Soils managed organically were found to harbor a greater diversity of soil microorganisms (Mäder et al., 2002; Li et al., 2012; Hartmann et al., 2015), but also to contain specific microbial communities, where certain taxa were more abundant than in conventionally managed soils (Li et al., 2012; Hartmann et al., 2015; Pershina et al., 2015; Bonanomi et al., 2016). In this context, it is of special interest how sustainable cropping systems influence beneficial microorganisms, i.e. fungi and bacteria which improve plant growth and health.

Bacteria of the genera *Pseudomonas* and *Bacillus*, for example, are considered among the important taxa for soil health, in particular for their ability to suppress soilborne fungal pathogens (Weller et al., 2002; McSpadden Gardener, 2004; Haas and Defago, 2005). The genus *Pseudomonas* comprises species ranging from human- and plant-pathogenic to plant-beneficial organisms. Similarly, within the genus *Bacillus*, only some species are considered to be plant-beneficial (McSpadden Gardener, 2004). A limitation of most studies so far performed on the diversity of soil bacteria was that the taxonomic resolution was not detailed enough to distinguish between beneficial and non-beneficial bacteria at the subgenus and subspecies level. Many species in the *Pseudomonas fluorescens* group (Gomila et al., 2015), but not all of them, exhibit multiple plant-beneficial properties, i.e. the induction of systemic resistance (Bakker et al., 2013), the competition with pathogens on the root surface (Haas and Defago, 2005; Lemanceau et al., 2006) and the production of metabolites with broad-spectrum antimicrobial activity (Haas and Keel, 2003; Haas and Defago, 2005; Weller et al., 2007). Certain *Pseudomonas* strains with antimicrobial activity have been
commercialized as biocontrol agents against a variety of plant diseases (Berg, 2009; Mosimann et al., 2016).

Among the most important antimicrobial metabolites that have an effect against fungal pathogens, are 2,4-diacetylphloroglucinol (DAPG) (Haas and Keel, 2003; Weller et al., 2007), phenazines (PHZ) (Thomashow and Weller, 1988; Mavrodi et al., 2006) and pyrrolnitrin (PRN) (Hwang et al., 2002). Antimicrobial metabolite-producing pseudomonads have been found in high abundances in suppressive soils, where specific pathogens are present but plants show little or no disease symptoms (Weller et al., 2002; Lemanceau et al., 2006). However, their presence cannot be used as sole indicator of disease suppressiveness since these bacteria are also present in disease conducive soils (Frapolli et al., 2010; Almario et al., 2013a; Kyselkova et al., 2014).

The effect of cropping systems on the abundance of antimicrobial pseudomonads is not well known. PRN producing bacteria were found to be more abundant in grassland compared to arable land (Garbeva et al., 2004) and DAPG producing pseudomonads to be more abundant in conventionally managed than in organically managed soils (Hiddink et al., 2005), but there is no study assessing, in the same field experiment, the effect of different cropping systems on abundance of different groups of antimicrobial pseudomonads. Moreover, there is only little knowledge on the resistance of soils to soilborne pathogens under different cropping systems. In studies by Van Bruggen (1995) and by Hiddink et al. (2005), soils from organic systems were more resistant to soilborne pathogens than soils from conventional systems. However, also here, to date there is no study comparing the influence of tillage and organic management on soil resistance to root pathogens in the same year and the same field site.

In this study, we made use of the Swiss farming systems and tillage experiment (FAST) which compares conventional and organic farming, each with intensive and with conservation tillage (Wittwer et al., 2017) to address the above mentioned gaps. We examined the impact of different cropping systems on i) the abundance of *Pseudomonas* spp. within the microbiomes of the wheat roots and of bulk soil; ii) the abundance of specific groups of beneficial pseudomonads harboring antimicrobial genes; iii) the ability of the soil to support the expression of antimicrobial genes in *Pseudomonas* reporter strains; iv) the abundance in soil of the two important soilborne pathogens *Pythium ultimum*, causing damping-off and root rot on various crop plants, and *Gaeumannomyces tritici*, causing take-all of wheat; and v) the soil
resistance to these two pathogens. The overall aim of this study was to better understand the relationships between the abundance, diversity and activity of *Pseudomonas* spp. and natural resistance to root pathogens in soils in response to different cropping systems. This knowledge will be important for the development of new strategies for the reduction of soilborne diseases.

3.3. Material and Methods

3.3.1 The Swiss Farming System and Tillage experiment (FAST)

The Swiss Farming System and Tillage experiment (FAST) was established in 2009 on a field site at the Agroscope research station Reckenholz in Zurich, Switzerland (latitude 47°26’N, longitude 8°31’E). The FAST experiment compares organic and conventional farming in combination with two levels of tillage intensity based on the following four cropping systems: Organic reduced tillage (O-RT), Organic intensive tillage (O-IT), Conventional no tillage (C-NT) and Conventional intensive tillage (C-IT). The conventional systems are managed according to the “Proof of Ecological Performance” (PEP) guidelines of the Swiss Federal Office for Agriculture. The organic systems are managed according to the guidelines of Bio Suisse, the governing body for organic producers in Switzerland. All systems are cultivated with a crop rotation of six years and the present study was performed during the fourth year of the experiment (Table 1). The FAST experiment consists of two replicate experiments (FAST I and II) that are located side by side on the same field but with the crop rotation staggered by one year. Each replicate experiment comprises 4 replicate blocks with the cropping systems as main plots. The main plots are further subdivided into 4 subplots of 3 m x 15 m, three of which were sown with different cover crops (non-legume, legume and mixture) between main crops and one subplot was a control without cover crop. The factor cover crop was not included in this study and all assessment were performed in the legume cover crop treatment. The conventional treatments were fertilized with mineral fertilizer according to the quantities allowed in Swiss agriculture (Flisch et al., 2009); while winter wheat in organic systems was fertilized with cattle slurry (1.4 livestock units ha⁻¹). The treatments are summarized in Table 1. The experiment is described in depth in the study by Wittwer et al. (2017).
Table 1: The Farming Systems and Tillage experiment (FAST, Wittwer et al., 2017)

<table>
<thead>
<tr>
<th>Treatment name</th>
<th>Treatment description</th>
<th>Tillage depth (cm)</th>
<th>Fertilization (ha⁻¹)¹</th>
<th>Crop rotation</th>
</tr>
</thead>
<tbody>
<tr>
<td>C-NT</td>
<td>Conventional, no tillage</td>
<td>No tillage</td>
<td>120 kg N, 88 kg P, 128 kg K</td>
<td>cover crop, wheat, cover crop, maize, field bean, wheat², grass-clover, grass-clover</td>
</tr>
<tr>
<td>C-IT</td>
<td>Conventional, intensive tillage</td>
<td>20-25</td>
<td>120 kg N, 88 kg P, 128 kg K</td>
<td>cover crop, wheat, cover crop, maize, field bean, wheat², grass-clover, grass-clover</td>
</tr>
<tr>
<td>O-RT</td>
<td>Organic, reduced tillage</td>
<td>5</td>
<td>Slurry 1.4 livestock units</td>
<td>cover crop, wheat, cover crop, maize, field bean, wheat², grass-clover, grass-clover</td>
</tr>
<tr>
<td>O-IT</td>
<td>Organic, intensive tillage</td>
<td>20-25</td>
<td>Slurry 1.4 livestock units</td>
<td>cover crop, wheat, cover crop, maize, field bean, wheat², grass-clover, grass-clover</td>
</tr>
</tbody>
</table>

¹Average fertilization for winter wheat in 2013 and 2014
²Sampling time point in the crop rotation

3.3.2 Sampling and DNA extraction

Both FAST replicated experiments where sampled, FAST I in 2013 and FAST II in 2014, in the fourth year of the crop rotation. Winter wheat roots and bulk soil were sampled from all 16 main plots (four plots each: O-RT, O-IT, C-NT, C-IT). The wheat variety in both years was “Titlis”. For each sampled plot, root systems from five plants were collected and pooled. Sampling was performed when the wheat plants were at flowering stage. The bulk soil samples were collected at 0-20 cm depth between wheat rows. Five soil cores were collected per plot and pooled. To collect bacteria from the root surface, the root systems were rinsed with tap water to remove bulk soil, incubated overnight at 3°C in sterile Erlenmeyer flasks in 50 mL 0.9% NaCl solution and subsequently shaken on an orbital shaker at 350 rpm for 30 min. Roots were then separated from the suspension and dried for 2 days at 100°C to determine dry weight. The suspensions were centrifuged at 3500 rpm for 20 min and 0.5 g of the obtained pellet was used for DNA extraction with the FastDNA Spin kit for soil (MP Biologicals, Illkirch, France). Bulk soil samples were thoroughly mixed and 0.5 g were used for
DNA extraction with the same kit as used for the root. DNA concentrations were measured with the Qubit fluorometer broad range dsDNA assay (Thermo Fisher Scientific, Waltham, USA).

Twenty-five liters of soil per plot were collected in 2014 for the disease resistance and gene expression experiments. Soil cores (0-25 cm) were collected randomly through the plots, sieved with a 1-cm-mesh sieve to remove stones and large plant debris, and thoroughly mixed. The soil samples were stored at 15°C.

### 3.3.3 Microbiome analysis using 16S rRNA gene amplicon sequencing

To study the relative abundance of *Pseudomonas* spp. and other bacterial taxa on the roots and in bulk soil, the V5-V7 regions of the 16S rRNA gene were sequenced using the DNA samples from the FAST II experiment (collected in 2014). We used the methodology described in Hartman et al. (2017). Briefly, PCR primers used were 799F (5’-AACMGGATTAGATACCCKG-3’, (Chelius and Triplett, 2001) and 1193R (5’-ACGTCATCCCCACCTTCC-3’, (Bodenhausen et al., 2013). Primers were fused at the 5’ end to an 8 bp barcode (Faircloth and Glenn, 2012) and a 5 bp padding sequence [5’-padding-barcode_xy-primer-3’]. PCR reactions consisted of 1x 5Prime Hot Mastermix (5Prime, Boulder, USA), 0.3% Bovine Serum Albumin (New England Biolabs, Ipswich MA, USA), 400 nM of each tagged primer (Microsynth, Balgach, Switzerland), and 10 ng template DNA in a total reaction volume of 20 µL. PCRs were performed on an iCycler instrument (BioRad, Hercules, CA, USA) with cycling conditions consisted of an initial denaturation of 3 min at 94°C, 30 cycles of 45 sec at 94°C, 30 sec at 55°C and 1 min 30 sec at 65°C, followed by a final elongation of 10 min at 65°C. Band size of the PCR products was verified by gel electrophoresis before purification with the NucleoSpin Gel and PCR cleanup kit (Macherey-Nagel, Oensingen, Switzerland). PCR product concentrations were measured with a Varian fluorescence plate reader (Varian, Palo Alto, USA) using the Quant-iT PicoGreen dsDNA Assay Kit (Invitrogen, Carlsbad, USA) and Herring Sperm DNA (Invitrogen, Carlsbad, USA) as standard solution. The samples were equimolarly pooled to a library containing 50 ng PCR products per sample. The library was purified with Agencourt AMPure XP beads (Beckman Coulter, Indianapolis, USA) and the concentration of the library was measured after purification with a Varian fluorescence plate reader (Varian, Palo Alto, USA). Sequencing adapters were ligated to the library by the Functional Genomics
Center Zurich (Zurich, Switzerland, http://www.fgcz.ch/) followed by sequencing on the Illumina MiSeq instrument in paired-end 2x 300 bp mode (Illumina, San Diego, USA).

Sequence processing was conducted according to Hartman et al. (2017). Briefly, the raw sequencing read data (available at European Nucleotide Archive database, accession no. PRJEB20139) were quality filtered using PRINSEQ v0.20.4 (Schmieder and Edwards, 2011), merged with FLASH v.1.2.9 (Magoč and Salzberg, 2011) and de-multiplexed employing Cutadapt v1.4.2 (Martin, 2011). The high-quality 16S rRNA gene sequences were trimmed to a fixed length of 360 bp, sorted by abundance, de-replicated, and clustered to operational taxonomic units (OTUs, ≥ 97% sequence similarity) with UPARSE v8.1.1812 (Edgar, 2013). Only OTUs with a minimal coverage of 5 sequences were included. Chimeric OTU sequences were removed after identification with UCHIME (Edgar et al., 2011) against the GOLD database (Reddy et al., 2014). Taxonomy assignment was performed using the SILVA 16S v119 database (Quast et al., 2013) with the RDP classifier implemented in QIIME v1.8 (Caporaso et al., 2010). Microbiome profiles were filtered to exclude OTUs classified as Cyanobacteria or assigned to mitochondria.

3.3.4 Quantitative real-time PCR

To quantify *Pseudomonas* spp. producing antimicrobial metabolites on roots and in bulk soil, quantitative real-time PCR (qPCR) was used, targeting the genes *phlD*, (biosynthesis pathway of 2,4-diacetylophloroglucinol) and *phzF* (biosynthesis pathway of phenazines) according to the methods described in Chapter 2, and *prnD* (biosynthesis pathway of pyrrolnitrin) as described by Garbeva et al. (2004). Primers and cycling conditions of the qPCR assays are described in Table S1 and Table S2. The assays targeting *phlD* and *phzF* are specific for *Pseudomonas* of the *P. fluorescens* lineage (see Chapter 2), while the assay targeting *prnD* additionally detects *Burkholderia* and *Serratia* (Garbeva et al., 2004). The functions of the genes mentioned above are summarized in Table S3. To quantify the plant pathogenic oomycete *P. ultimum*, a qPCR assay targeting the internal transcribed spacer (ITS) region was used (Cullen et al., 2007). Additionally, the pathogenic ascomycetes *G. tritici* and *Gaeumannomyces avenae* were quantified with a qPCR assay targeting the ITS region (Bithell et al., 2012b). All qPCR assays and preparation of standard curves for
quantification of fungal ITS regions and of *Pseudomonas* harboring antimicrobial genes on roots are described in detail in Chapter 2. Briefly, pseudomonads carrying antimicrobial genes were quantified with in-vivo standard curves prepared by adding defined numbers of cells to sterile wheat roots. This allows to directly relating the cycle threshold (Ct) values of the qPCR assays to cell numbers of *Pseudomonas* carrying antimicrobial genes. Moreover, since *Pseudomonas* carry only one copy per genome of antimicrobial biosynthesis genes *phlD*, *phzF* and *prnD*, cell numbers per gram of root are comparable to gene copies per gram of root (see Chapter 2). For quantification of *Pseudomonas* carrying antimicrobial genes in bulk soil, in-vitro standard curves with genomic DNA from strains *P. protegens* CHA0 (*phlD*, *prnD*) and *Pseudomonas synxantha* 2-79 (*phzF*) were performed, ranging from 2 x10⁶ to 2 genome copies reaction⁻¹ in six ten-fold dilutions. Three technical replicates were performed for each of the 16 main plots (four replicate plots for each treatment, i.e. O-RT, O-IT, C-NT and C-IT). Detection limits of the antimicrobial metabolite qPCR assays were 2 cells per reaction (*phzF* in-vivo standard curve), 20 cells per reaction (*phlD* and *prnD* in-vivo standard curves), 2 genome copies per reaction (*phzF* and *prnD* in-vitro standard curves) and 20 genome copies per reaction (*phlD* in-vitro standard curve). Detection limits of qPCR assays targeting the ITS regions of pathogens were 200 attograms DNA per reaction (*P. ultimum*) and 10³ attogram DNA per reaction (*G. tritici* and *G. avenae*).

### 3.3.5 In situ reporter strain assay for quantification of antimicrobial gene expression

The reporter assays were conducted as detailed in Chapter 2. Briefly, the expression of antimicrobial genes on the roots of wheat plants was quantified with GFP-marked variants of *P. protegens* CHA0 (CHA0::attTn7-gfp; Péchy-Tarr et al. (2013) and *P. chlororaphis* PCL1391 (PCL1391::attTn7-gfp; see Chapter 2, harboring mCherry-based reporter plasmids pME9012 (*phlA*-mcherry; Rochat et al. (2010), pME11011 and pME11017 (*prnA*-mcherry and *phzA*-mcherry, respectively; see Chapter 2. The expression of the reporter fusions *phlA*-mcherry and *prnA*-mcherry (genes involved in the biosynthesis of 2,4-diacetylphloroglucinol and the biosynthesis of pyrrolnitrin, respectively) was measured in strain *P. protegens* CHA0, whereas the expression of the reporter fusion *phzA*-mcherry (gene involved in the biosynthesis pathway of...
phenazines) was monitored in \textit{P. chlororaphis} PCL1391. The functions of the genes mentioned above are listed in Table S3. Spring wheat seeds of the variety "Rubli" (Delley Seeds, Delley, Switzerland) were surface disinfested for 12 min in 4\% v/v NaClO, washed with distilled water and germinated on soft agar (Agar, Agar SERVA, 9 g L$^{-1}$) by incubating for 48 h at room temperature in the dark. The germinated wheat seedlings were transferred to 200 mL Erlenmeyer flasks containing 60 g of soil. Soil sampled in 2014 as described above was used. Three seedlings per flask were planted. The \textit{Pseudomonas} reporter strains were grown overnight in 8 mL of NYB supplemented with gentamycin (10 µg mL$^{-1}$) and kanamycin (25 µg mL$^{-1}$), at 30°C and 180 rpm. Each wheat seedling was inoculated with 1 mL suspension of washed bacteria cells corresponding to 3-4 x 10$^8$ CFU. Control treatments were performed with wild type \textit{P. protegens} CHA0 and \textit{P. chlororaphis} PCL1391 and with GFP-tagged \textit{P. protegens} CHA0-gfp and \textit{P. chlororaphis} PCL1391-gfp with or without empty vector control (see Chapter 2). Flasks were incubated for 5 days in a growth chamber at 60\% relative humidity with a 16 h light period at 176 µE m$^{-2}$ s$^{-1}$ and 25°C and an 8 h dark period at 20°C. Wheat roots were harvested and cell suspensions from root washes prepared as described above. The suspensions were filtered using a 5.0 µm sterile syringe single-use filter (Sartorius Stedim Biotech GmbH, Goettingen, Germany), transferred on ice and immediately analyzed by FACS with a BD LSRFortessa flow cytometer (Becton-Dickinson, San Jose, USA). Gating and settings for detecting GFP and mCherry fluorescence emitted by reporter strains were the same as described previously (see Chapter 2). Fresh and dry weight of wheat roots were recorded and the number of GFP-marked \textit{Pseudomonas} cells present in root wash was determined by FACS and expressed as CFU g root$^{-1}$. The experiment was performed twice. Three technical replicates were performed for each of the 16 investigated main plots (four replicate plots for each treatment, i.e. O-RT, O-IT, C-NT and C-IT) and for each of the control treatments.

\textbf{3.3.6 Assessment of soil resistance to root pathogens}

The effect of the different cropping systems of the FAST experiment on the resistance of the soil to the two common soilborne plant pathogens \textit{P. ultimum} and \textit{G. tritici} was tested in a greenhouse experiment as described in detail in Chapter 2. Briefly, pathogen inoculum was prepared by growing \textit{P. ultimum} on autoclaved millet seeds.
and *G. tritici* on autoclaved oat seeds. Soil (200 g per pot) sampled from the 16 main plots (four replicate plots for each treatment, i.e. O-RT, O-IT, C-NT and C-IT) was filled in pots, amended with increasing quantities of pathogen inoculum and planted with three sterile-grown cucumber seedlings (*Cucumis sativa* var. “Chinese Snake”) in the *P. ultimum* system or three sterile-grown spring wheat seedlings (*Triticum aestivum* var. “Rubli”) in the *G. tritici* system. Six replicate pots were prepared per plot and pathogen concentration (four pathogen concentrations and one control treatment without inoculum). Plants were grown for 10 days (cucumber) or 21 days (wheat) in the greenhouse with a 16-h-day period (210 μmol m⁻²s⁻¹) at 22°C (cucumber) or 18°C (wheat) and an 8-h-night period at 18°C (cucumber) or 15°C (wheat) with an air moisture of 70%. At the end of the experiment, fresh shoot weights per pot were determined as a measure to assess the disease resistance of the soils.

### 3.3.7 Data analysis

All data were analyzed with the R software version 3.2.3 (RCoreTeam, 2015). The OTU and taxonomy tables were imported in R for further analysis. The difference between the number of reads from different sample groups and treatments was tested with non-parametric Kruskal-Wallis test (package “coin”). No significant difference was found; therefore, the data were not rarefied but normalized by the sampling depth. OTUs assigned to the genus *Pseudomonas* with a relative abundance greater than 0.1% were selected for further analysis. The differences between the relative abundances of *Pseudomonas* OTUs in the different treatments was calculated with Kruskal-Wallis test followed by Dunn’s post-hoc test (R package “dunn.test”). To determine the identity of the *Pseudomonas* OTUs more precisely, a phylogenetic tree was inferred with the V5-V7 16S rRNA gene regions from type strains and other well-known biocontrol strains of the *P. fluorescens* lineage, the *P. aeruginosa* lineage, and the *P. oryzihabitans* group (Mulet et al., 2010; Flury et al., 2016) and *Cellvibrio japonicum* Ueda 107 as an outgroup. A maximum-likelihood tree with 1000 bootstrap replicates was inferred with MEGA 4 (Tamura et al., 2007). Quantitative PCR (qPCR) cycle threshold (Ct) values of the assays targeting antimicrobial genes and fungal ITS were normalized for variation in DNA extraction by adding a specified quantity of APA9 plasmid prior to DNA extraction as described in Chapter 2 and in Von Felten et al. (2010).
Gene expression per gram of roots was calculated by multiplying the relative red fluorescence per cell with the number of detected events (cells) per gram of roots (dry weight). Data from the two experiments were pooled, since no significant difference was found between the results of experiment 1 and experiment 2 (linear mixed effect model with “experiment” as a fixed effect, function “lmer” from package “lme4”). Pathogen resistance in the different treatments was calculated by expressing the fresh shoot weight of the plants from inoculated pots as a percentage of the fresh shoot weight from control plants of the same treatment. Significant differences between treatments were determined with a linear mixed effect model (function “lmer” from package “lme4”) with “cropping system” and “block” as fixed effects and “plot” as a random effect. A post-hoc test was performed for “cropping system” (Tukey’s HSD, function “glht” from package “multcomp”).

3.4. Results
We determined the relative abundance of the genus *Pseudomonas* on the wheat root surface and in soil, and whether they differ between cropping systems, using 16S rRNA gene amplicon sequencing. We sequenced 32 samples and generated a total of 1’398’161 high quality sequences, of which 1’856 different OTUs were detected. On average, 43’717 high quality filtered reads per sample were obtained. The highest numbers of OTUs were assigned to the phyla Proteobacteria (741 OTUs, 52% relative abundance on roots and 38% in bulk soil), Actinobacteria (272 OTUs, 18% relative abundance on roots and 32% in bulk soil) and Bacteroidetes (173 OTUs, 18% relative abundance on roots and 13% in bulk soil). We found three OTUs with a relative abundance >0.1% that were assigned to the genus *Pseudomonas*. The most abundant was OTU1, being the second most abundant OTU in the entire dataset (Figure 1), with an average relative abundance of 7.6% on roots and 2.6% in bulk soil (Figure 2AB). The second *Pseudomonas* OTU152, had an average relative abundance of 0.9% on roots and 0.3% in bulk soil (Figure 2CD). The third, OTU140 had an average relative abundance of 0.18% on roots and 0.13% in bulk soil (Figure 2EF). OTU1 and OTU152 were significantly more abundant on roots compared to bulk soil, while for OTU140 this was only the case for the organic treatment with reduced tillage. Cropping system had no significant effect on relative abundances of *Pseudomonas* OTUs (Figure 2).
Overall, pseudomonads, together with *Flavobacterium* and *Variovorax*, were found to be among the most abundant taxa on wheat roots and in the soil of the FAST field experiment (Figure 1).

**Figure 1:** The twenty most abundant bacterial operational taxonomic units (OTUs) detected on wheat roots and in bulk soil based on 16S rRNA V5-V7 region amplicon sequencing. Taxonomic assignments were determined with the SILVA database. The highest assigned taxonomic rank is shown. Sequencing was performed with samples from the field experiment **FAST II (sampling in 2014)**. Data from different cropping systems (conventional without tillage (C-NT), conventional with tillage (C-IT), organic with reduced tillage (O-RT), organic with tillage (O-IT)) were pooled. Four replicates per treatment were sequenced. Bars show the average relative abundance and standard errors. Asterisks denote taxa that are significantly more abundant on roots than in bulk soil (Kruskal-Wallis test, p<0.05). The twenty most abundant taxa for each cropping system are shown in Figures S1 (C-NT), Figure S2 (C-IT), Figure S3 (O-RT) and Figure S4 (O-IT).

Because the SILVA taxonomy permitted only limited resolution within *Pseudomonas*, we mapped the V5-V7 16S rRNA gene sequence of the *Pseudomonas* OTUs onto a phylogenetic tree inferred from 46 published strains of the genus *Pseudomonas* (Fig. 3). OTU1 was most closely related to the *Pseudomonas thivervalensis* and *Pseudomonas kilonensis* type strains, while OTU152 was most closely related to the *Pseudomonas agarici* type strain and OTU140 to the *Pseudomonas entomophila* type strain. Although
the short length of the analyzed sequence does not allow a detailed phylogenetic positioning, the obtained results indicate that OTU1 and OTU152 belong to the *P. fluorescens* group whereas OTU140 belongs to the *P. putida* group according to Mulet et al. (2010).

**Figure 2:** Relative abundance of operational taxonomic units (OTUs) assigned to the genus *Pseudomonas* on the roots of wheat and in soil in different agricultural management systems. Amplicon sequencing of the 16S rRNA gene V5-V7 regions was performed on four replicates per cropping system. Taxonomic assignments were determined with the SILVA database. OTUs with a relative abundance greater than 0.1% are shown. Letters show significant differences (Kruskal-Wallis test followed by Dunn post-hoc test, p<0.05). Yellow squares show relative abundances of single replicates. Cropping systems: «C-NT» is conventional without tillage, «C-IT» is conventional with tillage, «O-RT» is organic with reduced tillage, «O-IT» is organic with tillage. Sequencing was performed with samples collected from the field experiment FAST II in 2014.
In a second step, we quantified pseudomonads carrying the well-known antimicrobial genes *phlD*, *phzF* and *prnD* (for description of genes and their function see Table S3).

**Figure 3:** Phylogenetic placement of the *Pseudomonas* OTUs detected in the FAST field experiment 2014 (FAST II). Reads from 16S rRNA V5-V7 region amplicon sequencing (344 bp) were used to infer a maximum likelihood tree with the Jukes–Cantor Model and 1000 bootstrap replications. Partial 16S rRNA sequences of reference strains were obtained from previously published 16S rRNA gene sequences (Mulet, et al. 2010) and genomes (Flury et al, 2016). Type strains for *Pseudomonas* species are indicated with a T.

While OTUs belonging to the genus *Pseudomonas* were not significantly influenced by cropping system at the taxonomic level, we found significant differences in the
abundance of *Pseudomonas* harboring antimicrobial genes between the different cropping systems. Pseudomonads carrying the antimicrobial genes *phlD* and *phzF* were quantified with a qPCR assay specific for the *P. fluorescens* lineage (see Chapter 2), while *prnD* carrying bacteria were quantified with a qPCR assay that detects *prnD*+ *Pseudomonas, Burkholderia* and *Serratia* (Garbeva et al., 2004). However, since the genera *Burkholderia* (average relative abundance: 0.07% on roots and 0.04% in bulk soil) and *Serratia* (average relative abundance: 0.02% on roots and 0.01% in bulk soil) were markedly less abundant than *Pseudomonas* (average relative abundance: 8.8% on roots and 3.13% in bulk soil) in the samples of this study, we assume that the measured *prnD*+ bacteria correspond mainly to *Pseudomonas*. Pseudomonads harboring the gene *phlD* (2,4-diacetylphloroglucinol biosynthesis) were significantly more abundant on roots in conventional farming with no tillage compared to organic farming with reduced tillage in both investigated years (Figure 4A, Figure S5A).
Figure 4: Abundance of bacterial cells harbouring biosynthesis genes for antimicrobial compounds in soils with different agricultural management systems: (A, B) *Pseudomonas* harbouring *phlD* (2,4-diacetylphloroglucinol biosynthesis), (C, D) *Pseudomonas* harbouring *phzF* (biosynthesis of phenazines) and (E, F) bacteria harbouring *prnD* (pyrrolnitrin biosynthesis), (A, C, E) wheat root, (B, D, F) bulk soil. Antimicrobial genes were quantified by qPCR. Detection limits of the qPCR assays are listed in the Materials and Methods section. The dotted line indicates $10^5$ cells per g of dry root. For each cropping system, four biological replicates (four replicate plots) with three technical replicates each were performed. Black lines show medians of pooled data from all replicates. The yellow squares show the averages for each biological replicate. Letters in the graphs indicate significant differences between cropping systems (linear mixed effect model and Tukey’s HSD post hoc test, $p<0.05$. Cropping systems: «C-NT» is conventional without tillage, «C-IT» is conventional with tillage, «O-RT» is organic with reduced tillage, «O-IT» is organic with tillage. This Figure shows results of FAST II, 2014. The abundance of antimicrobial genes on roots and in bulk soil was also measured in FAST I, 2013 (Figure S5).

Similarly, the bulk soil of C-NT harbored more *phlD*+ pseudomonads compared to the O-RT treatment, although here the difference was only significant in 2014 (Figure 4B, Figure S5B). The abundance of pseudomonads carrying *phzF* (biosynthesis of
phenazines) was not significantly different between treatments in both years of sampling (Figures 4C-D and S5C-D). For prnD (biosynthesis of pyrrolnitrin) obtained results differed between the two years. In 2014, there were no significant differences found for the roots (Figure 4E) while in bulk soil, the abundance of bacteria carrying prnD was significantly lower in C-NT compared to C-IT and O-RT (Figure 4F).

Figure 5: Relative expression of genes required for the biosynthesis of antimicrobial compounds (A) 2,4-diacetylphloroglucinol (phlA), (B) phenazines (phzA), (C) pyrrolnitrin (prnA) in soils from different cropping systems planted with spring wheat. Expression was monitored by fluorescence-activated cell-sorting based flow cytometry using GFP-tagged strains of *Pseudomonas protegens* (CHA0-gfp) carrying reporter plasmids pME9012 (phlA-mcherry), or pME11011 (prnA-mcherry) and *Pseudomonas chlororaphis* (PCL1391-gfp) carrying reporter plasmid pME11017 (phzA-mcherry). Seedlings inoculated with the reporter strains were grown in soils for five days prior to analysis of bacterial cells in root washes. Data are shown as relative fluorescence units (RFU) per gram of root dry weight, and were calculated as the median mCherry expression per GFP tagged *Pseudomonas* cell multiplied with the total number of GFP-tagged *Pseudomonas* cells per gram of root. Results from two independent experiments with three replicates each are presented. Since analysis with a linear mixed effect model did not reveal a significant experiment effect, data from the two experiments were pooled for statistical analysis. No significant differences between cropping systems were found (linear mixed effect model followed by Tukey's post-hoc test. \( p < 0.05 \)). Soils from four plots per cropping
system were tested. The yellow squares show average relative expression for each plot. Soils were sampled from FAST II in 2014. Cropping systems: «C-NT» is conventional without tillage, «C-IT» is conventional with tillage, «O-RT» is organic with reduced tillage, «O-IT» is organic with tillage.

However, in 2013 prnD+ bacteria abundances were significantly higher on the roots of C-IT compared to the both organic treatments and in that of C-NT compared to O-IT (Figure S5E) and in bulk soil of C-IT compared to O-RT (Figure S5F). Of the studied bacteria with antimicrobial activity, phlD+ Pseudomonas were on average the most abundant on the roots in 2014 (average abundance: 2.5 x 10^4 cells/g root, Figure 4) and in 2013 (average abundance: 1.9 x 10^5 cells/g root, Figure S5). phzF+ Pseudomonas on roots were on average 8-fold and 70-fold less frequent than phlD+ Pseudomonas in 2014 and 2013, respectively. prnD+ bacteria on roots were 41-fold less frequent than phlD+ Pseudomonas in 2014 and 6-fold less frequent in 2013.

Figure 6: Natural abundance of the pathogens *Pythium ultimum* and *Gaeumannomyces tritici/G. avenae* in soils from different cropping systems planted with winter wheat. (A) *P. ultimum* in bulk soil; (B) *G. tritici/G. avenae* in bulk soil; (C) *P. ultimum* on wheat roots; (D) *G. tritici/G. avenae* on wheat roots. Abundance of pathogens was determined with qPCR assays targeting the internal transcribed spacer region (ITS). Abundance is shown as quantity of pathogen DNA (attograms) per gram of soil or root (dry weight). The detection limits of the qPCR assays were 10 attogram DNA/ g of root or soil (*P. ultimum* assay) and 10^4 attogram/ g root or soil (*G. tritici/G. avenae* assay). For each cropping system, four biological replicates (four replicate plots) with three technical replicates each were analyzed. The yellow squares in the boxplots show the average abundance for each biological replicate. No significant differences between cropping systems could be found for both pathogens (linear mixed effect model and Tukey’s HSD post hoc test, p<0.05). Management system «C-NT» is conventional without tillage, «C-IT» is conventional with tillage, «O-RT» is organic with reduced tillage, «O-IT» is organic with tillage. Data of the sampling in 2014 (FAST II) are shown. The abundance of resident *P. ultimum* and *G. tritici/G. avenae* on roots and in soil was measured also in 2013 (Figure S6).
In addition to the abundance, the expression of antimicrobial biosynthesis genes was measured with a reporter strain based assay on the roots of wheat plants. The results of two experiments were pooled and are shown in Figure 5. The investigated cropping systems had no impact on the expression of the genes *phlA* (biosynthesis of 2,4-diacetylphloroglucinol), *phzA* (biosynthesis of phenazines) and *prnA* (biosynthesis of pyrrolnitrin). The levels of root colonization and gene expression at single cell level were measured as previously described in Chapter 2, but no differences could be observed in the different treatments (data not shown). These results indicate that the investigated cropping systems have not impact on antimicrobial activity.

![Figure 7: Relative resistance of soils from different cropping systems to the soil-borne pathogens (A) *Pythium ultimum* (*Pythium*) and (B) *Gaeumannomyces tritici* (*Gaeumannomyces*). Increasing concentrations of pathogen inoculum were added to the soil before planting with cucumber (*Pythium* experiment) or spring wheat (*Gaeumannomyces* experiment) seedlings. Data shown here are for 0.5 g *Pythium* and 2.0 g *Gaeumannomyces* per pot. Results for the other inoculum concentrations are shown in Figures S7 (*Pythium* experiment) and S9 (*Gaeumannomyces* experiment). Soil resistance is shown as fresh shoot weight of plants in artificially pathogen-infested soil compared to fresh shoot weight of control plants grown in non-infested soil. Soils from four replicate plots per cropping system were tested. For each plot, each pathogen concentration was tested in six replicate pots. Letters indicate significant differences between management systems (linear mixed effect model followed by Tukey's post-hoc test, p<0.05). The yellow squares show average resistance for each plot. Soils were sampled from FAST II in 2014. Cropping systems: «C-NT» is conventional without tillage, «C-IT» is conventional with tillage, «O-RT» is organic with reduced tillage, «O-IT» is organic with tillage.](image)

Complementary to the assessment of beneficial pseudomonads, we also investigated if cropping systems impacted the abundance of *P. ultimum* and *G. tritici* or the disease resistance of the soils to these pathogens. The abundance of naturally present *P. ultimum* and *G. tritici* was assessed with qPCR on roots and bulk soil.
While in 2014 *P. ultimum* could be detected in all biological replicates from all treatments, in bulk soil as well as on roots, *G. tritici* was only occasionally detected, in bulk soil more frequently than on roots (Figure 6). In 2013, both pathogens were only sporadically detected and at a lower abundances than in 2014 (Figure S6). For both years no significant differences in pathogen abundance was detected between cropping systems.

We tested the resistance of the soils to *P. ultimum* and *G. tritici* in a greenhouse experiment, where the pathogen load in the soils was manipulated. At lower *P. ultimum* concentrations, plants growing in soil from O-RT plots tended to have higher shoot weights compared to the other treatments (Figure S7). This difference was more pronounced under higher pathogen pressure. When 0.5 g *P. ultimum* had been added per pot, relative shoot weights of plants grown soil from O-RT plots were significantly higher than those of both conventional treatments (Figures 7A and S7). Moreover, in a similar experiment (see Supplementary Methods) testing the resistance to *P. ultimum* in soil samples from FAST I collected in 2016, organic cropping systems tended to support higher soil resistance to *P. ultimum* than conventional cropping systems (Figure S8). The soils sampled from all cropping systems were completely resistant to *G. tritici* and no reduction of shoot weight in comparison to untreated control plants was observed, even at the highest pathogen concentration (Figures 7B and S9). We excluded the possibility that this lack of plant infection was due to a lack of virulence of the inoculum by conducting an experiment with autoclaved soil (Figure S10). Adding *G. tritici* to autoclaved soil strongly reduced the shoot weight of wheat plants.
Figure 8: Heat map showing normalized medians of relative abundance of Pseudomonas OTUs, resistance to pathogens, pathogen abundance in soil, abundance of cells harboring antimicrobial genes on roots and expression of antimicrobial genes on roots. Medians were normalized by setting the highest median for each measured trait to 100% and showing the other medians of a given trait in % of the highest median. Values can only be compared within rows. Only data from 2014 (FAST II) were used. Soil resistance is the resistance of soils to Pythium ultimum and Gaeumannomyces tritici determined in a greenhouse assay (see Fig. 7). Natural abundance of P. ultimum in soil was determined by qPCR (see Fig. 6). Natural abundance of G. tritici/ G. avenae in soil is not shown as it was below the detection limit in the majority of samples (see Fig. 6). Abundance of antimicrobial gene harboring Pseudomonas was determined by qPCR on roots of wheat (see Figure 4), expression of antimicrobial genes on roots was determined by FACS using mCherry based reporter strains of Pseudomonas protegens CHA0 and Pseudomonas chlororaphis PCL1391 (see Fig. 5) and abundance of Pseudomonas OTUs is the relative abundance within the bacterial microbiome as determined by 16S rRNA amplicon sequencing (see Fig. 2). Cropping system «C-NT» is conventional without tillage, «C-IT» is conventional with tillage, «O-RT» is organic with reduced tillage, «O-IT» is organic with tillage.

To summarize the diverse information obtained in this study, we report the normalized medians for each measured trait in the four tested cropping systems (Figure 8). The heat map shows a trend that conventional cropping systems support higher levels of DAPG and PHZ producers, whereas PRN producers were especially abundant in the organic treatment with intensive tillage. Interestingly, the organic cropping system with reduced tillage displayed the highest resistance to P. ultimum and at the same time, harbored the lowest abundances of the investigated groups of antimicrobial pseudomonads.
This may indicate that DAPG, PRN and PHZ might not be involved in the suppression of this pathogen in the soil of the FAST experiment. No differences between organic and conventional treatments were detected for antimicrobial gene expression. There was no trend observed for conservation tillage systems (reduced and no tillage), where neither the abundance of antimicrobial pseudomonads on roots, nor expression of antimicrobial genes, nor the disease resistance to *P. ultimum* and *G. tritici* were significantly different from the respective intensive tillage treatment (Figure 4, Figure S5, Figure 7, Figures S7-S9).

**3.5. Discussion**

In this study, we investigated relationships between cropping systems, plant-beneficial pseudomonads and soil disease resistance. Our most important findings were the following: The genus *Pseudomonas* was among the three most frequent taxa on roots of field-grown wheat (Figure 1) and its abundance did not vary between the tested cropping systems. With respect to specific groups of pseudomonads with antimicrobial activity, it emerged that conventionally managed no tillage plots promote higher numbers of pseudomonads harboring DAPG genes, which are well known for disease-suppressive activity (Figure 4). A higher number of DAPG producers, however, did not result in enhanced resistance of the soil to the tested root diseases (Figure 7). In contrast, organically managed plots with reduced tillage, which harbored significantly less DAPG producers than conventionally managed plots, were most resistant to *P. ultimum*. These results indicate that in the investigated field experiment, there is no direct relationship between disease resistance and abundance of neither pseudomonads in general, nor specific groups producing the antimicrobial metabolites.

The 16S rRNA gene amplicon sequencing revealed that *Flavobacterium, Variovorax* and *Pseudomonas* were among the most abundant taxa on wheat roots (Fig. 1). In previous studies, *Flavobacterium* was found to be ubiquitous in soils and on roots of many different plants species, such as cereals, pepper, tomatoes and *Arabidopsis* (Schlaeppi et al., 2014) and its function was suggested to be related to organic matter decomposition (Kolton et al., 2016). *Variovorax* is a very common genus in water and soil (Satola et al., 2013) and comprises also strains with plant growth-promoting traits.
(Chen et al., 2013). Earlier studies reported *Pseudomonas* among the abundant bacteria on roots of various plant species, including *Arabidopsis* (Bulgarelli et al., 2012), barley (Bulgarelli et al., 2015), maize (Hacquard et al., 2015), clover (Hartman et al., 2017), as well as cucumber and wheat (Ofek-Lalzar et al., 2014). To our knowledge, the present study is the first to compare the relative abundances of *Pseudomonas* in soil and in wheat root microbiomes between different cropping systems in a common experimental setup under field conditions. We did not detect an impact of tillage or organic farming on the relative abundance of *Pseudomonas* on the roots or in bulk soil. However, in another study *Pseudomonas* were found to be more abundant in soil from a conventionally managed field, compared to soil from an adjacent organically managed field (Pershina et al., 2015).

We identified three OTUs that could be assigned to the genus *Pseudomonas* (OTU1, OTU152 and OTU140). Mapping them to a phylogenetic tree constructed based on the sequenced 16s rRNA gene fragment indicated that the closest relatives of OTU1 and OTU152 are pseudomonads of the *P. fluorescens* group (Figure 3), which are typical root-colonizing bacteria. Indeed, we found that OTU1 and OTU152 were significantly more abundant on roots than in bulk soil (Figure 2). Many type strains in the *P. fluorescens* group have been isolated from plant roots (Flury et al., 2016; Garrido-Sanz et al., 2016). In contrast, OTU140, which was equally abundant on roots and in soil, clustered to the *P. putida* group, i.e. bacteria which have been isolated from different environments such as soils and plant roots (Wu et al., 2011). It is important to mention that the 16S rRNA gene reads obtained from amplicon sequencing are too short to infer a reliable species assignment. In fact, the phylogeny obtained with these short fragments (Figure 3) differs from *Pseudomonas* phylogenies inferred with full length 16S rRNA genes, concatenated housekeeping gene sequences (Mulet et al., 2010), or whole genomes (Flury et al., 2016; Garrido-Sanz et al., 2016).

While the abundance of *Pseudomonas* OTUs, as determined by amplicon sequencing, was not influenced by tillage or organic management, the abundance of pseudomonads carrying antimicrobial genes differed between cropping systems. Our findings that *phlD*+ pseudomonads are more abundant in C-NT compared to O-RT are in agreement with a previous study (Hiddink et al., 2005), where DAPG producers were also more abundant in conventionally managed fields compared to organically managed fields. One reason might be that, in our study, conventionally managed plot
were fertilized with mineral fertilizer, while organically managed plots were fertilized with organic fertilizer (see Table 1). *Pseudomonas* spp. are generally considered to be copiotrophs (Smit et al., 2001; Fierer et al., 2007) and might therefore be favored by the higher concentrations of readily available nutrients in the conventionally managed plots. However, in a previous study, we could not detect differences in the abundance of *phlD*+ pseudomonads on roots between conventional and organic management (see Chapter 4). Also, we did not observe an effect of tillage on the abundance of *phlD* carrying pseudomonads on plant roots here, while Rotenberg et al. (2007) found that *phlD*+ pseudomonads were more abundant in the rhizosphere of maize grown in no tillage plots compared to moderately tilled plots. In contrast to our results for the roots, bulk soil from the conventional no tillage and organic reduced tillage treatments both harbored significantly higher numbers of *phlD*+ pseudomonads than the respective intensive tillage treatments (Figure 4). This suggests that cropping systems with reduced tillage intensity can favor the abundance of these bacteria in soil. For pseudomonads carrying the PHZ biosynthetic gene *phzF*, no significant differences between cropping systems were found, neither in soil nor on the root surface (Figures 4 and S5). While the abundance of PRN producers was previously compared in grassland and arable land (Garbeva et al., 2004), the effect of organic management or reduced tillage on *prnD*+ bacteria is not well known. In another study, we found *prnD*+ bacteria to be significantly less abundant in samples from organic compared to conventional soil (Chapter 4), similarly to the results obtained here for FAST I in 2013 (Figure S5). However, in 2014 (Figure 4) this trend was not confirmed, highlighting the need of studies over multiple growing seasons to understand the link between cropping systems and the abundance of specific groups of microorganisms. The quantification of antimicrobial genes from pseudomonads is an indication for the size of the bacterial population potentially able to produce certain antimicrobial metabolites, but it does not indicate if different cropping systems influence expression levels of these genes. Therefore, we monitored the expression of antimicrobial genes using FACS-based flow cytometry and GFP-marked *Pseudomonas* model strains carrying mCherry-based reporter plasmids. To our best knowledge, the present study is the first assessing expression of antimicrobial metabolite biosynthesis genes in response to different cropping systems. However, we did not detect any significant differences in the expression of DAPG, PHZ or PRN biosynthesis genes on roots of
wheat planted in soil from plots with different cropping systems (Figure 5), suggesting that at the FAST field site the investigated agricultural practices have at most minor impacts on antimicrobial gene expression. Nevertheless, our results only give first indications, since they are obtained with two reporter strains and not by quantifying the expression of naturally present *phlA*, *phzA* and *prnA* genes. Only little is known on expression of antimicrobial genes in agricultural soils, mainly because of methodological challenges associated with the recovery of sufficient quantities of the specific mRNAs from natural soil. Still, some of the factors influencing antimicrobial gene expression in *Pseudomonas* have already been identified. For instance, a recent study (Chapter 2 of this thesis) found correlations between *phlA*, *phzA* and *prnA* expression in reporter strains and organic matter, clay, silt, magnesium, potassium and manganese contents in soil. Another study by Almario et al. (2013b) also showed that expression of *phlA* was influenced by the type of clay present in an artificial soil. In addition, antimicrobial gene expression is strongly influenced by the plant species and even by the plant cultivar, as determined in different studies (Notz et al., 2001; de Werra et al., 2008; Rochat et al., 2010). All these results indicate that soil physical and chemical properties might have a stronger impact on antimicrobial gene expression than the cropping system. However, the expression of antimicrobial genes will have to be addressed in additional field experiments in order to obtain a deeper insight into the interplay of agricultural practices and activity of plant-beneficial soil bacteria.

Dissecting bacterial communities and analysis of known plant-beneficial bacteria in soil delivers information on how certain environmental factors, in this study cropping systems, influence soil ecology. But most important from an agronomical point of view and a prerequisite for the implementation of conservation biocontrol strategies is the knowledge on how cropping systems impact on plant performance and plant health. To this end, we tested the resistance of soils sampled in the FAST experiment to two soilborne pathogens, *P. ultimum* which is major seedling and root pathogen for many different crops, and *G. tritici*, a pathogen attacking wheat roots and causing the take-all disease. While the soil from organically managed plots with reduced tillage was significantly more resistant to *P. ultimum* than soil from conventionally managed plots (Figure 7), the *P. ultimum* qPCR data showed that there were no differences in abundance of resident *P. ultimum* between FAST treatments (Figure 6). This indicates that the naturally present *P. ultimum* population did not affect the outcome of our
disease resistance tests and that the investigated cropping systems do not impact on the numbers of this pathogen in soil. The increased *P. ultimum* resistance of the O-RT plots cannot be accredited to pseudomonads since these plots neither harbored higher numbers of total pseudomonads, nor of antimicrobial *Pseudomonas* groups, nor did soils of these plots support elevated levels of antimicrobial gene expression. We assume that other microorganisms than pseudomonads were responsible for the higher resistance to *P. ultimum* in the organic reduced tillage treatment. This hypothesis is supported by a recent study investigating the abundance of *Pseudomonas* harboring DAPG, and PHZ biosynthetic genes in ten representative Swiss agricultural soils; where no significant correlation between the level of soil disease resistance to *P. ultimum* and the abundance of DAPG+ and PHZ+ *Pseudomonas* was found (see Chapter 2).

Other indications that organically managed soils are more resistant to soilborne pathogens than conventionally managed soils are summarized in a review by van Bruggen and Finckh (2016), describing a reduced disease severity in organically managed plots for *Fusarium* infections, damping off caused by *Rhizoctonia solani* and stalk rot caused by *Sclerotinia sclerotiorum*. In addition, Hiddink et al. (2005) found that take-all disease severity was lower in organically managed compared to conventionally managed fields. In the FAST experiment, we did not observe such an effect, since all the soils sampled from all treatments were completely resistant to *G. tritici* (Figure 7 and Figure S9). Similarly as for *P. ultimum*, the *G. tritici/G. avenae* qPCR results showed that cropping systems had no impact on abundance of naturally present *G. tritici*. Abundance of naturally present *G. tritici* was lower in most samples from our study (Figures 6 and S6), compared to other studies on soils from New Zealand (Bithell et al., 2012a; Keenan et al., 2015). Our experiment comparing autoclaved with natural soils from all FAST treatments with and without addition of *G. tritici*, showed that first, the pathogen inoculum we used was virulent, and second, that autoclaved soils had lost their *G. tritici* resistance (Figure S10). This indicates that the soil of the FAST experiment is indeed resistant to *G. tritici* and that the soil resistance is probably due to biological factors. Whether DAPG producing pseudomonads, which are known to be play a key-role in take-all decline soils (Weller et al., 2002) and which we found to be abundant in the FAST experiment, are involved in the *G. tritici* resistance, remains subject to further studies.
We did not detect any differences in disease resistance between no- or reduced tillage systems and the respective intensive tillage treatments. The influence of reduced tillage on the severity of root diseases is not well studied, although there are indications that no tillage might favor soilborne pathogens by helping them persist on crop residues and roots of volunteer plants (Paulitz, 2006). Moreover, in a study by Steinkellner and Langer (2004) it was found that *Fusarium* spp. were more abundant and diverse in soils managed with conservation tillage than in soils managed with conventional tillage.

Taken together, our results suggest that *Pseudomonas* are among the dominant taxa in the soil as well as on wheat roots in all the studied cropping systems (Figures 1, S1-S4). The *Pseudomonas* OTU with the highest relative abundance probably belongs to the *P. fluorescens* group (Figure 3), which harbors many plant growth-promoting and biocontrol strains (Gomila et al., 2015; Flury et al., 2016). While 16S rRNA gene metagenome sequencing did not reveal any differences in the relative abundance of *Pseudomonas* on wheat roots between cropping systems (Figure 2), *Pseudomonas* spp. producing specific antimicrobial metabolites, i.e. DAPG were more abundant on the roots of wheat grown in conventional systems (Figures 4, 8, S5). These results highlight that it may indeed be possible to selectively favor specific groups of plant-beneficial *Pseudomonas* by adapting the cropping system. However, resistance to *P. ultimum* was highest in O-RT soils, which supported the lowest abundance of DAPG-producing *Pseudomonas* on roots (Figure 8) and was not supportive of PHZ and PRN producers either. This indicates that single taxa of known biocontrol microorganisms cannot be used as bio-indicators for the evaluation of conservation biocontrol strategies. Disease resistance, respectively natural biocontrol of soilborne pathogens is most probably based on the interplay of several beneficial microorganisms and their complex interaction with plant pathogens is influenced by a multitude of biotic and abiotic factors, such as soil physical and chemical characteristics (Chapter 2), plant species (Latz et al., 2015) and cropping history (Landa et al., 2006). Despite the complex interactions that determine disease resistance in soils, our results indicate that certain cropping systems might increase the resistance of soils to specific pathogens. Studies over multiple cropping seasons and field sites, which focus on various plant-beneficial functions within the root-associated microbiome, are needed to identify strategies for conservation biocontrol of soilborne plant pathogens.
3.6. Acknowledgements

We thank Michele Gusberti, Beat Ruffner, Alain Held, Christelle Velatta and Ursula Oggenfuss for technical assistance with field and lab work. We thank Pascale Flury for providing 16S rRNA gene sequences of reference strains. Quantitative PCR data were obtained at the Genetic Diversity Centre Zurich (GDC). Amplicon sequencing data were obtained at the Functional Genomic Centre Zurich (FGCZ). This project was funded by the National Research Program 68 “Sustainable Use of Soil as a Resource” of the Swiss National Science Foundation (grant no. NRP68 406840_143141 awarded to MM and CK; grant no. NRP68 406840-143144 to THMS).

3.7. References


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Faircloth, B.C., and Glenn, T.C. (2012). Not all sequence tags are created equal: designing and validating sequence identification tags robust to indels. *PloS ONE* 7(8), e42543. doi: 10.1371/journal.pone.0042543.


3.8. Supplementary Material

3.8.1 Supplementary Methods

An additional *Pythium ultimum* resistance test was performed with soil samples collected from FAST I in April 2016. Soil samples were collected from all plots of the experiments and samples from the same cropping system were pooled. Soil samples were sieved with a 0.5 cm mesh sieve and stored at 4°C until further use.

The *P. ultimum* resistance test was performed as described in the Materials and Methods section of this study, with some modifications.

In particular, 400 g of soil per pot were used (instead of 200 g per pot), resulting in a lower *P. ultimum* inoculum concentration in soil, compared to the experiment described in the Materials and Methods section. Moreover, 10 technical replicates for each inoculum concentration and cropping system were performed (instead of 6 technical replicates). The results of this experiment are shown in Fig. S8.
### 3.8.2 Supplementary Tables

**Table S1: Primers and probes used for quantitative PCR (qPCR) in this study**

<table>
<thead>
<tr>
<th>Target, gene</th>
<th>Primers and probes</th>
<th>Sequence (5’-3’)</th>
<th>Annealing (°C)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>DAPG(^2), (\text{phlD})</td>
<td>PhlD(_{65F_DEG})</td>
<td>GGT RTG GAA GAT GAA RAA RTC</td>
<td>50°C</td>
<td>Chapter 2</td>
</tr>
<tr>
<td></td>
<td>PhlD(_{153P_DEG})</td>
<td>FAM-ATG GAG TTC ATS ACV GCY TTG TC-BHQ1</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>PhlD(_{236R_DEG})</td>
<td>GCC YRA BAG YGA GCA YTA C</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phenazine, (\text{phzf})</td>
<td>PhzF(_2Fm)</td>
<td>ACC GGC TGT ATC TGG AAA CC</td>
<td>62°C</td>
<td>Chapter 2</td>
</tr>
<tr>
<td></td>
<td>PhzF(_2Pm)</td>
<td>FAM-GCC GCC AGC ATG GAC CAG CCG AT-BHQ1</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>PhzF(_2Rm)</td>
<td>TGA TAG ATC TCG ATG GGA AAG GTC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pyrrolnitrin, (\text{prnD})</td>
<td>PrnD(_F)</td>
<td>TGC ACT TCG CGT TCG AGA C</td>
<td>60°C</td>
<td>Garbeva, et al. (2004)</td>
</tr>
<tr>
<td></td>
<td>PrnD(_P)</td>
<td>FAM-CGA CGG CCG TCT TGC GGA TC-BHQ1</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>PrnD(_R)</td>
<td>GTT GCG CGT CGT AGA AGT TCT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(\text{Pythium ultimum}) (ITS)</td>
<td>92F</td>
<td>TGT TTT CAT TTT TGG ACA CTG GA</td>
<td>60°C</td>
<td>Cullen, et al. (2007)</td>
</tr>
<tr>
<td></td>
<td>116T</td>
<td>FAM-CGG GAG TCA GCA GGA CGA AGG TTG-BHQ1</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>166R</td>
<td>TCC ATC ATA ACT TCG ATT ACA ACA GA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(G.\ tritici/\ G.\ avenae) (ITS)</td>
<td>tritici(_{avenae_F})</td>
<td>AAC TCC AAC CCC TGT GAC CA</td>
<td>60°C</td>
<td>Bithell, et al. (2012)</td>
</tr>
<tr>
<td></td>
<td>tritici(_{avenae_P})</td>
<td>FAM-TCG TCC GCC GAA GCA GCA-BHQ1</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>tritici(_{avenae_R})</td>
<td>CGC TGC GTT CTT CAT CGA TGC C</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Internal control, APA9 plasmid</td>
<td>CMV(_{1F})</td>
<td>TCA TCA TTT CCA CTC CAG GCT C</td>
<td>62°C</td>
<td>Von Felten, et al. (2010)</td>
</tr>
<tr>
<td></td>
<td>CMV(_{1R})</td>
<td>TCA TCC CTC TGC TCA TAC GAC TG</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\(^1\)TaqMan probes were labelled with Fluorescin (FAM) at the 5’ end and with the black hole quencher 1 (BHQ-1) at the 3’ end

\(^2\)DAPG: 2,4-diacetylphloroglucinol
Table S2: Reaction setup and cycling conditions of qPCR assays

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Quantity in reaction mix (final reaction volume=20 µL)</th>
<th>Concentration of stock</th>
<th>Manufacturer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Forward primer</td>
<td>2 µL</td>
<td>10 µM</td>
<td>Microsynth,</td>
</tr>
<tr>
<td>Reverse Primer</td>
<td>2 µL</td>
<td>10 µM</td>
<td>Microsynth</td>
</tr>
<tr>
<td>TaqMan Probe</td>
<td>2 µL</td>
<td>2.5 µM</td>
<td>Microsynth</td>
</tr>
<tr>
<td>Bovine Serum Albumin</td>
<td>0.5 µL</td>
<td>20 mg mL⁻¹</td>
<td>New England</td>
</tr>
<tr>
<td>GeneExpression Mastermix</td>
<td>10 µL</td>
<td>According to manufacturer’s indications</td>
<td>Applied Biosystems, Foster City,</td>
</tr>
<tr>
<td>DNA</td>
<td>2 µL</td>
<td>10-50 ng µL⁻¹</td>
<td></td>
</tr>
<tr>
<td>H₂O</td>
<td>1.5 µL</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Cycling conditions

<table>
<thead>
<tr>
<th>Step</th>
<th>Temperature</th>
<th>Duration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Uracyl Glycosylase Activation</td>
<td>50°C</td>
<td>2 min.</td>
</tr>
<tr>
<td>Initial Denaturation</td>
<td>95°C</td>
<td>10 min.</td>
</tr>
<tr>
<td>Denaturation</td>
<td>95°C</td>
<td>15 sec.</td>
</tr>
<tr>
<td>Annealing</td>
<td>See Table S1</td>
<td>30 sec.</td>
</tr>
<tr>
<td>Elongation</td>
<td>72°C</td>
<td>30 sec.</td>
</tr>
</tbody>
</table>

Reaction mix and cycling conditions were the same for all qPCR assays used in this study targeting the following genes: *phlD* (2,4-diacetylphloroglucinol biosynthesis), *phzF* (biosynthesis of phenazines), *prnD* (pyrrolnitrin biosynthesis), ITS (*P. ultimum*), ITS (*G. tritici/avenae*).

Table S3: Function of genes studied with quantitative PCR and in in-situ reporter strain assay

<table>
<thead>
<tr>
<th>Antimicrobial metabolite(s)</th>
<th>Experiment¹</th>
<th>Gene</th>
<th>Function²</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>DAPG³</td>
<td>Abundance-quantitative real-time PCR</td>
<td><em>phlD</em></td>
<td>Synthesis of phloroglucinols from malonyl-CoA</td>
<td>Bangera and Thomashow (1996); Achkar, et al. (2005)</td>
</tr>
<tr>
<td>DAPG</td>
<td>Expression-in situ reporter strain assay</td>
<td><em>phlA</em></td>
<td>Condensation of monoacetylphloroglucinol to DAPG⁵</td>
<td>Bangera and Thomashow (1996)</td>
</tr>
<tr>
<td>Phenazines</td>
<td>Abundance-quantitative real-time PCR</td>
<td><em>phzF</em></td>
<td>Synthesis of phenazine-1-carboxylic acid</td>
<td>Mavrodi, et al. (1998); Blankenfeldt, et al. (2004); Mentel, et al. (2009)</td>
</tr>
<tr>
<td>Phenazines</td>
<td>Expression-in situ reporter strain assay</td>
<td><em>phzA</em></td>
<td>Synthesis of the intermediate product 6-amino-5-oxocyclohex-2-ene-1-carboxylic acid</td>
<td></td>
</tr>
<tr>
<td>Pyrrolnitrin</td>
<td>Abundance-quantitative real-time PCR</td>
<td><em>prnD</em></td>
<td>Catalyzation of the oxidation in the final step of pyrrolnitrin biosynthesis</td>
<td>Kirner, et al. (1998)</td>
</tr>
</tbody>
</table>

¹Experiment in which the gene was studied (see chapter Material and Methods).
²Function of the gene in the biosynthesis pathway of the antimicrobial metabolite.
³DAPG: 2,4-diacetylphloroglucinol.
3.8.3 Supplementary Figures

**Figure S1:** The twenty most abundant bacterial operational taxonomic units (OTUs) detected on wheat roots and in bulk soil in conventional no tillage (C-NT) soil. Amplicon sequencing of the 16S rRNA V5-V7 region was performed with samples from the field trial FAST II (sampling in 2014). Four replicates were sequenced. Taxonomic assignments were determined with the SILVA database. The highest assigned taxonomic rank is shown. Bars show the average relative abundance and standard errors.

**Figure S2:** The twenty most abundant bacterial operational taxonomic units (OTUs) detected on wheat roots and in bulk soil in conventional with tillage (C-IT) soil. Amplicon sequencing of the 16S rRNA V5-V7 region was performed with samples from the field trial FAST II (sampling in 2014). Four replicates were sequenced. Taxonomic assignments were determined with the SILVA database. The highest assigned taxonomic rank is shown. Bars show the average relative abundance and standard errors.
Figure S3: The twenty most abundant bacterial operational taxonomic units (OTUs) detected on wheat roots and in bulk soil in organic with reduced tillage (O-RT) soil. Amplicon sequencing of the 16S rRNA V5-V7 region was performed with samples from the field trial FAST II (sampling in 2014). Four replicates were sequenced. Taxonomic assignments were determined with the SILVA database. The highest assigned taxonomic rank is shown. Bars show the average relative abundance and standard errors.

Figure S4: The twenty most abundant bacterial operational taxonomic units (OTUs) detected on wheat roots and in bulk soil in organic with tillage (O-IT) soil. Amplicon sequencing of the 16S rRNA V5-V7 region was performed with samples from the field trial FAST II (sampling in 2014). Four replicates were sequenced. Taxonomic assignments were determined with the SILVA database. The highest assigned taxonomic rank is shown. Bars show the average relative abundance and standard errors.
Figure S5: Abundance of bacterial cells harbouring biosynthesis genes of antimicrobial compounds in soils with different agricultural management systems: (A, B) *Pseudomonas* harbouring *phlD* (2,4-diacetylphloroglucinol biosynthesis), (C, D) *Pseudomonas* harbouring *phzF* (biosynthesis of phenazines) and (E, F) bacteria harbouring *prnD* (pyrrolnitrin biosynthesis), (A, C, E) wheat root, (B, D, F) bulk soil. Antimicrobial genes were quantified by qPCR. For detection limits of the qPCR assays, see the Material and Methods section. The dotted line indicates $10^5$ cells per g of root. For each cropping system, four biological replicates (four replicate field plots) with three technical replicates each were performed. The yellow squares show the average abundance for each biological replicate. Letters in the graphs indicate significant differences between cropping systems (linear mixed effect model and Tukey’s HSD post hoc test, $p<0.05$. Cropping systems: «C-NT» is conventional without tillage, «C-IT» is conventional with tillage, «O-RT» is organic with reduced tillage, «O-IT» is organic with tillage. This Figure shows results of FAST I, 2013.
Figure S6: Natural abundance of the pathogens *Pythium ultimum* and *Gaeumannomyces tritici/G. avenae* in soils from different cropping systems planted with winter wheat. (A) *P. ultimum* in bulk soil; (B) *G. tritici/G. avenae* in bulk soil; (C) *P. ultimum* on wheat roots; (D) *G. tritici/G. avenae* on wheat roots. Abundance of pathogens was determined with qPCR assays targeting the internal transcribed spacer (ITS). Abundance is expressed as quantity of pathogen DNA (attograms) per gram of soil or root (dry weight). The detection limits of the qPCR assays were 10 attogram DNA/g of root or soil (*P. ultimum* assay) and $10^4$ attogram/g root or soil (*G. tritici/G. avenae* assay). For each cropping system, four biological replicates (four replicate field plots) with three technical replicates each were analyzed. The yellow squares in the boxplots show the average abundance of each biological replicate. No significant differences between cropping systems could be found for both pathogens (linear mixed effect model and Tukey’s HSD post hoc test, p<0.05). Cropping system «C-NT» is conventional without tillage, «C-IT» is conventional with tillage, «O-RT» is organic with reduced tillage, «O-IT» is organic with tillage. Data of the sampling in 2013 (FAST I) are shown.
Figure S7: Relative resistance of soils from different cropping systems to the soil-borne pathogen *Pythium ultimum* at different inoculum quantities. Increasing concentrations of pathogen inoculum were added to the soil before planting with cucumber seedlings. (A) 0.125 g/pot, (B) 0.25 g/pot, (C) 0.5 g/pot, (D) 1.0 g/pot. Soil resistance is shown as fresh shoot weight of plants in artificially pathogen-infested soil compared to fresh shoot weight of control plants grown in non-infested soil. Soils from four replicate plots per cropping system were tested. For each plot, each pathogen concentration was tested in six replicate pots. Letters indicate significant differences between cropping systems (linear mixed effect model followed by Tukey’s post-hoc test, p<0.05). The yellow squares show average resistance for each biological replicate. Soils were sampled from FAST II in 2014. Cropping systems: «C-NT» is conventional without tillage, «C-IT» is conventional with tillage, «O-RT» is organic with reduced tillage, «O-IT» is organic with tillage.
Figure S8: Relative resistance of soils from different cropping systems to the soil-borne pathogen *Pythium ultimum* at different inoculum quantities, second experiment with soil from FAST I, 2016. Increasing concentrations of pathogen inoculum were added to the soil before planting with cucumber seedlings. (A) 0.125 g/pot, (B) 0.25 g/pot, (C) 0.5 g/pot, (D) 1.0 g/pot. 400 g of soil per pot were used. Soil resistance is shown as fresh shoot weight of plants in artificially pathogen-infested soil compared to fresh shoot weight of control plants grown in non-infested soil. Pooled soil samples from all plots were tested. For each cropping system and pathogen concentration, 10 technical replicates were performed. No significant differences between cropping systems were found (Kruskal-Wallis test, p<0.05). The yellow squares show individual technical replicates. Cropping systems: «C-NT» is conventional without tillage, «C-IT» is conventional with tillage, «O-RT» is organic with reduced tillage, «O-IT» is organic with tillage.
Figure 59: Relative resistance of soils from different cropping systems to the soil-borne pathogen *Gaeumannomyces tritici* at different inoculum quantities. Increasing concentrations of pathogen inoculum were added to the soil before planting with spring wheat seedlings. (A) 0.6 g/pot, (B) 2.0 g/pot, (C) 4.0 g/pot, (D) 8.0 g/pot. Soil resistance is shown as fresh shoot weight of plants in artificially pathogen-infested soil compared to fresh shoot weight of control plants grown in non-infested soil. Soils from four replicate plots per cropping system were tested. For each plot, each pathogen concentration was tested in six replicate pots. Letters indicate significant differences between management systems (linear mixed effect model followed by Tukey's post-hoc test, $p<0.05$). The yellow squares show average resistance for each biological replicate. Soils were sampled from FAST II in 2014. Cropping systems: «C-NT» is conventional without tillage, «C-IT» is conventional with tillage, «O-RT» is organic with reduced tillage, «O-IT» is organic with tillage.
Figure S10: Virulence of *Gaeumannomyces tritici* inoculum used for greenhouse assays. Autoclaved (green bar) or natural (yellow bar) soil was infested with 2 g/pot of *G. tritici* strain I-17 inoculum, and planted with spring wheat var. "Rubli". The fresh shoot weight was measured after 21 days and compared to the fresh shoot weight of spring wheat plants grown in non-infested autoclaved (blue bar) or natural (orange bar) soil. In autoclaved soils, plants grown in pots inoculated with *G. tritici* had a markedly reduced shoot weight compared to plants from autoclaved control pots. In natural soils, the shoot weight was not reduced by *G. tritici* inoculation. Cropping systems: «C-NT» is conventional without tillage, «C-IT» is conventional with tillage, «O-RT» is organic with reduced tillage, «O-IT» is organic with tillage.
CHAPTER 4

Abundance of plant beneficial pseudomonads in the rhizosphere of winter wheat grown in different agricultural management systems

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4.1. Abstract

Sustainable soil management systems, such as organic fertilization and reduced tillage, are increasingly adopted by farmers to protect soils and to decrease the application of mineral fertilizers. However, it is still not well known how these practices influence the presence and abundance of key groups of soil microorganisms, such as fluorescent pseudomonads. This group of bacteria can improve plant health by protecting roots against the attack of soil borne fungal pathogens through the production of antifungal metabolites and by activating plant defence mechanisms.

In this study, the abundance of fluorescent pseudomonads producing the antifungals 2,4-diacyethylphloroglucinol (DAPG), phenazines and pyrrolnitrin was measured in soil and rhizosphere of wheat with a qPCR based approach in two long term trials that compare conventional to organic cultivation, reduced tillage to conventional tillage and monoculture to crop rotation.

DAPG and phenazine producers were significantly less abundant in unfertilized plots compared to plots under conventional or organic cultivation. Phenazine and pyrrolnitrin producers were less abundant in organic plots compared to conventional plots. Monoculture, which had been found to favour the build-up of pseudomonads populations in past studies, had no significant effect on the abundance of any of the three quantified pseudomonads groups. Our results indicate that the quantity of fertilization, rather than the form of fertilization, influences the abundance of plant beneficial pseudomonads in wheat cultivation systems. Further research is needed to identify soil management systems favouring the growth of plant beneficial pseudomonads populations in different cropping systems.

4.2. Introduction

Increasing awareness of the importance of soil protection for agricultural production is leading to the adoption of cultivation practices designed to decrease erosion and compaction, such as organic fertilization and reduced tillage. However, it is not known how these soil management practices affect the abundance of key groups of plant beneficial soil microorganisms, for example fluorescent pseudomonads. Fluorescent pseudomonads, a group of soil bacteria belonging mainly to the species *Pseudomonas protegens*, *Pseudomonas corrugata*, *Pseudomonas fluorescens* and *Pseudomonas chlororaphis*, have many plant beneficial effects. They can directly promote plant
growth or they can improve plant health either by activating plant defence mechanisms or by protecting roots against the attack of soil borne fungal pathogens through the production of secondary metabolites with antifungal effects (reviewed by Haas and Defago (2005). Fluorescent pseudomonads were found to be abundant in suppressive soils, where pathogens were present but plants showed reduced or no symptoms (Weller et al., 2002). Monoculture can lead to an increase in the abundance of certain groups of plant beneficial pseudomonads, therefore leading to a shift from conducive to suppressive soil (Weller et al., 2002; Berendsen et al., 2012). While the effect of tillage and fertilization on the soil microbiome have been studied recently (Hartmann et al., 2015; Degrune et al., 2016), their effect on fluorescent pseudomonads remains largely unknown. Rotenberg et al. (2007) quantified pseudomonads producing the antifungal 2,4-diacetylphloroglucinol (DAPG) in maize fields under different tillage and crop rotation managements, but no clear effects were found.

Therefore, the aim of our study was to compare the abundance of plant beneficial pseudomonads that produce antifungal metabolites in two long-term field trials. The management systems compared in the trials are organic versus conventional fertilization, monoculture versus crop rotation, and conventional tillage versus reduced tillage. Knowledge about management systems that favour plant beneficial pseudomonads could help to limit the damage caused by soilborne fungal pathogens with a conservation biocontrol approach.

4.3. Material and Methods

4.3.1 Long term experiments

Rhizosphere samples were collected in 2013 and 2014 in two long-term experiments in Switzerland. Samples were collected from plots cropped with winter wheat in the year of sampling. Trial DOK, started in 1978 and located in Therwil, Switzerland, compares different fertilization regimes (Mäder et al., 2002). For this study, samples were collected in plots fertilized with mineral fertilizer only (treatment “Mineral”), plots fertilized with organic and mineral fertilizers (treatment “Conventional”), plots fertilized with organic fertilizers only (treatment “Organic”, cultivation according to Swiss regulations for organic agriculture), and plots that have not been fertilized since the start of the experiment (treatment “Unfertilized”). Trial Plot 20, started in 1967 in
Nyon, Switzerland, compares reduced tillage (15 cm depth) to conventional tillage (25 cm depth) in a wheat monoculture and in a 4 year-crop rotation system (Charles et al., 2011).

### 4.3.2 Real-time quantitative PCR

Key groups of biocontrol pseudomonads were quantified in the rhizosphere of winter wheat. Abundance was measured with qPCR targeting biosynthesis genes of metabolites known to have a biocontrol effect against fungal pathogens. Assays for the following genes were developed and tested for specificity: \textit{phzF} (biosynthesis pathway of phenazines) and \textit{phlD} (biosynthesis pathway of 2,4-diacetylphloroglucinol), as described in Chapter 2. A previously published method (Garbeva et al., 2004) was used to quantify \textit{prnD} (biosynthesis pathway of pyrrolnitrin). One pooled sample (five plants) per replicate (four replicates per treatment) was analyzed.

### 4.4. Results and Discussion

Plant beneficial pseudomonads producing the antifungal metabolites DAPG, phenazines and pyrrolnitrin could be detected in all treatments of the DOK trial and in all treatments of the Plot 20 trial. In the DOK trial (Figure 1), the abundance of DAPG producers and phenazine producers was significantly higher in all fertilized treatments compared to the unfertilized treatment. There was no significant difference between samples from conventional and organic plots for the DAPG producers, but phenazine producers were significantly less abundant in the organic treatment. Furthermore no significant difference could be found between conventional (where a mixture of organic and mineral fertilizer was amended) and mineral treatments for any of the three quantified groups of plant beneficial pseudomonads. According to Mäder et al. (2002), the amended amount of soluble nitrogen was approximately 70% lower in the organic treatment compared to the conventional and mineral treatments, while the amended amount of total nitrogen was in a similar range. This result indicates that the final quantity of nutrients in the soil, and not the form in which they are applied, impacts on the abundance of DAPG and phenazine producing pseudomonads in the wheat rhizosphere. In a third long term trial (FAST trial) performed at Agroscope, Zürich Switzerland, where the organic treatment received less nitrogen than the conventional treatment, the abundances of
phenazine and DAPG producers were consistently lower in the organic treatments (Chapter 3). Pyrrolnitrin producing pseudomonads were apparently less influenced by the quantity of nutrients in the soil, since they were not significantly less abundant in the unfertilized treatment compared to the conventional and mineral treatments. Interestingly, lower numbers of pyrrolnitrin producing bacteria were detected in the organic treatments compared to the other three treatments. The qPCR assay used in this study detects also pyrrolnitrin producers belonging to the genera *Burkholderia* and *Serratia* (Garbeva et al., 2004), which might respond differently to the nutrient levels than *Pseudomonas*.

In field trial on plot 20, 47 years of wheat monoculture had no significant effect on any of the three quantified groups of plant beneficial pseudomonads (data not shown), despite the many reports indicating that monoculture can lead to take-all suppressive soils and a higher abundance of plant beneficial pseudomonads (Weller et al., 2002). Reduced tillage also did not lead to any significant differences in the abundance of plant beneficial pseudomonads. It is possible that the close proximity of plots belonging to different treatments led to the transfer of a certain quantity of soil between plots, for example through rain, thus minimizing the differences between treatments.

Our results indicate that soil management systems, in particular fertilization, indeed can have a strong impact on the abundance of plant beneficial pseudomonads in the wheat rhizosphere. However, collection of data over more cropping seasons, at more field sites and with different plant species are needed to better understand which soil management type is best to favour the growth of plant beneficial pseudomonads for a certain crop or environment.
Figure 1: Abundance of antimicrobial metabolite biosynthesis genes from plant beneficial pseudomonads in soils from different agricultural management systems. Abundance of DAPG biosynthesis gene *phlD* (A); abundance of phenazines biosynthesis gene *phzF* (B); and abundance of pyrrolnitrin biosynthesis gene *prnD* (C) in the rhizosphere of wheat harvested from the DOK trial. The dotted line indicates the threshold of $10^5$ gene copies/g root, required for biocontrol effects. Letters indicate significant differences between treatments (linear mixed effect model and Tukey’s HSD, p<0.05).

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4.6. References


CHAPTER 5

Interactions within bacterial and fungal communities in agricultural soils with different levels of disease resistance

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FD performed qPCR assays and amplicon library preparation, analysed the data and wrote the manuscript with critical revisions from KS and MM. KS planned the amplicon sequencing experiment, and provided initial bioinformatics scripts, as well as help with amplicon sequencing data analysis.
5.1. Abstract

Soil and root microbial communities play an important role in defending plants against soilborne pathogens. In particular soils, defined as suppressive soils, pathogens are present but plants show no disease symptoms. The microbiome of these soils has been compared to adjacent conducive soils in previous studies, and certain taxa have been found to be more abundant in suppressive than in conducive soils. However, there is a lack of studies comparing the microbiomes of a larger number of agricultural soils with different levels of disease resistance (e.g. high, low, intermediate) and not much is known about relations between bacterial and fungal members of microbial communities in response to soil disease resistance.

In this study, we analysed nine representative Swiss agricultural soils planted with wheat for co-occurrence patterns in the microbial community, focusing on bacterial genera known to include biocontrol bacteria and fungal genera known to include soil-borne pathogenic fungi, using 16S rRNA gene, respectively ITS amplicon sequencing. We further studied the links between the abundances of bacterial genera as well as of bacterial OTUs and soil resistance to the pathogens *Pythium ultimum* and *Gaeumannomyces tritici*. Moreover, we quantified three functional *Pseudomonas* traits known to be involved in suppression of fungal pathogens, i.e. biosynthesis genes for the antimicrobial metabolites 2,4-diacetylphoroglucinol, phenazines and pyrrolnitrin. Network analysis revealed that keystone taxa displaying most positive or negative relations to other taxa within the microbial community were neither the most abundant fungal and bacterial taxa, nor taxa comprising biocontrol bacteria or fungal pathogens. We detected significant correlations between abundances of certain genera comprising biocontrol bacteria, but very few significant correlations between genera containing biocontrol bacteria and fungal genera containing plant pathogens. Further, we found no significant correlations between abundances of genera containing biocontrol bacteria, such as *Pseudomonas* and *Bacillus*, and soil disease resistance. However, our microbial community analysis showed, if we focus on the OTU level, we find that individual bacterial OTUs, e.g. belonging to the genus *Pseudomonas*, *Streptomyces* and *Flavobacterium*, are significantly positively correlated with soil resistance to *P. ultimum* or *G. tritici*. Moreover, we found significant negative correlations between abundances of specific *Pseudomonas* antimicrobial genes and the oomycete pathogen *Pythium ultimum*, as revealed by qPCR analysis. Our findings
suggest that soil resistance to plant diseases is shaped by specific interactions within the bacterial and fungal soil microbial communities, and that the focus in exploring soil resistance should mostly be on soil microbiome functions, i.e. on abundance and expression of traits known to be involved in soil disease resistance.

5.2. Introduction

The soil and root microbiomes provide a variety of functions for plants, ranging from nutrient acquisition to the defence of plants against fungal and oomycete plant pathogens (Berendsen et al., 2012; Schlaeppi and Bulgarelli, 2015). In particular, the bacterial and fungal community in the soil layer on the root surface (called rhizosphere), form very tight associations with plants, and have been compared to the intestinal microbiome in animals in terms of importance for the host (Berendsen et al., 2012; Mendes and Raaijmakers, 2015; Berg et al., 2017). Soil and root microorganisms that protect plants from pathogenic fungi have been widely studied (Haas and Defago, 2005), and have also been commercialised as biocontrol products (Berg, 2009). Widely used bacterial biocontrol strains belong to a restricted number of bacterial genera (Berg, 2009), mainly *Pseudomonas*, *Bacillus* and *Serratia* (Haas and Defago, 2005; Berg, 2009). Research on soil and root bacteria which are effective against pathogens was first carried out in disease suppressive soils, where plant pathogens are present but plants are healthy (Weller et al., 2002; Lemanceau et al., 2006). While a small number of soils is suppressive to a certain disease (i.e. no disease symptoms are found on plants), in general natural soils show a certain degree of unspecific resistance to several pathogens, if compared to sterilized soils, likely because of the protective effect of the soil microbiome (Haas and Defago, 2005). It has been postulated that specific microbial communities are responsible for the disease resistance of suppressive soils (Weller et al., 2002; Haas and Defago, 2005; Mendes et al., 2011; Klein et al., 2013; Kyselkova et al., 2014; Cha et al., 2016). Especially one group of bacteria, *Pseudomonas* spp. of the *P. fluorescens* group, known for their capacity to produce antimicrobial metabolites, such as 2,4-diacetylphloroglucinol and phenazines, have been suggested to contribute to disease suppressiveness in certain soils (Garbeva et al., 2006; Lemanceau et al., 2006; Weller, 2007; Mazurier et al., 2009). However, these bacterial groups have been found to be present also in disease conducive soils (Mazurier et al., 2009; Kyselkova et al., 2014), and a recent study
(Chapter 2 of this thesis) did not find any significant correlation between the abundance on roots of antimicrobial metabolite producing *Pseudomonas* spp. and soil diseases resistance. These results highlight the need of additional research to understand the soil and root microbiota involved in disease suppressiveness.

Next-generation techniques have opened new perspectives to study the soil bacterial communities involved in interactions with soilborne plant pathogenic fungi (Mauchline and Malone, 2017), in particular to explore the non-culturable part of microbial communities (Busby et al., 2017). Recent studies have characterized the rhizosphere and soil microbiomes in disease suppressive soils and in nearby conducive soils (Mendes et al., 2011; Kyselkova et al., 2014; Xiong et al., 2017). They identified taxa that were associated with suppressive soils, for example Proteobacteria (in particular Pseudomonadaceae, Burkholderiaceae and Xanthomonadales), Firmicutes (Lactobacillaceae) and Actinobacteria in a *Rhizoctonia* suppressive soil (Mendes et al., 2011), Sphingomonadaceae and *Azospirillum* in *Thielaviopsis* suppressive soils (Kyselkova et al., 2014), and the fungal genus *Mortierella* in *Fusarium* suppressive soils (Xiong et al., 2017). Only a small number of studies have analysed and compared the taxa associated with pathogen resistance in different agricultural soils (Köberl et al., 2017; Trivedi et al., 2017). The first of these two studies compared the γ-Proteobacteria populations on healthy and *Fusarium* infested roots of banana plants at different sampling locations in two countries, and found that *Pseudomonas* spp. were more abundant on healthy than on diseased plants (Köberl et al., 2017). The second study compared the microbiomes of 24 agricultural soils in Australia, and identified bacterial taxa associated with inhibition in soils of *Fusarium oxysporum* (Trivedi et al., 2017). Studies comparing microbiomes, or abundances of specific taxa suggested to be involved in plant protection against fungal diseases, such as *Pseudomonas*, in a larger number of agricultural soils with different levels (not only suppressive or conducive) of disease resistance, are missing to date.

In this study, we aimed to increase our knowledge on the relations within the fungal and bacterial communities in agricultural soils and on relations between abundances of bacterial taxa and soil disease resistance. Our investigations focused on nine representative Swiss agricultural soils with a cereal-oriented crop rotation history, displaying different levels of disease resistance/susceptibility to two soilborne pathogens (Chapter 2). At first, a network analysis based on bacterial 16S rRNA gene
and fungal ITS amplicon sequencing was performed to identify keystone taxa in the microbiome of Swiss agricultural soils. Secondly, we investigated whether abundances of certain bacterial taxa, with special emphasis on taxa known to harbour biocontrol bacteria, correlate with soils resistance against the pathogens *Pythium ultimum* and *Gaeumannomyces graminis var. tritici*, recently renamed *G. tritici* (Hernández-Restrepo et al., 2016), or with abundance of certain fungal taxa, with emphasis on those harbouring plant pathogens. At last, we focused on a specific group of biocontrol bacteria, i.e. *Pseudomonas* spp. producing antimicrobial metabolites, and we investigated, using qPCR, whether the abundances of genes required for the biosynthesis of these metabolites in soil and on roots correlate with the abundance of the soilborne pathogen *P. ultimum*, and with soil disease resistance against *P. ultimum* and *G. tritici*.

5.3. Material and Methods

5.3.1 Soil and root sampling

Samples were collected in spring 2013 from nine fields across Switzerland. Sampling sites are described in depth in Chapter 2. Briefly, all sampled fields were planted with winter wheat in the year of sampling, but subjected to crop rotation. Sampling sites were chosen to have different soil physical and chemical characteristics. In each sampling site, five wheat root systems were collected randomly. In parallel, a soil core of 8 cm diameter and 15 cm length was collected between plant rows beside each collected root sample. Soil physical and chemical characteristics were determined in a pooled sample consisting of 1 kg of soil collected randomly at different points in each sampled field, and are given in Table 1 of Chapter 2. Of the sampling sites described in Chapter 2, the site “Witzwil” was excluded in the present study.

5.3.2 DNA extraction

Wheat roots were rinsed with tap water, to keep only soil particles tightly attached to the roots; then blotted with paper and incubated in 50 mL 0.9% NaCl solution overnight at 3°C. The samples were then shaken on a horizontal shaker at 350 rpm for 30 min. to detach root surface material. The roots were separated from the solution and dried at 100°C for 2 days to determine dry weight. The NaCl suspension containing root surface material was centrifuged at 3500 rpm for 20 min. After centrifugation, the
pellet of root surface material that had formed at the bottom of the tube was kept for DNA extraction, while the supernatant was discarded. The root surface material pellet was resuspended and its volume adjusted to 1 mL with 0.9% NaCl. DNA extraction was carried out with the FastDNA extraction kit for soil (MP Biomedicals, Illkirch, France) using 0.5 mL of root surface material pellet or 0.5 g of soil. DNA was extracted following the manufacturer’s protocol with small modifications (in step 1 the quantity of phosphate buffer was reduced to 700 µL, the time of the binding step was increased to 3 min.). DNA concentration was measured with the Qbit broad range dsDNA assay (Thermo Fisher Scientific, Waltham, USA).

5.3.3 Amplicon sequencing
The bacterial root surface and bulk soil microbiome, as well as the fungal soil microbiome, were studied with Illumina amplicon sequencing. To study the bacterial community, the V5-V7 region of the 16S rRNA gene was sequenced, following the method described in Hartman et al. (2017). The same methods for DNA amplification, library preparation, sequencing and sequence processing as described in detail in Chapter 3 were used. The same sequencing run contained both the samples from Chapter 3 and the samples of this chapter.

The fungal soil microbiome was assessed by sequencing a fragment of approximately 300 bp of the Internal Transcribed Spacer (ITS1). Primers used for ITS amplification were fITS7 (5’-GTG ART CAT CGA ATC TTT G-3’) from Ihrmark et al. (2012), and ITS4 (5’-TCC TCC GCT TAT TGA TAT GC-3’) (White et al., 1990). PCRs were carried out with the KAPA HiFi ready mix (KAPA, Wilmington, USA) according to the manufacturer’s protocol. PCR products were purified with Agencourt AMPure XP beads (Beckman Coulter, Indianapolis, USA). Indexing was performed with the Nextera index kit (Illumina, San Diego, USA). Indexed PCR products were pooled to a library containing 2 nM PCR product per sample. Quality and concentration of the obtained libraries were verified with a TapeStation instrument (Agilent, Santa Clara, USA), by Qbit (Thermo Fisher Scientific, Waltham, USA) and by qPCR (Library standard quantification kit, KAPA), after a second cleanup step with Agencourt AMPure XP beads (Beckman Coulter, Indianapolis, USA). A library with a final concentration of 6 nM PCR product was obtained and spiked with 20% PhiX control library (Illumina, San Diego, USA), prior to sequencing. Sequencing was performed on a MiSeq instrument.
(Illumina, San Diego, USA) in paired-end 2 x 300 bp mode, at the Genetic Diversity Centre Zurich (Zurich, Switzerland, http://www.gdc.ethz.ch/).

The raw ITS sequencing read data (available at European Nucleotide Archive database, accession no. PRJEBXXX) were quality filtered with FastQC v0.11.2 (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/), and merged with Usearch v8.1.1812 (Edgar, 2010). Primers were trimmed with Cutadapt v1.4.2 (Martin, 2011). Reads were then quality filtered with PRINSEQ-lite v0.20.4 (Schmieder and Edwards, 2011). The high-quality ITS sequences were trimmed to a fixed length of 230 bp, and clustered to operational taxonomic units (OTUs, ≥ 97% sequence similarity) with UPARSE (Edgar, 2013). Only OTUs with a minimal coverage of two sequences were included. Chimeric OTU sequences were removed with UPARSE. Taxonomy assignment was performed using UTAX (www.drive5.com/usearch/manual/utax_algo.html) and the Unite ITS v7 reference database (http://www2.dpes.gu.se/project/unite/UNITE_intro.htm). The taxonomy of frequent OTUs was additionally hand annotated with BLAST (Altschul et al., 1990).

5.3.4 qPCR-based quantification of Pseudomonas antimicrobial genes and of resident Pythium and Gaeumannomyces

Biosynthesis genes of the antimicrobial metabolites phlD (2,4-diacetylphloroglucinol), phzF (phenazines) and prnD (pyrrolnitrin), were quantified with specific qPCR assays as described in Chapter 2 and Garbeva et al. (2004). The assays for phlD and phzF specifically amplify these genes in Pseudomonas from the P. fluorescens group; while the assay for prnD additionally amplifies prnD genes from Burkholderia and Serratia (see Chapter 2). Two important soilborne pathogens of wheat, Pythium ultimum and Gaeumannomyces tritici/Gaeumannomyces avenae, were quantified with specific qPCR assays targeting the internal transcribed spacer (ITS) gene region, as described in Cullen et al. (2007) and Bithell et al. (2012). Briefly, either DNA extracted from root surface washes (as described above) or from bulk soil was used as template DNA. The reaction setup and the primers used for each reaction are given in Chapter 2. For the quantification of antimicrobial metabolite biosynthesis genes on roots, in-vivo standard curves with cells of strains harboring the respective biosynthesis gene, were serially diluted and inoculated on roots grown in sterilized soil, as described in detail in Chapter 2. This method allows quantifying the number of cells harboring a specific
metabolite biosynthesis gene per gram of root. Since Pseudomonas only harbor one copy per genome of the quantified genes, the number of Pseudomonas harboring antimicrobial genes also corresponds to the number of antimicrobial gene copies (see Chapter 2). For the quantification of antimicrobial biosynthesis genes in bulk soil samples and for the quantification of pathogen ITS, in vitro standard curves with genomic DNA were used. Detection limits of the qPCR assays targeting antimicrobial metabolite genes were 2 genome copies per reaction (phzF in-vivo standard curve), 20 genome copies per reaction (phlD and prnD in-vivo standard curves), 2 genome copies per reaction (phzF and prnD in-vitro standard curves) and 20 genome copies per reaction (phlD in-vitro standard curve). Detection limits of qPCR assays targeting the ITS regions of pathogens were 200 attograms DNA per reaction (P. ultimum) and 10³ attograms DNA per reaction (G. tritici and G. avenae). Samples were always run in triplicates. qPCR reactions were run on a 7500 Fast cycler (Applied Biosystems).

5.3.5 Data analysis

All data were analyzed with the open source software R version 3.3.2 (R Core Team, 2016).

The OTU count and taxonomy tables of the 16S and ITS sequencing were imported in R for further analysis. The difference between the number of reads from different sampling locations and sample types (root surface or soil) was assessed with a non-parametric Kruskal-Wallis test (package “coin”). No significant difference was found; therefore, the data were not rarefied but normalized by the sampling depth. An unconstrained principal coordinate analysis (Bray-Curtis PCoA) was performed with function “ordinate” of the package “vegan”. Alpha diversity indices (observed alpha diversity, Shannon index and inverted Simpson index) were calculated with the function “estimate_richness” of the package “Phyloseq”. Correlation plots were obtained with the package “corrplot”, using a Spearman's rho rank correlation. Network analysis was performed with the package “igraph”, displaying strong Spearman's rho rank correlations with p < 0.01 and rho > 0.7 or rho < (-0.7). P-values were adjusted with the Benjamini and Hochberg method (Benjamini and Hochberg, 1995). OTUs with a relative abundance higher than 0.1% were used for network analysis. Correlations between OTUs containing biocontrol bacteria and OTUs containing plant pathogens were performed with the same methods as the
correlations for the network analyses, except that all OTUs assigned to these genera were included, also OTUs with a relative abundance below 0.1%. For the correlations between OTUs and disease resistance, the median of six technical replicates was used (normalized plant weights from six pots each amended with 0.125 g/pot *P. ultimum* and 0.6 g/pot *G. tritici*, respectively, data from Chapter 2).

The abundances of *phlD*, *phzF*, and *prnD* gene copies in rhizosphere and soil were calculated with the in-vivo standard curves described in section above. Cycle threshold values were normalized for differences in DNA extractions as described in Von Felten et al. (2010). Normalized values were used to calculate the number of gene copies per g of bulk soil or per g of dry root weight. The average values obtained from the three technical replicates for each sample of the qPCR assays were used for statistical analysis. Data were checked for normal distribution with the Shapiro-Wilk test and by plotting QQ-Plots. Equality of variance was verified with Bartlett’s test. Depending on the data distribution, analysis of variance was carried out with a parametric test (ANOVA, significance level p < 0.05) or with a non-parametric test (Kruskal-Wallis test, significance level p < 0.05), followed by a post-hoc test (Tukey’s HSD or kruskalmc from the R package “Pqirmess”). Correlations were inferred with Spearman’s rho rank correlation (significance level p < 0.05).

5.4. Results

5.4.1 Amplicon sequencing of bacterial and fungal microbiomes

Soil and winter wheat root samples were collected in nine farmer’s fields across Switzerland. These field sites are cultivated with crop rotation and were planted with winter wheat in the year of sampling. Soil characteristics at the sampling locations and the resistance of soils to the two fungal pathogens *Pythium ultimum* and *Gaeumannomyces tritici* are described in depth in Chapter 2, and are summarized in Table S1.

To obtain the composition of the bacterial microbiome, three biological replicate samples each of bulk soil, and of a suspension washed from the root surface layer (containing bacteria, exudates and soil particles tightly attached to the roots, hereafter called root samples), were sequenced. The 16S rRNA gene regions V5-V7 were sequenced with Illumina MiSeq (see Materials and Methods section). In total, 2'792'125 high quality, 380 bp reads, were obtained, corresponding to 51'706 reads
per sample on average. A total of 3,210 operational taxonomic units (OTUs) were identified in the dataset, belonging to 31 different phyla (Figure 1). Proteobacteria was the most abundant phylum in soil and in root samples. In soil samples, Bacteroidetes, Actinobacteria, Acidobacteria and Firmicutes were further dominant fila, the latter three were much more abundant than in root samples, which were dominated by Proteobacteria and Bacteroidetes (Figure 1). The dominant phyla were the same for all nine soils. The 20 most abundant OTUs in the bacterial dataset are shown in Figure S1. The dominant OTU was Flavobacterium OTU1 with an average relative abundance of 9.9% in soil and 36.0% on roots. A total of 24 different Flavobacterium OTUs were detected in the dataset, with a cumulative relative abundance of 12.5% in soil and 41.4% on roots, making Flavobacterium the most abundant taxon at the genus level.

Figure 1: Phyla composition of 16S rRNA gene dataset. Amplicon sequencing of the 16S rRNA V5-V7 region was performed with samples from nine Swiss agricultural soils. The sampled fields were under crop rotation and cropped with winter wheat in the year of sampling. The relative abundance of each phylum is shown in % of the total number of quality-filtered reads for a given sampling site and sample type. The average relative abundance of the three biological replicates is shown for each phylum. Taxonomy assignments were determined with the SILVA database.

Bray-Curtis principal coordinate analysis (PCoA) indicated that the bacterial soil microbiomes differ more between sampling sites than the root microbiomes (Figure
However, the greatest variation (54.7% of the total variation) was explained by the difference between root and soil samples. Accordingly, alpha diversity indices indicated a greater diversity in soil samples compared to root samples (Figure S2). Clustering of soils was not related to resistance levels to *P. ultimum* or *G. tritici*. Cadenazzo, Grangeneuve and Eschikon soils, which clustered apart from the others, are soils with a rather low micro and macro nutrient content (Table 1 in Chapter 2 of this thesis). It is however, worth to note that the Vouvry soil, which is a soil highly susceptible to *P. ultimum* and *G. tritici*, displayed the lowest alpha diversity regarding all indices (Figure S2).

**Figure 2: Principal coordinate analysis of the 16S rRNA gene dataset.** Amplicon sequencing of the 16S rRNA V5-V7 region was performed with samples from nine Swiss agricultural soils. The sampled fields were under crop rotation and cropped with winter wheat in the year of sampling. Three replicates for each sampling site and sample type were sequenced. The Bray-Curtis dissimilarity is shown. Colours denote sampling sites and shapes denote the sample type (circles: root samples, triangles: soil samples).

The fungal soil microbiome was characterized by sequencing three biological replicate soil samples for each sampling site, with primers targeting the internal transcribed spacer (ITS). In total, 2’305’699 high quality, 230 bp, reads were obtained, corresponding to 85’396 reads per sample on average. In the entire fungal dataset, 3’844 different OTUs were detected, belonging to six different phyla (Figure 3). The dominant phylum at all sampling sites was Ascomycota, followed by Basidiomycota and Zygomycota. The most abundant OTU in the dataset was *Mortierella ITS_OTU_2*
with an average relative abundance of 4.0% (Figure S3). With 99 different OTUs and a relative abundance of 14.2%, Mortierella was the dominant taxon at the genus level.

Bray-Curtis PCoA indicated that fungal soil microbiomes differed by sampling sites, in particular samples from soils such as Cadenazzo, the soil with the lowest pH, and with a markedly different composition than other soils, clustered separately (Table 1 in Chapter 2). In addition, the Vouvry and Cazis soils, two soils with a high sand, but a low clay content (Table 1, Chapter 2) differed from the others. Alpha diversity indices show different results for particular indices, however, certain soils, such as Taenikon, had a high alpha diversity regarding all indices used, while Vouvry, had consistently low alpha diversity indices (Figure S4).
Figure 4: Principal coordinate analysis of the internal transcribed spacer (ITS) dataset. Amplicon sequencing of the ITS4 region was performed with samples from nine Swiss agricultural soils. The sampled fields were under crop rotation and cropped with winter wheat in the year of sampling. Three replicates for each sampling site were sequenced. The Bray-Curtis dissimilarity is shown. Colours denote sampling sites and shapes denote the sample type (circles: soil samples, root samples were not sequenced).

5.4.2 Network analysis of bacterial and fungal soil microbial communities

The bacterial and fungal dataset of soil samples were combined to infer positive and negative co-occurrence networks. Only bacterial and fungal OTUs with relative abundancies of >0.1% were considered. Strong correlations were considered to construct networks (Spearman’s rho > 0.7 or <-0.7 and p-value <0.01). Keystone taxa, i.e. OTUs with the highest number of strong correlations, were calculated by inferring the node score, and defined as the 1% of OTUs with the highest node scores. In general, a higher number of positive than negative correlations were detected, indicating that co-occurrence relationships were more frequent than exclusion relationships in the combined bacterial and fungal soil microbial community. The positive co-occurrence network (Figure 5), contained 16 clusters, of which two major clusters (consisting of 70 respectively 58 OTUs) contained the highest number of OTUs. These two major clusters contained both bacterial and fungal taxa, while the other clusters consisted mostly of either bacteria or fungi. Keystone taxa in this network were Acidobacteria OTU61, Sphingomonas OTU764 and Jatrophihabitans OTU182. Very frequent OTUs such as Flavobacterium OTU1, Pseudomonas OTU2 or Mortierella ITS_OTU_2 were not among the taxa with high node degrees, indicating that their abundance does not
strongly correlate with the abundance of many other OTUs. This could be due to the high relative abundance of these OTUs in all the analysed soil samples.

The negative correlations network was subdivided in 15 clusters, of which the two biggest clusters contained 53 and 34 OTUs (Figure 6). Of the five major clusters, two clusters, including the biggest one, contained only or almost only bacteria, one cluster only fungi, and two clusters contained both, bacterial and fungal OTUs. The keystone OTUs were *Bradyrhizobium* OTU9 and Acidobacteriaceae OTU56, indicating that these OTUs had the highest number of exclusion relationships compared to other OTUs. As with the positive correlations network, dominant OTUs *Flavobacterium* OTU1 and *Mortierella* ITS_OTU_2 were not among the taxa with the highest node degrees.
Moreover, in both the positive correlations network and in the negative correlation network, neither OTUs assigned to genera containing biocontrol bacteria (Table 1), nor OTUs assigned to fungal genera containing pathogens (Table 2), were among the keystone taxa.

**Figure 6: Network of the combined bacterial and fungal soil community, negative correlations.** Edges (lines) show Spearman's correlations with rho < (-0.7) and p-value <0.01. Vertices (circles) show single operational taxonomic units (OTUs). Vertex size is proportional to the relative abundance of the OTU in the dataset. Vertex colour denotes clusters of OTUs. For the five clusters containing the greatest number of OTUs, the number of bacterial and fungal OTUs is given in brackets. Keystone OTUs were defined as the OTUs with the top 1% number of edges. Keystone OTUs are labelled in red. Additional OTUs belonging to the top ten with the highest number of edges are labelled in black. Selected frequent OTUs are labelled in blue. The highest taxonomic rank is shown. Taxonomic assignments were determined with the SILVA database for bacteria and the UTAX database for fungi.
5.4.3 Genera containing biocontrol bacteria and plant pathogenic fungi in the microbiome

Bacterial genera containing known biocontrol strains (Table 1) and fungal genera containing known soil- and seedborne plant pathogens (Table 2) were selected for further analysis. The selected genera do not exclusively contain biocontrol bacteria or plant pathogenic fungi. For example, *Pseudomonas* can contain biocontrol strains, plant pathogenic strains and human pathogenic strains (Rumbaugh, 2014). Another example is the genus *Fusarium*, which contains both plant pathogenic and plant beneficial strains (Alabouvette, 1986). Of the genera containing biocontrol bacteria, the most abundant genus in the root and soil microbiome was *Pseudomonas*, with seven OTUs in the dataset and a relative abundance ranging from 7.9% to 23.2% on roots and from 2.6% to 6.5% in soil (Table 1 and Table S2). In contrast, other genera containing biocontrol bacteria were less abundant, such as *Bacillus* (14 OTUs, 0.2-1.5% on roots, and 1.8-5.9% in soil), or *Serratia* (1 OTU, 0.02-0.6% on roots, 0.007-0.046% in soil). It has to be noted that the sequences obtained with 16S rRNA gene amplicon sequencing (380 bp) are too short to infer a reliable phylogeny at the species, or sub-species level, therefore the obtained OTUs cannot be assigned to individual species. Genera containing pathogens with the highest relative abundance were *Fusarium* (and its teleomorphs *Michrodochium* and *Gibberella*) as well as *Phoma*. However, the genera containing pathogens were not among the dominant taxa in the dataset, since the most frequent genera containing pathogens, *Phoma* and *Gibberella*, had an average abundance of 0.9% and 0.5% in the dataset, which is low compared to the average abundance of the dominant genus *Mortierella* (14.2%). It has to be considered, however, that it is possible that some of the dominant OTUs, which could not be assigned to a genus, such as Sordariomycetes OTU4 or Hypocreales OTU1804 (Figure S3), could belong to a genus comprising plant pathogenic fungi. Similarly as for bacteria, the ITS fragment obtained with amplicon sequencing (230 bp) is too short to infer a phylogeny at the species or sub-species level, and single OTUs can therefore not be assigned at these taxonomic levels.
Table 1: Bacterial OTUs assigned to genera containing biocontrol bacteria: average relative abundance in soil and on roots, reference reporting biocontrol activity of the genera.

<table>
<thead>
<tr>
<th>Genus (number of OTUs in the dataset)</th>
<th>Range relative abundance root</th>
<th>Range relative abundance soil</th>
<th>Example of species containing biocontrol strains</th>
<th>Pathogens against which there is a biocontrol effect</th>
<th>References that report or review biocontrol effect</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pseudomonas (7 OTUs)</td>
<td>7.87% - 23.15%</td>
<td>2.60% - 6.52%</td>
<td>P. protegens, P. fluorescens, P. chlororaphis and many others</td>
<td>Pythium, Gaeumannomyces, Rhizoctonia, Thielaviopsis and others</td>
<td>Haas and Defago (2005)</td>
</tr>
<tr>
<td>Bacillus (14 OTUs)</td>
<td>0.24% - 1.52%</td>
<td>1.87% - 5.89%</td>
<td>B. subtilis</td>
<td>Rhizoctonia, Fusarium, Pythium</td>
<td>Berg (2009)</td>
</tr>
<tr>
<td>Burkholderia (2 OTUs)</td>
<td>0.009% - 0.29%</td>
<td>0.018% - 0.222%</td>
<td>B. cepacia</td>
<td>Fusarium</td>
<td>Bach et al. (2016)</td>
</tr>
<tr>
<td>Stenotrophomonas (1 OTU)</td>
<td>0.010% - 0.18%</td>
<td>0.005% - 0.036%</td>
<td>S. maltophilia</td>
<td>Verticilliun</td>
<td>Berg et al. (2006)</td>
</tr>
<tr>
<td>Serratia (1 OTU)</td>
<td>0.02% - 0.58%</td>
<td>0.007% - 0.046%</td>
<td>S. plymuthica</td>
<td>Verticilliun, Rhizoctonia and others</td>
<td>Vleesschauwer (2007)</td>
</tr>
<tr>
<td>Streptomyces (10 OTUs)</td>
<td>0.011% - 0.096%</td>
<td>0.11% - 0.37%</td>
<td>S. griseoviridis</td>
<td>Phomopsis, Botrytis, Phytophtora, Pythium</td>
<td>Berg (2009)</td>
</tr>
<tr>
<td>Paenibacillus (27 OTUs)</td>
<td>0.082% - 0.197%</td>
<td>0.20% - 0.67%</td>
<td>P. polymyxa</td>
<td>Fusarium, Leptosphaeria and others</td>
<td>Rybakova et al. (2016)</td>
</tr>
<tr>
<td>Achromobacter (2 OTUs)</td>
<td>0.081% - 0.200%</td>
<td>0.027% - 0.326%</td>
<td>Achromobacter sp.</td>
<td>Rhizoctonia</td>
<td>de Boer et al. (2015)</td>
</tr>
<tr>
<td>Lysobacter (1 OTU)</td>
<td>0.0012% - 0.016%</td>
<td>0.006% - 0.043%</td>
<td>L. antibioticus</td>
<td>Phytophtora</td>
<td>Ko et al. (2009)</td>
</tr>
</tbody>
</table>

In Figure 7, we focused on correlations between bacterial genera potentially containing biocontrol bacteria. On roots, we did not detect many significant correlations, with the exception of some positive correlations, i.e. Pseudomonas with Serratia, and Bacillus with Streptomyces and Paenibacillus. In contrast, in soil, a higher number of significant negative correlations was found between the selected genera; i.e. Pseudomonas with Streptomyces, Stenotrophomonas with Bacillus and
**Paenibacillus**, and *Burkholderia* with *Streptomyces* and *Lysobacter*. These results indicate that competitive relationships might occur between these genera. Only two significant positive correlations were detected in soil, i.e. between *Bacillus* and *Paenibacillus* and between *Burkholderia* and *Achromobacter*.

**Table 2: Fungal OTUs assigned to genera containing plant pathogens, average relative abundance in soil, host plants and disease name**

<table>
<thead>
<tr>
<th>Genus (number of OTUs in dataset)</th>
<th>Range relative abundance</th>
<th>Example species containing pathogenic strains</th>
<th>Host plants of example pathogenic species</th>
<th>Common disease name</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Fusarium</strong> (8 OTUs)</td>
<td>0.11% - 0.68%</td>
<td><em>Fusarium oxysporum</em> f.sp. <em>lycopersici</em></td>
<td>Tomato</td>
<td><em>Fusarium</em> wilt of tomato</td>
</tr>
<tr>
<td><strong>Thielaviopsis</strong> (1 OTU)</td>
<td>0% - 0.010%</td>
<td><em>Thielaviopsis basicola</em></td>
<td>Tobacco</td>
<td>Tobacco black root rot</td>
</tr>
<tr>
<td><strong>Gaeumannomyces</strong> (0 OTUs)</td>
<td>Not detected</td>
<td><em>Gaeumannomyces tritic</em></td>
<td>Wheat</td>
<td>Take-all disease</td>
</tr>
<tr>
<td><strong>Rhizoctonia</strong> (0 OTUs)</td>
<td>Not detected</td>
<td><em>Rhizoctonia solani</em></td>
<td>Various, e.g. wheat</td>
<td>Damping-off, bare patch of cereals etc.</td>
</tr>
<tr>
<td><strong>Phoma</strong> (2 OTUs)</td>
<td>0.029% - 1.48%</td>
<td><em>Phoma beta</em></td>
<td>Beets</td>
<td><em>Phoma</em> blight of beets</td>
</tr>
<tr>
<td><strong>Colletotrichum</strong> (3 OTUs)</td>
<td>0% - 0.028%</td>
<td><em>Colletotrichum coccodes</em></td>
<td>Various, e.g. tomato</td>
<td>Anthracnose of tomato, black dot of potato, etc.</td>
</tr>
<tr>
<td><strong>Verticillium</strong> (5 OTUs)</td>
<td>0% - 1.02%</td>
<td><em>Verticillium dahliae</em></td>
<td>Various, e.g. cucumber</td>
<td><em>Verticillium</em> wilt</td>
</tr>
<tr>
<td><strong>Microdochium</strong> (1 OTU)</td>
<td>0.028% - 0.55%</td>
<td><em>Microdochium nivale</em></td>
<td>Cereals and grasses</td>
<td>Snow molds</td>
</tr>
<tr>
<td><strong>Alternaria</strong> (1 OTU)</td>
<td>0% - 0.87%</td>
<td><em>Alternaria radicina</em></td>
<td>Carrot</td>
<td>Carrot decay</td>
</tr>
<tr>
<td><strong>Ustilago</strong> (1 OTU)</td>
<td>0% - 0.13%</td>
<td><em>Ustilago maydis</em></td>
<td>Maize</td>
<td>Smut</td>
</tr>
<tr>
<td><strong>Bipolaris</strong> (2 OTUs)</td>
<td>0% - 0.035%</td>
<td><em>Bipolaris sorokiniana</em></td>
<td>Cereals</td>
<td>Common root rot</td>
</tr>
<tr>
<td><strong>Gibberella</strong> (anamorph: <em>Fusarium</em>) (1 OTU)</td>
<td>0.13% - 0.94%</td>
<td><em>Gibberella zeae</em></td>
<td>Cereals</td>
<td><em>Fusarium</em> head blight</td>
</tr>
<tr>
<td><strong>Leptosphaeria</strong> (anamorph: <em>Phoma</em>) (4 OTUs)</td>
<td>0.024% - 1.15%</td>
<td><em>Leptosphaeria maculans</em></td>
<td><em>Brassica</em> species</td>
<td>Blackleg disease</td>
</tr>
<tr>
<td><strong>Thanatephorus</strong> (anamorph: <em>Rhizoctonia</em>) (2 OTUs)</td>
<td>0% - 0.071%</td>
<td><em>Thanatephorus curcumensis</em></td>
<td>Various, e.g. wheat</td>
<td>Damping-off, bare patch of cereals etc.</td>
</tr>
<tr>
<td><strong>Tilletia</strong> (5 OTUs)</td>
<td>0% - 0.014%</td>
<td><em>Tilletia caries</em></td>
<td>cereals</td>
<td>Stinking smut of wheat</td>
</tr>
</tbody>
</table>
In Figure 8, we show correlations between the relative abundances of genera containing plant pathogens and the relative abundances of genera containing biocontrol bacteria. However, only a small number of significant negative correlations were detected: between *Paenibacillus* and *Bipolaris/Alternaria*, as well as between *Ustilago* and *Achromobacter*. These correlations were relatively weak, with Spearman’s rho lower than 0.7 for positive correlations and higher than -0.7 for negative correlations.

![Heat map showing correlations between relative abundances of genera containing biocontrol bacteria on the roots and in soil.](image)

**Figure 7: Heat map showing correlations between relative abundances of genera containing biocontrol bacteria on the roots and in soil.** Bacterial genera containing known biocontrol strains (see Table 1) were selected. Spearman’s rho rank correlations between relative abundances of single genera (sum of all operational taxonomic units assigned to a single genus) are shown. Colours indicate positive (green) or negative (red) correlations. Asterisks denote significant correlations (p-value <0.01). Taxonomic assignments were determined with the SILVA database.

When correlations were inferred between the relative abundances of all OTUs assigned either to genera containing biocontrol bacteria, or to genera containing plant pathogens, 55 significant correlations (p <0.01) were detected (Table S3). *Bacillus* OTU36 and *Paenibacillus* OTU2171 had the highest number of correlations with OTUs assigned to genera containing plant pathogens (five correlation each). *Bacillus* OTU36
was either positively or negatively correlated with OTUs belonging to the Ascomycete genera *Colletotrichum* and *Verticillium*, respectively *Microdochium*, *Alternaria* and *Giberella*, while *Paenibacillus* OTU2171 was positively correlated to OTUs belonging to the Ascomycete genera *Fusarium*, *Colletotrichum* and the Basidiomycete genera *Ustilago* and *Tilletia*. Interestingly, no significant correlation was detected between the relative abundances of *Pseudomonas* OTUs and fungal OTUs containing pathogens. However, of the detected 55 correlations, only two were strong correlations with Spearman’s rho higher than 0.7 for positive correlations or lower than -0.7 for negative correlations; these were a negative correlation between *Fusarium* ITS_OTU_345 and *Streptomyces* OTU806; and a positive correlation between *Colletotrichum* ITS_OTU_1611 and *Paenibacillus* OTU2171.

Figure 8: Heat map showing correlations between relative abundances of genera containing biocontrol bacteria and between fungal genera containing plant pathogens in soil. Bacterial genera containing known biocontrol strains (see Table 1) and fungal genera containing soil- and seedborne plant pathogens (see Table 2) were selected. Spearman’s rho rank correlations between relative abundances of single genera (sum of all operational taxonomic units assigned to a single genus) are shown. Colours indicate positive (green) or negative (red) correlations. Asterisks denote significant correlations (p-value < 0.01) Taxonomic assignments were determined with the SILVA database for bacteria and the UTAX database for fungi.
We have further investigated correlations between the relative abundances of bacterial genera known to comprise biocontrol bacteria and soil disease resistance, using the resistance data generated in greenhouse tests in our earlier study (Chapter 2), where we have evaluated the resistance/susceptibility of the nine soils to the two soilborne plant pathogens *P. ultimum* and *G. tritici*. Our results indicate that at the genus level, none of these bacterial taxa is significantly linked to disease resistance of soils (Figure 9).

**Figure 9 | Heat map showing correlations between relative abundances of genera containing biocontrol bacteria and resistance of soils to pathogens.** Bacterial genera containing known biocontrol strains (Table 1) were correlated with the resistance of soils to the pathogens *Pythium ultimum* and *Gaeumannomyces tritici*. Resistance to *P. ultimum* and *G. tritici* was determined in greenhouse assays by artificially inoculating soils with pathogens and comparing the shoot weight of plants grown in control soils and in inoculated soils. Six technical replicates (pots) were performed. Spearman’s rho rank correlations between relative abundances of single genera (sum of all operational taxonomic units assigned to a single genus) and resistance to pathogens are shown. Medians were used for analysis. Taxonomic assignments were determined with the SILVA database. Colours indicate positive (green) or negative (red) correlations. No significant correlations were found (p-value <0.01).

However, analysing the correlation between abundances of frequent bacterial OTUs in the microbial community (relative abundance >0.1%), and disease resistance of soils, revealed significant correlations, which were pathogen-specific (Table 3). For
instance, resistance to *P. ultimum*, but not to *G. tritici* was linked to the relative abundance of Oxalobacteraceae OTU5 and *Pseudomonas* OTU102 on roots (but not to the more frequent *Pseudomonas* OTU2, Table S2), and to the relative abundance of four different bacterial OTUs in soil. On the other hand, soil resistance to *G. tritici*, but not to *P. ultimum* was linked to abundance of Streptomyces OTU538 in soil. In Table 3 we have listed all significant correlations between disease resistance of soils and abundance of frequent bacterial OTUs; for genera containing biocontrol bacteria also less frequent OTUs were included. Indeed most significant positive correlations were detected between soil disease resistance and OTUs not assigned to “biocontrol” genera. For example, resistance to *G. tritici* was linked to the relative abundance of *Flavobacterium* OTU640 on roots (but not to other *Flavobacterium* OTUs). Moreover, resistance to *P. ultimum* was correlated to OTUs belonging to genera such as *Planifilum* or *Roseiflexus*, not known for disease suppressive activity. Taken together, we did not find any significant correlations between soil disease resistance and abundance of bacteria at the genus level, however there were significant correlations at the OTU level.
Table 3: Bacterial OTUs of the abundant community (relative abundance >0.1%), and OTUs from genera containing biocontrol bacteria, strongly correlated (Spearman’s rho > 0.7 or rho < -0.7 and p ≤0.01) with soil resistance to *Pythium ultimum* or *Gaeumannomyces tritici*

<table>
<thead>
<tr>
<th>Type of correlation</th>
<th>OTU</th>
<th>Closest taxonomic assignment</th>
<th>Spearman’s rho (p-value)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Root OTUs positively correlated with resistance to <em>P. ultimum</em></td>
<td>OTU102</td>
<td><em>Pseudomonas</em></td>
<td>0.81 (0.010)</td>
</tr>
<tr>
<td></td>
<td>OTU5</td>
<td>Oxalobacteraceae</td>
<td>0.90 (0.002)</td>
</tr>
<tr>
<td>Root OTUs negatively correlated with resistance to <em>P. ultimum</em></td>
<td>none</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Soil OTUs positively correlated with resistance to <em>P. ultimum</em></td>
<td>OTU2742</td>
<td><em>Paenibacillus</em></td>
<td>0.83 (0.00555)</td>
</tr>
<tr>
<td></td>
<td>OTU203</td>
<td><em>Planifilum</em></td>
<td>0.83 (0.00826)</td>
</tr>
<tr>
<td></td>
<td>OTU171</td>
<td>Chloroflexi TK10</td>
<td>0.93 (0.00074)</td>
</tr>
<tr>
<td></td>
<td>OTU188</td>
<td><em>Roseiflexus</em></td>
<td>0.83 (0.00826)</td>
</tr>
<tr>
<td>Soil OTUs negatively correlated with resistance to <em>P. ultimum</em></td>
<td>none</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Root OTUs positively correlated with resistance to <em>G. tritici</em></td>
<td>OTU826</td>
<td><em>Flavobacterium</em></td>
<td>0.85 (0.00607)</td>
</tr>
<tr>
<td>Root OTUs negatively correlated with resistance to <em>G. tritici</em></td>
<td>none</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Soil OTUs positively correlated with resistance to <em>G. tritici</em></td>
<td>OTU538</td>
<td><em>Streptomyces</em></td>
<td>0.89 (0.00094)</td>
</tr>
<tr>
<td>Soil OTUs negatively correlated with resistance to <em>G. tritici</em></td>
<td>none</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

5.4.4 Relations between *Pseudomonas* biocontrol traits and pathogen abundance/soil disease resistance

In addition to investigating relations between soil bacteria and disease resistance at the taxonomic level, we also analysed the link between abundance of specific biocontrol traits (biosynthesis genes of antimicrobial metabolites) and pathogen abundance respectively soil disease resistance. To this end, we quantified the biosynthetic genes *phlD*, *phzF* and *prnD*, required for the biosynthesis of the antimicrobial compounds 2,4-diacetylphloroglucinol, phenazines and pyrrolnitrin, respectively, in the nine sampled soils using qPCR. Results are shown in Figures S5 and S6. Significant differences were found between soils for all three genes and a soil supporting high amounts of one gene did not necessarily support equally high amounts of the other investigated genes. E.g. the Grangeneuve soil harboured up to $10^6$ *prnD* copies per g of soil, whereas the *phlD* cells were around the detection limit in
this soil. On average, \( \text{phlD} \) was the most abundant of the investigated genes on roots, but the least abundant in soil, where \( \text{prnD} \) was the most abundant. In particular, \( \text{phlD} \) was below or close to the detection limit in soil samples from four soils. In addition, the pathogens \( \text{P. ultimum} \) and \( \text{G. tritici} \) were quantified by qPCR (Figure S7).

![Figure 10](image)

**Figure 10** | Heat map showing correlations between abundances of antimicrobial genes on roots and in soils, \( \text{Pythium ultimum} \) abundance on roots and in soils, and soil resistance to \( \text{Pythium ultimum} \) and \( \text{Gaeumannomyces tritici} \) in greenhouse tests. The abundance of bacterial biosynthesis genes \( \text{phlD} \) (2,4-diacetylphloroglucinol), \( \text{phzF} \) (phenazines) and \( \text{prnD} \) (pyrrolnitrin) were quantified by qPCR. \( \text{P. ultimum} \) and \( \text{G. tritici/avenae} \) were quantified with specific qPCR assays targeting the internal transcribed spacer (ITS) region. \( \text{G. tritici/avenae} \) abundance data were excluded from the analysis because \( \text{G. tritici/avenae} \) was below the detection limit in most samples. Heat maps show Spearman’s rho rank correlations. Colour indicates rho values (positive correlations: green, negative correlations: red). Asterisks indicate significant correlations (\( p < 0.05 \)). **A** Medians of qPCR results were correlated with disease resistance greenhouse assays (medians). **B** qPCR data correlated alone (five biological replicates per sampling site for each root and soil samples).
P. ultimum was detected in root and soil samples from all sampling sites, but significant differences between sites were found. G. tritici was detected only in soil samples and only in samples from three sites (Cadenazzo, Courtedoux and Eschikon). No significant correlation was found between disease resistance of soils and the abundance of biosynthesis genes of antimicrobial metabolites (Figure 10A), although there was a tendency of disease resistance being positively correlated with phlD and phzF, but not prnD abundance. Accordingly, P. ultimum abundance in soil (determined by qPCR) was significantly negatively correlated with phlD and phzF abundance on roots, and with all three genes in soil (Figure 10B). Two Pseudomonas OTUs correlated with abundances of Pseudomonas antimicrobial genes: OTU423 with abundance of phlD in soil and OTU890 with abundance of phzF on roots (Figure S8).

5.5. Discussion

In this study, we analysed the co-occurrence patterns of the bacterial genera and fungal genera in the microbial communities of nine representative Swiss agricultural soils, with emphasis on genera comprising biocontrol bacteria, or plant pathogenic fungi. Furthermore, we investigated links between bacterial abundances and soil disease resistance. To our best knowledge, this is the first study looking at interactions within fungal and bacterial communities in relation to soil disease resistance based on investigations on a larger number of agricultural soils. Moreover, it is the first study analysing specifically the links between soil disease resistance, and genera containing biocontrol bacteria and genera containing pathogenic fungi at the OTU level. Principal coordinate analysis revealed that some of the nine soils harbour distinct fungal and bacterial communities, compared to other soils. These differences are probably due to different soil characteristics (Table 1 in Chapter 2). Regarding bacterial communities, soils with low macro and micronutrient content harbour a different composition of bacteria (Figure 2). In fungal communities, soils which have, compared to the other investigates soils, a low clay but rather high sand content, or the soil with the lowest pH, cluster separately (Figure 4). In particular, pH has been found to shape microbial communities in soils (Fierer and Jackson, 2006; Rousk et al., 2010; de Menezes et al., 2015), while clay and sand soil contents had less important effects on bacterial communities (Fierer and Jackson, 2006). Root samples did not form distinct clusters based on the soil type in our study (Figure 2). In contrast, in
other studies, the soil type has been found to strongly shape the rhizosphere microbiome (Berg and Smalla, 2009; Inceoglu et al., 2012; Schreiter et al., 2014). Interestingly, the Vouvry soil, which is the soil with the highest susceptibility to soilborne diseases, displays the lowest diversity (Figure S2, Table S1). This is in accordance with previous studies, where a high microbial diversity has been associated with disease suppressiveness (Postma et al., 2008; Hu et al., 2016; Berg et al., 2017). We could, however, comparing all soils, not detect any link between disease resistance and community structure or diversity.

Of the genera containing biocontrol bacteria, *Pseudomonas* was the most abundant, followed by *Bacillus* (Table 1). OTUs of both genera were among the twenty most abundant (Fig. S1). *Pseudomonas* and Pseudomonadaceae have been found to be abundant on roots and in soils in previous microbiome studies (Bulgarelli et al., 2012; Ofek-Lalzar et al., 2014). Similarly, *Bacillus* has been recovered frequently from agricultural soils and from plant roots (McSpadden Gardener, 2004). Of the examined genera containing soil- or seedborne fungal plant pathogens, *Phoma*, as well as *Fusarium* and its teleomorphs, were the most abundant (Table 2). *Fusarium* abundance determined in our study was rather comparable to the suppressive soil in Xiong et al. (2017), whereas in their conducive soil it was the dominant genus, with 17.2% relative abundance. Surprisingly, common soilborne pathogens such as *Rhizoctonia* or *Gaeumannomyces* could not be detected in our dataset. One reason that could explain these results is that all sampled soils were under crop rotation, and therefore certain pathogens could not establish themselves and become dominant in the microbial community (Larkin, 2015). Accordingly, no disease symptoms were observed on wheat roots at sampling. Moreover, a limitation of our study was that the used primers for ITS amplicon sequencing did not amplify oomycetes, therefore we have no data on some common plant pathogens in the microbial community of agricultural soils, such as *Pythium* and *Phytophthora* (Agrios, 2005). It must be kept in mind, that our analysis was based on only 380 bp sequences of the 16S rRNA gene for bacteria, and on 230 bp sequences of the ITS region for fungi, and did therefore not allow to identify biocontrol bacteria or plant pathogenic fungi at the species level. Because of these short sequences, we could not compare specific beneficial or pathogenic bacterial and fungal species in communities of different soils.
Network analysis of the combined bacterial and fungal datasets in the soil revealed that neither most abundant fungal and bacterial OTUs, nor OTUs assigned to genera containing biocontrol bacteria, nor OTUs assigned to genera containing pathogenic fungi were among the keystone taxa (Figure 5 and Figure 6). Keystone taxa are taxa that have the highest number of strong correlations with other taxa in a dataset (Berry and Widder, 2014), therefore these results suggest that OTUs assigned to genera comprising biocontrol bacteria or pathogenic fungi are not among the organisms shaping the microbial community. In the investigated agricultural soils, higher numbers of strong bacteria-bacteria and fungi-fungi correlations were observed, compared to correlations between bacteria and fungi. This result is in contrast with a previous study in forest and pasture soils, where the microbial community network clusters were composed of a balanced number of bacterial and fungal taxa (de Menezes et al., 2015).

Correlation analysis detected a small number of significant correlations between individual genera containing biocontrol bacteria (Fig. 7); and between genera containing biocontrol bacteria and genera containing pathogenic fungi (Fig. 8). Although our analysis did not allow to investigate correlations at the species or subspecies level, our results indicate that abundances of some bacterial genera harbouring known biocontrol isolates are negatively correlated, e.g. *Pseudomonas* with *Streptomyces* or *Bacillus* with *Stenotrophomonas*. These results suggest that it could be difficult to adapt agricultural management systems in a way that favours different genera containing biocontrol bacteria, since they might require different soil conditions, or might occupy the same niches and therefore compete with each other (Hol et al., 2013). Accordingly, in field experiments combining different biocontrol bacteria, synergistic as well as antagonistic relationships could be observed (Hol et al., 2013).

A major focus of our study was to investigate whether certain bacterial taxa or OTUs are indicators for soil disease resistance. Interactions in the soil between pathogenic fungi and biocontrol bacteria are likely to be very specific, with specific pathogenic strains being inhibited by specific bacterial strains. Therefore, it was not surprising that we did not detect any significant correlations between the abundance of bacterial genera, or higher taxonomic levels, and soil resistance. However, in other studies, such links were identified. For instance, a *Rhizoctonia* suppressive soil was associated with
a higher relative abundance in the microbial community of Pseudomonadaceae, Burkholderiaceae, Xanthomonadales and Lactobacillaceae (Mendes et al., 2011). Similarly, also a Fusarium suppressive soil was found to harbour a high abundance of Burkholderiaceae and Xanthomonadales (Campos et al., 2016). In another study, Fusarium suppressiveness was associated with a higher abundance of Bacillus, Paenibacillus and Streptomyces (Klein et al., 2013). In contrast to the genus level, at the OTU level, we found that certain bacterial OTUs significantly correlate with P. ultimum or G. tritici soil disease resistance (Table 3). Interestingly, the same OTUs were never associated with resistance to both pathogens, but distinct OTUs were associated to the resistance of each pathogen. Similar to the relations between soil resistance and bacteria, also for relations between fungi and bacteria many more significant correlations were detected at the OTU level than at the genus, or higher taxonomic levels, indicating very specific interactions (Table S3). Some bacterial OTUs, for example Bacillus OTU36 were positively linked with certain fungal OTUs from genera containing pathogens but negatively with others. These findings further indicate that we cannot link higher taxonomic units, such as genera, and probably not even species, to soil disease resistance. However, it has to be considered that the correlations between bacterial and fungal OTUs found in this study might be due to similar or opposite links to soil abiotic factors rather than to direct interactions. Bacterial interactions with fungal pathogens are probably rather dependent on microbial functions and not on taxonomy, which was also suggested by Lemanceau et al. (2017). A proposed hypothesis is that in the core microbiome of plants, bacteria that have essential functions for plants are always present, but can belong to different taxa depending on the soil conditions or the location (Lemanceau et al., 2017). In this study, we quantified genes required for the biosynthesis of three different antimicrobial metabolites, representing traits known to play a role in the suppression of soilborne plant diseases. We found significant, negative correlations between the abundance of antimicrobial genes and P. ultimum abundance in soil (Figure 10). However, soil resistance against both pathogens, P. ultimum and G. tritici, was positively, but not significantly correlated with the abundance of genes phlD and phzF on roots, (Figure 10A). This might indicate that other bacterial functional traits are involved in disease resistance in soils. For instance, Lemanceau et al. (2017) lists genes involved in biosynthesis of 21 different secondary metabolites involved in interactions
with pathogens, a number which will certainly increase, since constantly new microbial compounds with potential to contribute to disease resistance are discovered (Nguyen et al., 2016).

In conclusion, the interactions between antagonistic bacteria and pathogenic fungi in the soil microbiome are very specific, and probably can be better characterized by studying bacterial functional traits than taxonomical assignments (Morrissey et al., 2016). Therefore, it is difficult to identify a microbiome composition indicative of disease resistance based on taxonomic studies (Schlaeppi and Bulgarelli, 2015; Lemanceau et al., 2017). Functional studies, where the abundance and expression of plant protecting traits are combined with bioassays based on different pathogen, host plant and soil combinations, are likely better suited to understand how beneficial bacteria and pathogens interact in the microbiome of agricultural soils.

5.6. Acknowledgements
We thank farmers and research station managers for access to their fields: Mario Bertossa (Cadenazzo), Padruot Salzgeber (Cazis), Michel Petitat (Courtedoux), Karl Camp (Delley), Hanspeter Renfer (Eschikon), André Chassot (Grangeneuve), Thomas Anken (Taenikon), Jürg Hiltbrunner (Utzenstorf), Quentin Lassueur (Vouvry). We thank Michele Gusberti for assistance with fieldwork. 16S rRNA amplicon sequencing data were obtained at the Functional Genomics Centre Zurich (FGCZ) and ITS amplicon sequencing data were obtained at the Genetic Diversity Centre Zürich (GDC). We thank Lucy Poveda (FGCZ) and Silvia Kobel (GDC) for assistance with library preparation and sequencing. This project was funded by the National research Programme 68: “Soil as a Resource” of the Swiss National Science Foundation (grant number: 406840_143141).

5.7. References


5.8. Supplementary Tables and Figures

5.8.1 Supplementary Tables

Table S1: Soil disease resistance against *P. ultimum* and *G. tritici* (determined in Chapter 2)

<table>
<thead>
<tr>
<th>Sampling site</th>
<th>Resistance against <em>P. ultimum</em> (%)&lt;sup&gt;1&lt;/sup&gt;</th>
<th>Resistance against <em>G. tritici</em> (%)&lt;sup&gt;2&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cadenazzo</td>
<td>22.2</td>
<td>71.7</td>
</tr>
<tr>
<td>Cazis</td>
<td>106.1</td>
<td>68.4</td>
</tr>
<tr>
<td>Courtedoux</td>
<td>25.6</td>
<td>66.9</td>
</tr>
<tr>
<td>Delley</td>
<td>26.0</td>
<td>82.6</td>
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<td>Eschikon</td>
<td>66.7</td>
<td>49.7</td>
</tr>
<tr>
<td>Grangeneuve</td>
<td>57.8</td>
<td>31.4</td>
</tr>
<tr>
<td>Taenikon</td>
<td>34.6</td>
<td>107.8</td>
</tr>
<tr>
<td>Utzenstorf</td>
<td>68.8</td>
<td>68.6</td>
</tr>
<tr>
<td>Vouvry</td>
<td>0</td>
<td>38.1</td>
</tr>
</tbody>
</table>

<sup>1</sup>Resistance is given as shoot weight of *P. ultimum*-inoculated plants, compared to shoot weight of control plants, in %. The inoculated plants were collected from pots with 0.125 g inoculum/pot. The medians from six technical replicates per sampling site are given.

<sup>2</sup>Resistance is given as shoot weight of *G. tritici*-inoculated plants, compared to shoot weight of control plants, in %. The inoculated plants were collected from pots with 0.6 g inoculum/pot. The medians from six technical replicates per sampling site are given.

Table S2: Relative abundance of *Pseudomonas* OTUs in root and soil samples

<table>
<thead>
<tr>
<th><em>Pseudomonas</em> OTU</th>
<th>Range relative abundance root (%)</th>
<th>Range relative abundance soil (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>OTU2</td>
<td>6.28-19.1</td>
<td>2.05-5.08</td>
</tr>
<tr>
<td>OTU102</td>
<td>0.99-3.51</td>
<td>0.36-1.31</td>
</tr>
<tr>
<td>OTU2321</td>
<td>0.13-1.96</td>
<td>0.032-0.193</td>
</tr>
<tr>
<td>OTU23</td>
<td>0.15-0.54</td>
<td>0.095-0.323</td>
</tr>
<tr>
<td>OTU423</td>
<td>0.007-0.092</td>
<td>0.006-0.035</td>
</tr>
<tr>
<td>OTU890</td>
<td>0-0.026</td>
<td>0.0006-0.017</td>
</tr>
<tr>
<td>OTU3029</td>
<td>0.001-0.010</td>
<td>0-0.007</td>
</tr>
</tbody>
</table>
Table S3: Significant correlations (p < 0.01) between relative abundances of OTUs assigned to genera containing pathogens and OTUs assigned to genera containing biocontrol bacteria in soil.

Strong correlations (Spearman's rho > 0.7 or < -0.7 and p < 0.01) are in bold.

<table>
<thead>
<tr>
<th>OTU of genus containing pathogens</th>
<th>OTU of genus containing biocontrol bacteria</th>
<th>Spearman's rho</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fusarium ITS_OTU_2313</td>
<td>Serratia OTU57</td>
<td>-0.492673993</td>
<td>0.009032326</td>
</tr>
<tr>
<td>Fusarium ITS_OTU_2313</td>
<td>Streptomyces OTU670</td>
<td>0.511599541</td>
<td>0.006379238</td>
</tr>
<tr>
<td>Fusarium ITS_OTU_2313</td>
<td>Streptomyces OTU2397</td>
<td>0.53107655</td>
<td>0.004367835</td>
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<tr>
<td><strong>Fusarium ITS_OTU_345</strong></td>
<td><strong>Streptomyces OTU806</strong></td>
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<td><strong>4.69E-05</strong></td>
</tr>
<tr>
<td>Fusarium ITS_OTU_345</td>
<td>Streptomyces OTU2837</td>
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<td>Fusarium ITS_OTU_2059</td>
<td>Streptomyces OTU2097</td>
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</tr>
<tr>
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<td>Paenibacillus OTU282</td>
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<td>Bacillus OTU719</td>
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<td>Spearman’s rho</td>
<td>p-value</td>
</tr>
<tr>
<td>----------------------------------</td>
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5.8.2 Supplementary Figures

**Figure S1**: The twenty most abundant bacterial operational taxonomic units (OTUs) detected on wheat roots and in bulk soil. Amplicon sequencing of the 16S rRNA V5-V7 region was performed with samples from nine Swiss agricultural soils. The sampled fields were under crop rotation and cropped with winter wheat in the year of sampling. Three replicates for each sampling site and sample type were sequenced. Taxonomic assignments were determined with the SILVA database. The highest assigned taxonomic rank is shown. Bars show the average relative abundance and standard errors.
Figure S2: Alpha diversity measures of the 16S rRNA gene dataset. Amplicon sequencing of the 16S rRNA V5-V7 region was performed with samples from nine Swiss agricultural soils. The sampled fields were under crop rotation and cropped with winter wheat in the year of sampling. Three replicates for each sampling site and sample type were sequenced. The observed alpha diversity, the Shannon index and the inverse Simpson index are shown. Colours denote sampling sites and shapes denote the sample type (circles: root samples, triangles: soil samples).
Figure S3: The twenty most abundant fungal operational taxonomic units (OTUs) detected on wheat roots and in bulk soil. Amplicon sequencing of the ITS4 region was performed with samples from nine Swiss agricultural soils. The sampled fields were under crop rotation and cropped with winter wheat in the year of sampling. Three replicates for each sampling site were sequenced. Taxonomic assignments were determined with the UTAX database. The highest assigned taxonomic rank is shown. Bars show the average relative abundance and standard errors.
Figure S4: Alpha diversity measures of the internal transcribed spacer (ITS) dataset. Amplicon sequencing of the ITS4 region was performed with samples from nine Swiss agricultural soils. The sampled fields were under crop rotation and cropped with winter wheat in the year of sampling. Three replicates for each sampling site were sequenced. Root samples were not sequenced. The observed alpha diversity, the Shannon index and the inverse Simpson index are shown. Colours denote sampling sites.
Figure S5: Abundance of bacterial biosynthesis genes for antimicrobial compounds on root samples from different sampling sites: (A) *phlD* (2,4-diacetylphloroglucinol biosynthesis), (B) *phzF* (phenazine biosynthesis) and (C) *prnD* (pyrrolnitrin biosynthesis). Antimicrobial genes were quantified by qPCR. Detection limits of the qPCR assays are listed in the Materials and Methods section. The dotted line indicates $10^5$ gene copies per g of dry root. For each sampling site, five biological replicates with three technical replicates each were performed. Black lines show medians of pooled data from all replicates. The grey squares show the averages of the technical replicates for each biological replicate. Letters in the graphs indicate significant differences between sampling sites (Kruskal-Wallis and Dunn post hoc test, $p<0.05$. Sampling sites: Cd: Cadenazzo, Cx: Courtedoux, Cz: Cazis, De: Delley, Es: Eschikon, Gr: Grangeneuve, Ta: Taenikon, Ut: Utzenstorf, Vo: Vouvry.)
Figure S6: Abundance of bacterial biosynthesis genes for antimicrobial compounds in soil samples from different sampling sites: (A) *phlD* (2,4-diacetylphloroglucinol biosynthesis), (B) *phzF* (phenazine biosynthesis) and (C) *prnD* (pyrrolnitrin biosynthesis). Antimicrobial genes were quantified by qPCR. Detection limits of the qPCR assays are listed in the Supplementary Materials and Methods section. The dotted line indicates $10^5$ gene copies per g of soil. For each sampling site, five biological replicates with three technical replicates each were performed. Black lines show medians of pooled data from all replicates. The grey squares show the averages of the technical replicates for each biological replicate. Letters in the graphs indicate significant differences between sampling sites (Kruskal-Wallis and Dunn post hoc test, $p<0.05$). Sampling sites: Cd: Cadenazzo, Cx: Courtedoux, Cz: Cazis, De: Delley, Es: Eschikon, Gr: Grangeneuve, Ta: Taenikon, Ut: Utzenstorf, Vo: Vouvry.
Figure S7: Abundance of pathogens *Pythium ultimum* and *Gaeumannomyces tritici/avenae* on roots and in soils. *P. ultimum* and *G. tritici/avenae* were quantified with specific qPCR assays targeting the internal transcribed spacer (ITS). Detection limits of the qPCR assays are listed in the Materials and Methods section. For each sampling site, five biological replicates with three technical replicates each were performed. Black lines show medians of pooled data from all replicates. The grey squares show the averages of the technical replicates for each biological replicate. Letters in the graphs indicate significant differences between sampling sites (Kruskal-Wallis and Dunn post hoc test, p<0.05. *G. tritici/avenae* abundance data for root samples were not analysed statistically, since *G. tritici/avenae* was below the detection limit. Sampling sites: Cd: Cadenazzo, Cx: Courtedoux, Cz: Cazis, De: Delley, Es: Eschikon, Gr: Grangeneuve, Ta: Taenikon, Ut: Utzenstorf, Vo: Vouvry
Figure S8: Heat map showing correlations between the relative abundances of OTUs assigned to the genus *Pseudomonas* and the abundance of biosynthesis genes of antimicrobial metabolites in root and soil samples. The abundance of antimicrobial metabolite biosynthesis genes was determined by qPCR. Antimicrobial biosynthesis genes phiD (biosynthesis of 2,4-diacetylphloroglucinol), phzF (biosynthesis of phenazines) and prnD (biosynthesis of pyrrolnitrin) were quantified. Taxonomic assignments were determined with the SILVA database. The heatmap shows Spearman’s rho rank correlations. Three biological replicates for each sampling site and sample type were randomly selected to infer correlations. Colour indicates rho values (positive correlations: green, negative correlations: red). Asterisks indicate significant correlations (p < 0.01).
CHAPTER 6

General conclusion
6.1. General conclusion

The interactions between plant beneficial bacteria and pathogens in agricultural soils are influenced by a multitude of biotic and abiotic factors. Plant beneficial bacteria have often been studied in suppressive soils, but their role in natural soil microbial communities in a range of different agricultural soils remains largely unknown. This thesis aimed to address this gap by studying the presence and abundance of *Pseudomonas* producing different antimicrobial metabolites in a range of different soils, and their link to the resistance against the two pathogens *Pythium ultimum* and *Gaeumannomyces tritici* (Figure 1 shows the *P. ultimum* soil resistance assay). Moreover, the link between taxa containing potential biocontrol bacteria and fungal taxa containing potential pathogens in soil microbial communities was studied.

In the second chapter of this thesis, we investigated the abundances of antimicrobial metabolite producing *Pseudomonas* on wheat roots and how they are linked, firstly to soil resistance against *Pythium ultimum* and *Gaeumannomyces tritici*, and secondly, to physical and chemical soil characteristics. We found that there is no significant correlation between the abundance of antimicrobial metabolite producing *Pseudomonas* and the soil resistance to the two tested pathogens, while certain soil characteristics had an effect on both, antimicrobial metabolite producing *Pseudomonas* abundance and disease resistance. These results suggest that *Pseudomonas* harbouring antimicrobial metabolite biosynthesis genes may contribute to, but cannot be considered as indicators of soil disease resistance.

In the third and fourth chapters of the thesis, we studied how the abundance of antimicrobial metabolite producing *Pseudomonas* and disease resistance is influenced by soils under different cropping systems, such as organic agriculture and conservation tillage. While DAPG producing *Pseudomonas* tended to be more abundant in conventional treatments compared to organic treatments in one of the two studied long-term trials, no general link was observed between the cropping system and the abundance of antimicrobial metabolite producing *Pseudomonas*. Similarly, no clear links were found between disease resistance and cropping system. Organic, reduced tillage soils were more resistant to *P. ultimum*, but no difference between cropping systems was found for *G. tritici* resistance. These results suggest that adapting cropping systems does not generally lead to an increase in antimicrobial metabolite
producing *Pseudomonas* or soil disease resistance, but specific cropping systems can favour specific groups of *Pseudomonas*, or soil resistance against specific pathogens.

![Image of greenhouse assay](image)

**Figure 1: Greenhouse assay to test the resistance of soils against *Pythium ultimum* in the 10 sampled Swiss agricultural soils of Chapter 2.** Pots inoculated with 0.125 g of *P. ultimum* and planted with cucumber seedlings are shown. For each agricultural soil and pathogen concentration, six technical replicates were performed. Soil disease resistance against *P. ultimum* was inferred by measuring cucumber shoot weights at the end of the experiment and normalizing them with the shoot weights from the respective control (not inoculated) treatment.

The results of the first part of the thesis suggest that complex interactions in microbial communities shape the resistance of soils against pathogens. These interactions were further explored in chapter five, where the interactions between bacterial and fungal soil communities, and disease resistance were studied. The results showed that at the genus level, the relative abundances of taxa containing biocontrol bacteria, such as *Pseudomonas*, *Bacillus* and *Stenotrophomonas*, were not linked to a higher disease resistance of soils. Moreover, at the genus level, only few significant links were found between the relative abundances of fungal taxa containing plant pathogens and taxa containing biocontrol bacteria. Significant links between plant beneficial bacteria and plant pathogenic fungi were detected at lower taxonomic levels, where single bacterial operational taxonomic units (OTUs) were associated with disease resistance. These results suggest that the interaction between plant beneficial bacteria and pathogenic fungi is very specific, and show the limitations of current microbial community studies based on taxonomy.

Overall, the results from this thesis strengthen the hypothesis that *Pseudomonas* producing antimicrobial metabolites are not indicative of soil disease resistance in
different agricultural soils. Although specific associations could be found between the abundance of certain groups of *Pseudomonas* producing antimicrobial metabolites, such as DAPG+ *Pseudomonas*, and the abundance of certain pathogens, such as *Pythium ultimum*, no general trends were found (Chapters 2 and 5).

Also, when summarizing earlier studies, the link between abundance of antimicrobial producing *Pseudomonas* and soil disease resistance remains unclear. The abundance of *Pseudomonas* harbouring antimicrobial biosynthesis genes has been considered an indicator for suppressive soils in some studies, where DAPG+ *Pseudomonas* were more abundant in suppressive than in conducive soils (Raaijmakers et al., 1997; de Souza et al., 2003). However, the link between suppressiveness and antimicrobial *Pseudomonas* abundance is controversial, since in other studies similar abundances have been found in conducive and suppressive soils (Mazurier et al., 2009; Almario et al., 2013; Kyselkova et al., 2014). The results of this thesis indicate that within natural microbial communities of agricultural soils, more complex interactions regulate soil disease resistance and the abundance of antimicrobial metabolite producing pseudomonads. *Pseudomonas* is not the only taxon producing antimicrobial metabolites in soil and on roots. Lemanceau et al. (2017) list 18 compounds produced by various root-colonizing known biocontrol taxa, which are involved in pathogen inhibition, and most likely many more will be discovered in the future. Moreover, the abundance of *Pseudomonas* harbouring antimicrobial genes only indicates the potential ability of these groups to produce these metabolites, but not under which conditions they are produced. Assessing the expression of antimicrobial metabolite biosynthesis genes in different soils with different levels of resistance against pathogens could lead to a better understanding of the role of antimicrobial metabolites produced by *Pseudomonas* in natural bacterial communities. The expression of *Pseudomonas* biosynthesis genes of antimicrobial metabolites was assessed by our project partners within the frame of the NFP68, with a fluorescent protein-based reporter strain system and well-known model *Pseudomonas* spp. strains (Chapter 2 and 3). However, this method has several limitations, the main limitation being that selected model strains are used, which might not have the same characteristics as the strains present in the natural field population. Moreover, since experiments assessing gene expression and experiments assessing soil disease resistance were conducted under different conditions, it was not possible to directly calculate the link between
antimicrobial metabolite biosynthesis gene expression and soil disease resistance. To avoid these limitations, methods based on RNA extraction from soil or root material could be used (DeCoste et al., 2011). Although soil RNA extraction is challenging (Novinski and Filion, 2011), these methods have already been used to measure the expression of DAPG, HCN and phenazines biosynthesis genes in soil (DeCoste et al., 2011; Arseneault et al., 2016; Paulin et al., 2017). Ideally, in future studies DNA and RNA could be extracted at the same time from aliquots of the same soil or root sample, thus allowing understanding how abundance and expression of antimicrobial metabolite biosynthesis genes are linked, and how they are influenced by different biotic factors, such as pathogen abundance and soil disease resistance.

The results from this thesis suggest that soil physical and chemical characteristics are an important factor influencing the abundance of antimicrobial metabolite producing *Pseudomonas*. Previous studies investigating the influence of different chemical soil elements on the production of antimicrobial metabolites (Duffy and Défago, 1999) and effects of soil characteristics on biocontrol (Ownley et al., 2003) were based on model strains. This is the first study where the links between soil characteristics and the abundance of antimicrobial metabolite producing *Pseudomonas* were assessed in natural populations from different soils. However, the results obtained here have several limitations. The most important limitation is the relatively balanced plant nutrient content of the studied soils (Chapter 2), meaning that it was not possible to study the effects of soils depleted or enriched in certain nutrients. Moreover, pH, which was found to influence the growth of *Pseudomonas protegens* CHA0 in soils (Mascher et al., 2013), was close to neutral in all the studied soils, therefore possible effects of pH on natural populations of *Pseudomonas* producing antimicrobial metabolites were not detected in this thesis. Thus, future studies on interaction of soil factors and pseudomonads, or other members of the microbial community, should specifically include soils depleted or enriched in plant nutrients, and with acidic or basic pH. Moreover, in this thesis, only ten soils were sampled. While this allows a first overview of how abiotic factors influence antimicrobial metabolite producing *Pseudomonas*, studies in a greater number of soils with different composition are needed to gain an in-depth understanding of the effect of single abiotic factors. Knowing how soil abiotic factors influence the abundance of antimicrobial metabolite producing *Pseudomonas* and other beneficial soil microbes is necessary in order to
predict the survival of biocontrol strains in specific soils, which is crucial for the
development of new biocontrol strategies and products.
Although physical and chemical soil characteristics had an effect on the abundance of
antimicrobial metabolite producing *Pseudomonas*, different cropping systems, such as
conservation tillage and organic agriculture, had a limited and not consistent effect in
the two studied long-term field trials. This indicates that it could be difficult to
implement conservation biocontrol strategies based on natural populations of
biocontrol pseudomonads. Other advantages of sustainable cropping systems for
farmers might have a higher weight than the implementation of conservation
biocontrol strategies. For instance, no tillage increases yields in regions with low
rainfalls because it increases soil water retention (Pittelkow et al., 2014), while
organic agriculture improves soil fertility and allows to considerably reduce fertilizer
input (Mäder et al., 2002).
From the second, third and fourth chapter of this thesis, it emerged that antimicrobial
metabolite producing *Pseudomonas* are likely only one microbial group among many
involved in soil disease resistance. The results from the fifth chapter on the
interactions between genera containing fungal pathogens and genera containing
biocontrol bacteria in the microbial community of soils, suggest that specific
interactions shape soil disease resistance in natural communities. These interactions
probably take place at the sub-species or strain level. Possibly, the interactions
between beneficial bacteria and plant pathogenic fungi could be better understood by
analysing microbial communities at the functional, rather than at the taxonomic level
(Lemanceau et al., 2017). As an example, the genus *Pseudomonas* contains plant
beneficial strains, plant pathogenic strains and human pathogenic strains (Rumbaugh,
2014). Similarly, genera containing plant pathogenic fungi, such as *Fusarium*, comprise
plant pathogenic and apathogenic or even beneficial strains (Alabouvette, 1986).
Moreover, even within phylogenetic closely related sub-groups or species, different
strains show different degrees of biocontrol activity against fungal pathogens (Flury
et al., 2016). It is therefore not surprising that the relative abundance of the genus
*Pseudomonas*, and of other genera containing biocontrol bacteria, in the microbial
community was not indicative of soil disease resistance. Despite the functional
variability of strains within bacterial taxa, previous studies have found that certain
taxa are more abundant in suppressive compared to conducive soils, such as
Pseudomonadaceae, Burkholderiaceae, Xanthomonadales and Lactobacillaceae in *Rhizoctonia* suppressive soils (Mendes et al., 2011). Moreover, in a study with *Fusarium* infested banana plants, the relative abundance of *Pseudomonas* was higher on the roots of healthy plants than on the roots of diseased plants (Köberl et al., 2017). The knowledge on which taxa are associated with suppressive soils and healthy plants could be useful to develop new cropping strategies, such as breeding plants which harbour a microbiome that is protective against pathogens or has plant beneficial traits (Schlaeppi and Bulgarelli, 2015; Wallenstein, 2017). However, taxonomic studies of microbial communities might not be effective to obtain the needed knowledge on plant protective microbiomes, since they are subject to different methodological biases, particularly if they are based on the diversity of the 16S rRNA and fungal ITS genes (Vetrovsky and Baldrian, 2013; Schlaeppi and Bulgarelli, 2015; Ranjan et al., 2016). Challenges include the multi-copy nature of the 16Sr RNA and ITS genes, which causes biases in relative abundance data (Vetrovsky and Baldrian, 2013). Additionally, biases due to DNA extraction methods and primer choice (Frank et al., 2008; Schlaeppi and Bulgarelli, 2015; Beckers et al., 2016; Nesme et al., 2016), as well as the quality of the database used for taxonomic assignments (Choi et al., 2016; Nesme et al., 2016) have to be considered. Therefore, it is difficult to interpret data obtained in taxonomical microbiome studies. Moreover, it is increasingly noted that the functional traits of microorganisms in communities are more important than the taxonomic composition for pathogen control strategies (Massart et al., 2015; Mazzola and Freilich, 2016; Lemanceau et al., 2017). Microorganisms that are taxonomically different can possess the same antimicrobial traits, and on the other hand, closely related microorganisms can have very different traits (Lemanceau et al., 2017). To understand which antimicrobial traits are involved in specific interactions between bacteria and pathogens, cultivation-dependent studies with different model organisms and consortia will be needed (Busby et al., 2017). Once antimicrobial traits are identified, the influence of different factors on these traits can be studied in the field with cultivation-independent methods such as qPCR or metatranscriptomics, and certain antimicrobial traits can be selected as indicators for plant health, for example in breeding programs (Massart et al., 2015; Busby et al., 2017; Lemanceau et al., 2017). Even once the functional traits of protective microbiomes will be known, it will be difficult to develop breeding or biocontrol strategies that protect plants against all
pathogens for all crop plants and soil types. Rather, it is likely that the beneficial microbial traits are specific for each pathogen, crop plant and soil type. Therefore, especially conservation biocontrol strategies will be difficult to implement, since adapting cropping conditions to favour microorganisms with beneficial traits against one pathogen could negatively affect microorganisms with beneficial traits against other pathogens. On the other hand, knowledge on beneficial microbial traits could be useful to implement augmentative biocontrol strategies, which are specifically targeted to a certain pathogen, crop plant and soil type.

6.2. References


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PUBLICATIONS AND PRESENTATIONS

1. Publications in peer-reviewed journals

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Pascale Flury, Pilar Vesga, Maria Péchy-Tarr, Nora Aellen, Francesca Dennert, Nicolas Hofer, Karent Paola Kupferschmied, Peter Kupferschmied, Zane Metla, Zongwang Ma, Sandra Siegfried, Sandra de Weert, Guido Bloemberg, Monica Höfte, Christoph Keel and Monika Maurhofer (2017): Antimicrobial and insecticidal: cyclic lipopeptides and hydrogen cyanide produced by plant-beneficial Pseudomonas strains CHA0, CMR12a and PCL1391 contribute to insect killing. Frontiers in Microbiology, 8, DOI: 10.3389/fmicb.2017.00100


2. Unpublished work

Francesca Dennert*, Nicola Imperiali*, Cornelia Staub, Jana Schneider, Raphaël Wittwer, Marcel van der Heijden, Theo H.M. Smits, Klaus Schlapepi, Christoph Keel, Monika Maurhofer (2017): Conservation tillage and organic farming induce minor variation in Pseudomonas abundance, their antimicrobial function and soil disease resistance. In preparation to be submitted to FEMS Microbiology Ecology *These authors contributed equally

Francesca Dennert*, Klaus Schlapepi*, Marie Fesselet, Ursula Oggenfuss, Kyle Hartman, Nicola Imperiali, Fabio Mascher, Theo H.M. Smits, Marcel van der Heijden, Jean-Claude Walser, Christoph Keel, Monika Maurhofer (2017): Interactions within bacterial and fungal communities in agricultural soils with different levels of disease resistance. In preparation to be submitted to Plos One *These authors contributed equally

Nicola Imperiali*, Xavier Chiriboga*, Klaus Schlapepi, Marie Fesselet, Daniela Villacrés, Geoffrey Jaffuel, S. Franz Bender, Francesca Dennert, Ruben Blanco-Pérez, Marcel G.A. van der Heijden, Monika Maurhofer, Fabio Mascher, Ted C.J. Turlings, Christoph Keel, and Raquel Campos-Herrera (2017): Combined field inoculations of Pseudomonas bacteria, arbuscular mycorrhizal fungi and entomopathogenic nematodes and their effects on wheat performance. Submitted to Frontiers in Microbiology *These authors contributed equally
3. Other publications

Francesca Dennert, Jana Schneider, Nicola Imperiali, Dmitri V. Mavrodi, Olga V. Mavrodi, Paul Mäder, Fabio Mascher, Raphaël Charles, Christoph Keel, Monika Maurhofer (2016): Abundance of plant beneficial pseudomonads in the rhizosphere of winter wheat grown in different agricultural management systems IOBC-WPRS Bulletin, Volume 117, pp.144-147


4. Oral presentation at international meetings

XIV Meeting of the IOBC-WPRS Working Group Biological Control of Fungal and Bacterial Plant Pathogens, Berlin 12-15 September 2016: Abundance of plant beneficial pseudomonads in the rhizosphere of winter wheat grown in different agricultural management systems

Rhizosphere 4 Conference, Maastricht, The Netherlands, 21-25 June 2015: How is the abundance of plant beneficial pseudomonads influenced by soil management practices?

10th International PGPR Workshop, Liège, Belgium, 16-19 June 2015: Is resistance to soilborne pathogens linked to the abundance of plant beneficial pseudomonads in Swiss agricultural soils?