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THE ROOT ROT COMPLEX OF PEA

Screening for resistance and quantification of microbial key players in the rhizosphere

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Use of germplasm exhibiting resistance to [...] one pathogen does not guarantee its success in the field where several other pathogens may be present.

F. J. Muehlbauer & J. M. Kraft (1973)

ABSTRACT

Pea (*Pisum sativum* L.) is a valuable and healthy protein source for food and feed. In addition to the nutritional benefits, pea is an invaluable agro-ecological asset for sustainable cropping systems through positive effects on soil fertility and soil microbial diversity. The symbiosis with nitrogen-fixing bacteria allows pea and other legume crops to supply the soil with nitrogen and, therefore, to significantly reduce the application of external nitrogen fertilisers. Therefore, pea plays an important role especially in low-input farming systems. The growing market for plant-based protein supply is likely to promote pea cultivation in the near future. However, pea production is severely challenged by various soil-borne pathogens that form a *Pea Root Rot Complex* (PRRC) causing root-rot diseases. Despite considerable progress in resistance breeding against individual pathogens, current pea varieties lack resistance against multiple interacting pathogens. The overall goal of this thesis was to contribute to the understanding of resistance against root rot pathogen complexes in pea.

Chapter 1 gives an overview of the importance of pea as a future key player in agricultural systems and the food sector before introducing the pea root rot complex concept and its relevance for research on resistance. Furthermore, the most recent developments in molecular biology relevant for molecular plant breeding of pea are briefly summarised and an overview of quantitative real-time PCR relevant for research on microbial interactions in the pea root rot complex is given.

Chapter 2 reviews the current knowledge of resistance against root-rot pathogens in major grain legumes, highlights the importance of the host genotype in determining the composition of plant-associated microbial communities and how the root associated microbiome relates to plant health. In addition, major findings on the role of root exudation in disease susceptibility and resistance of grain legumes are summarised.

Finally, it delineates how this knowledge could be integrated in resistance breeding of grain legumes.

In *Chapter 3*, a resistance screening assay was established based on infested soil from an agricultural field that showed severe pea root rot pressure. This approach was chosen in order to account for the whole rhizosphere microbiome - including the naturally occuring pathogen complex - in the assessment of root rot resistance in pea. The initial ITS-amplicon sequencing of the fungal rhizosphere community of diseased pea roots grown in the infested soil showed a root community of evenly abundant fungal taxonomic units not dominated by a few taxa. This finding points at complex interactions within the PRRC. Two hundred and sixty-one pea cultivars, landraces and breeding lines were screened for resistance on the naturally infested field soil in a controlled conditions experiment. The screening system allowed for a reproducible assessment of disease parameters among the tested genotypes. Broad sense heritabilities on the infested soil were $H^2 = 0.89$ for plant emergence, $H^2 = 0.43$ for root rot index and $H^2 = 0.51$ for relative shoot dry weight. The resistance ranking was verified in an on-farm experiment with nine pea genotypes in two field sites: The controlled conditions root rot index showed a significant correlation with the resistance ranking in the field site with high PRRC infestation (Spearman's $\rho = 0.73$, $p = .03$). The screening system offers a tool for selection at early stages of the plant development, and for the study of plant resistance in the light of complex plant-microbe interactions.

For *Chapter 4*, a subset of five resistant and three susceptible pea genotypes was selected based on the initial screening. In analogy to the previous experiment, a controlled conditions experiment was setup up in order to assess and validate resistance of the eight pea genotypes on four soils. Plant growth was significantly reduced on the three sick soils compared to the healthy soil. Despite the significantly different levels of disease pressure in the three infested soils (ANOVA: $p < .001$) and the strong genotype effect ($p < .001$), no significant soil × genotype interaction ($p < .342$) was found for plant growth reduction. In addition to disease assessments, ten key microbial taxa (eight putative pea pathogens and two putative beneficials) were quantified in the roots by quantitative real-time PCR (qPCR). *Fusarium solani, F. oxysporum* and *Aphanomyces euteiches* were the most abundant pathogens in diseased

roots from the three sick soils. Further, various levels of the pathogens *F. avenaceum*, *F. redolens*, *Rhizoctonia solani*, *D. pinodella* and *Pythium sp*. as well as the potential antagonist *Clonostachys rosea* were quantified by qPCR. The contribution of individual pathogens to root rot and growth reduction differed among the three sick soils: *F. solani* and *F. oxysporum* showed significant correlations (Spearman correlations; $p < 0.05$) with root rot index and relative shoot dry weight in the two soils with the highest infestation level; *A. euteiches* showed significant relations with disease in two sick soils from Germany. The quantities of arbuscular mycorrhizal fungi were negatively correlated with root rot index and positively correlated with relative shoot dry weight in all sick soils. Furthermore, the root microbial composition differed significantly among the pea genotypes (PERMANOVA; $p < .0001$) and the soils ($p < .0001$) and a significant pea genotype × soil interaction was evidenced ($p < .0001$). In addition, resistant pea genotypes showed significantly lower *F. solani* and *A. euteiches*, and higher arbuscular mycorrhizal fungi abundance in the roots (Wilcoxon rank-sum test; $p < .05$). These results give insights into the complex interaction between key microorganisms of the PRRC and the plant, by pointing out potential key microorganisms in the root rot pathobiome. Further disentanglement of this complex and the validation of key microbial players can be harnessed by resistance breeding.

Chapter 5 reviews the experimental approaches and results from the previous chapters before discussing the major findings and implications for future research and resistance breeding. I also raise the question if and how knowledge about complex soil microorganisms-plant feedbacks can be incorporated in resistance screenings and breeding efforts to conclude that today we are at a point where information on microbial complexes could indeed assist resistance breeding. However, our current state of knowledge does not yet allow to design specific microbiome-enabled selection-tools. This last chapter will also give short outlooks and indicate possible future lines of research in the field of microbe-mediated plant resistance.

ZUSAMMENFASSUNG

Die Erbse (*Pisum sativum* L.) ist eine wertvolle und gesunde Proteinquelle für Mensch und Tier. Zusätzlich ist die Erbse durch ihre positiven Auswirkungen auf die Bodenfruchtbarkeit und die mikrobielle Vielfalt des Bodens ein unschätzbarer agro-ökologischer Vorteil für nachhaltige Anbausysteme. Durch die Symbiose mit stickstoff-fixierenden Bakterien versorgen Erbsen und anderen Leguminosen den Boden mit Stickstoff und erlauben es so, den Einsatz von externem Stickstoffdünger deutlich reduzieren. Daher spielen Erbsen vor allem in extensiven Anbausystemen eine wichtige Rolle. Der wachsende Markt für die pflanzliche Proteinversorgung wird den Erbsenanbau höchstwahrscheinlich in naher Zukunft fördern. Eine intensivere Erbsenproduktion wird jedoch durch verschiedene bodenbürtige Krankheitserreger, die einen Erbsen-Wurzelfäule-Komplex bilden und schwere Wurzelfäulekrankheiten verursachen, stark beeinträchtigt. Trotz wichtiger Fortschritte in der Resistenzzüchtung gegenüber einzelnen Pathogenen sind die derzeitigen Erbsensorten nicht gegen mehrere interagierende Pathogene resistent. Das übergeordnete Ziel dieser Doktor-Arbeit war es deshalb, zu einem besseren Verständnis von der Resistenz gegen Wurzelfäule-Pathogenkomplexe bei der Erbse beizutragen.

Kapitel 1 gibt einen Überblick über die Bedeutung der Erbse in landwirtschaftlichen Systemen und ihr Potenzial im Lebensmittelsektor. Danach wird das Konzept des Erbsen-Wurzelfäule-Komplexes und seine Bedeutung für die Resistenzforschung vorgestellt. Darüber hinaus werden die jüngsten Entwicklungen in der Molekularbiologie, die für die molekulare Pflanzenzüchtung der Erbse relevant sind, kurz zusammengefasst und ein Überblick über die quantitative real-time PCR (qPCR) gegeben, die für die Forschung über mikrobielle Wechsel-wirkungen im Erbsen-WurzelfäuleKomplex von Bedeutung ist.

Kapitel 2 gibt einen Überblick über den aktuellen Wissensstand zur Resistenz gegen Wurzelfäuleerreger bei den wichtigsten Körnerleguminosen und hebt die Bedeutung des Wirtsgenotyps bei der Bestimmung der Zusammensetzung der pflanzenassoziierten Mikrobengemeinschaften hervor, indem die wichtigsten Forschungsergebnisse auf diesem Gebiet vorgestellt werden. Darüber hinaus werden die wichtigsten Erkenntnisse der Rolle von Wurzelexsudaten bei der Krankheitsanfälligkeit und -resistenz von Körnerleguminosen zusammengefasst. Schliesslich wird beschrieben, wie dieses Wissen in die Resistenzzüchtung von Körnerleguminosen integriert werden könnte.

In *Kapitel 3* wurde ein Resistenz-Screening entwickelt, basierend auf einem Ackerboden, der in der Vergangenheit starke Erbsenwurzelfäule gezeigt hat. Dieser Ansatz wurde gewählt, um das gesamte Mikrobiom der Rhizosphäre bei der Beurteilung der Wurzelfäule-Resistenz zu berücksichtigen. Eingangs wurde die Pilzgemeinschaft erkrankter, in besagtem Boden gewachsener Erbsenwurzeln mittels ITS-Amplikonsequenzierung charakterisiert. Dabei zeigte sich eine vielfältige Pilzgemeinschaft, bestehend aus gleichmäßig vorhandenen Pilzgruppen, ohne deutlich dominanter Taxa. Dieser Befund weist auf komplexe Interaktionen innerhalb des Wurzelfäulekomplexes hin. Darauf wurden 261 Erbsensorten, Landrassen und Zuchtlinien in einem Versuch unter kontrollierten Bedingungen auf dem natürlich befallenen Feldboden auf Resistenz untersucht. Das Screening-System ermöglichte eine reproduzierbare Bonitur von Krankheitsparametern unter den getesteten Genotypen: Die Heritabilität für den Pflanzenaufgang auf dem befallenen Boden war $H^2 = 0.89$, $H^2 = 0.43$ für den eigens erarbeiteten Wurzelfäuleindex und $H^2 = 0.51$ für das relative Sprosstrockengewicht. Die Resistenzeinstufung wurde in einem Feldversuch an zwei Standorten mit neun Erbsengenotypen verifiziert: Der unter kontrollierten Bedingungen erhobene Wurzelfäuleindex korrelierte signifikant mit der Resistenz in demjenigen Feld mit hohem Befall (Spearman's $\rho = 0.73$, $p = .03$). Das Screening-System bietet somit ein Instrument zur Selektion in frühen Stadien der Pflanzenentwicklung und darüber hinaus zur Erforschung der Pflanzenresistenz im Zusammenhang mit komplexen Pflanzen-Mikroben-Interaktionen.

Für *Kapitel 4* wurden fünf resistente und drei anfällige Erbsengenotypen auf der Grundlage des anfänglichen Screenings

ausgewählt. In Anlehnung an den vorherigen Versuch wurde ein Versuch unter kontrollierten Bedingungen durchgeführt, um die Resistenz der acht Erbsen-Genotypen auf vier Böden zu validieren. Im Vergleich zum gesunden Boden war das Pflanzenwachstum auf allen drei kranken Böden signifikant reduziert. Trotz des signifikant unterschiedlichen Krankheitsdrucks in den drei kranken Böden (ANOVA: $p < .001$) und des signifikanten Genotypeffekts ($p < .001$) wurde keine signifikante Boden × Genotyp-Interaktion ($p < .342$) für die Reduktion des Pflanzenwachstums gefunden. Zusätzlich zu den Krankheitsbonituren wurden zehn mikrobielle Taxa (acht mutmaßliche Erbsenpathogene und zwei mutmaßliche Nützlinge) in den Wurzeln mittels qPCR quantifiziert. *Fusarium solani*, *F. oxysporum* und *Aphanomyces euteiches* waren die häufigsten Pathogene in erkrankten Wurzeln aus den drei kranken Böden. Weiter wurden verschiedene Konzentrationen der Erreger *F. avenaceum*, *F. redolens*, *Rhizoctonia solani*, *D. pinodella* und *Pythium* sp. sowie des potentiellen Antagonisten Clonostachys rosea mittels qPCR quantifiziert. Die Beziehung der einzelnen Erreger und der phänotypischen Merkmalen Wurzelfäule und Wachstumsreduktion unterschied sich in den drei kranken Böden: So zeigten zum Beispiel *F. solani* und *F. oxysporum* signifikante Korrelationen (Spearman; $p < .05$) mit dem Wurzelfäuleindex beziehungsweise dem relativen Sprosstrockengewicht in den beiden am stärksten befallenen Böden; A. euteiches zeigte signifikante Zusammenhänge mit der Krankheit in zwei kranken Böden aus Deutschland; *R. solani* zeigte signifikante Zusammenhänge mit der Krankheit in allen drei Böden. Weiter korrelierte die in den Wurzeln gemessene Menge an arbuskulären Mykorrhizapilzen in allen kranken Böden negativ mit dem Wurzelfäuleindex und positiv mit dem relativen Sposstrockengewicht korreliert. Die mikrobielle Zusammensetzung der Wurzeln unterschied sich signifikant zwischen den Erbsen-Genotypen (PERMANOVA: $p < .0001$) und den Böden ($p < .0001$), und es konnte eine signifikante Erbsengenotyp-Boden-Interaktion nachgewiesen werden ($p < .0001$). Darüber hinaus zeigten resistente Erbsen-Genotypen eine signifikant niedrigere Menge an *F. solani* und *A. euteiches* und eine höhere Menge arbuskulärer Mykorrhizapilze in den Wurzeln. Diese Ergebnisse geben Einblicke in die komplexe Interaktion zwischen wichtigen Mikroorganismen des Erbsen-Wurzelfäulekomplexes und der

Wirtspflanze. Eine weitere Entflechtung dieses Komplexes und die Identifizierung der wichtigsten mikrobiellen Akteure könnte in der Zukunft die Resistenzzüchtung unterstützen.

Kapitel 5 fasst die experimentellen Ansätze und Ergebnisse aus den vorangegangenen Kapiteln zusammen, bevor die wichtigsten Erkenntnisse daraus und Folgen für die zukünftige Forschung und die Resistenzzüchtung diskutiert werden. Ich werfe dabei auch die Frage auf, ob und wie das Wissen über komplexe Bodenmikroorganismen-Pflanzen-Feedbacks in Resistenz-screenings und Züchtungsbemühungen einbezogen werden kann, um zu dem Schluss zu kommen, dass wir heute an einem Punkt angelangt sind, an dem Informationen über mikrobielle Komplexe tatsächlich die Resistenzzüchtung unterstützen könnten – unser derzeitiger Wissensstand erlaubt es jedoch noch nicht, eine spezifisch mikrobiombasierte Selektion zu entwickeln. Wo möglich werden Ausblicke auf die künftige Forschung auf dem Gebiet der Interaktion zwischen Pflanzenresistenz und der mikrobiellen Gemeinschaft gegeben.

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1 | INTRODUCTION

1.1 PEA – A REEMERGING CROP FOR SUSTAINABLE FOOD PRODUCTION

Pea (*Pisum sativum* L.) is an important legume plant, cultivated for its protein-rich grain, as a vegetable or fodder plant. With an annual world production of 35 million tons[1], it is the second most important pulse after common bean (FAOSTAT, 2019). Whereas pea has been an important part of the diet in the Mediterranean region, India and China, it has been doomed to a dreary, canned existence in many industrialised countries. While food habits are shifting towards plant-based proteins with the growing consumer awareness on climate change impact, peas get rediscovered as healthy food. At the same time, innovative brands such as Switzerland-based start-up *planted.*, *Beyond Meat* from California or *Raised & Rooted* by Tyson Foods Inc., one of the largest meat marketers in the U.S., enter the market with pea protein-based ersatz meat. Concerns regarding gluten or lactose intolerances are expected to stimulate the demand for alternative protein sources. Consequently, a whole new pea protein industry has emerged in the last few years, with a current market size of USD 216 millions and a forecasted significant expansion in the coming years (Grand View Research, 2020).

In addition to the nutritional benefits, pea is an invaluable agro-ecological asset for sustainable cropping systems. The positive effect on soil fertility through the symbiosis with N_2-fixing rhizobacteria has long been recognised; therefore, pea and other legume species have been included in crop rotations to provide subsequent crops with nitrogen

[1] According to the *Definition and classification of commodities* edited by the FAO, *green peas* are classified as vegetable crops and do not fall under the term *pulse*. The number given here, however, comprises dry and green peas. Production quantity of dry peas for 2018 was 21 million tons, and 14 million tons for green peas, with China producing roughly 90% of the latter.

(Drinkwater *et al.*, 1998; Iannetta *et al.*, 2016; Zimmer *et al.*, 2016). Legumes have additional positive effects on the soil, by promoting diversity in microbial populations and biological processes such as residue decomposition, nutrient cycling and breaking crop pest cycles (Lupwayi & Kennedy, 2007). Legume-based systems have the potential to significantly mitigate greenhouse gas emissions, mainly through the reduced input of N-fertilisers and its associated CO_2 and N_2O emissions (Stagnari *et al.*, 2017). Throwing in the weight of these benefits shows that the inclusion of legumes in cropping systems has the potential to make sustainable crop production not least also economically competitive (Reckling *et al.*, 2016). Recently, intercropping legumes with cereals – e.g. pea-barley (*Hordeum vulgare* L.) – has regained attention[2]. Crop diversification through intercropping can contribute to yield stability, reduce land use and is thought to increase N_2-fixation from the atmosphere compared to legume sole cropping (Bedoussac *et al.*, 2015; Jensen *et al.*, 2020). Growing pea in mixtures with cereals presents an effective means for the suppression of weeds, and therefore allows for a significant reduction of herbicide applications.

Pea could contribute to reducing the dependency on overseas import of soy (*Glycine max* L.) derived feed in Europe[3]. The import of plant-based proteins in Europe produces an excess of nitrogen that is mainly lost as animal waste and released as nitrogen oxides in the air or as nitrate leached into the aquifer and rivers (Zander *et al.*, 2016). The introduction of legumes in intensive, cereal-based crop rotations in Europe has the potential to significantly contribute to a reduction of greenhouse gas emissions, non-renewable energy consumption and global warming potential (Nemecek *et al.*, 2008).

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[2] The multi-actor project *ReMIX*, funded by the EU Horizon 2020 Research and Innovation Programme, carries out research on species mixtures with the goal to strengthen diversified and resilient agro-ecological arable cropping systems (https://www.remix-intercrops.eu). The EU imports about 14 million tonnes of soya beans per year as a source of protein to feed our animals, including chicken, pigs and cattle, as well as for milk production.

[3] According to the European Commission, the EU imports about 14 million tonnes a^{-1} of soy per year as a source of protein to feed animals, including chicken, pigs and cattle, as well as for milk production. It is mainly imported from the U.S. and South America. *https://ec.europa.eu/commission/presscorner/detail/en/IP_19_161*. Accessed on 23 March 2020.

The harvested area of pea was only ~3 million ha in 2018 in Europe; in Switzerland, the harvested area of pea was ~4000 ha in 2018 (total grain legumes: ~7700 ha; ~5% of the total arable land). Around 500 ha were sown under organic certification[4]. Zander *et al.* (2016) have identified specialization and simplification in the agro-system as driving forces of the decline of grain legumes in Europe. They point out that externalities of legumes, such as nitrogen fixation, crop diversification and positive impacts on the environment, need to be monetised in order to increase acreage of legumes. Furthermore, adaptation of policies, such as the omission of subsidies for competing crops or import restrictions on soy imports, could have a major impact on the future of grain legume cultivation in Europe.

Three major factors will promote the economic and environmental interest in pea and lead to an increase of the acreage of pea in the future: (i) the shifting consumer behaviour and the expansion of the market for plant-based proteins, (ii) the undeniable impact of the global agro-system on the ecological future and the role legumes can play in it and (iii) planned actions and incentives to promote legume-based cropping systems (EU: European Parliament, 2018).

These developments call for improvements and reorganisation along the whole value chain of pulses in general, and pea in particular, as the most important pulse in temperate regions. Crop genetics, plant breeding and cultivation technique are at the very basis of any agricultural value chain. Research in these topics is of utmost importance to foster the future development of grain legumes. In comparison with wheat, for example, pulses can be considered as “orphan” crops: Whereas in Europe, the mean yield of wheat has increased by 175% since 1961, the increase for pea has been only 64% (Our World in data, 2020). This is also reflected by a lack of research dedicated to pulses, as pointed out by Magrini *et al.* (2018).

Various abiotic and biotic stresses are challenging the cultivation of pea and other pulses. Whereas, on the abiotic side, drought is one of the most important constraint of pea cultivation, on the biotic side, it is the high vulnerability of pea to various diseases (Rubiales *et al.*, 2015; Rubiales *et al.*, 2018). This thesis is focusing on soil-borne diseases with

[4] Benedikt Haug, *Research Institute of Organic Agriculture* (FiBL), Switzerland (personal communication, Feb. 2020)

an emphasis on root-rot causing fungal and oomycete pathogens and aims at contributing, with basic research in the field of plant pathology and plant breeding, to the improvement of this valuable crop.

1.2 PATHOGEN COMPLEXES – MORE THAN THE SUM OF ITS PARTS

The epigraph felicitously summarizes the scope of the present thesis. In 1973 Fred Muehlbauer, a grain legumes breeder and genetics researcher, published a research article in the journal *Crop Science* with the title "Evidence of heritable resistance to *Fusarium solani* f. sp. *pisi* and *Pythium ultimum* in peas" together with the phytopathologist John Kraft (Muehlbauer & Kraft, 1973). Besides being succinctly written, the publication clearly demonstrates a "lack of agreement" between resistance assessed on artificial, single-pathogen inoculated soil and resistance levels observed on naturally infested field soil. The authors conclude, that various organisms must be present in the naturally infested soil, forming a complex of interacting pathogens thus challenging the plants resistance capacities.

Ten years earlier, Kerr (1963) showed how the co-infection of pea by multiple root rot pathogens reinforces disease symptoms. Since then, research on root rot resistance has swung back and forth between confirming the concept of pathogen complexes and reductionistic approaches based on single pathogen-plant interactions aiming at understanding resistance mechanisms and the underlying genetics on, both, the plant and the pathogen side. Inoculations of plants with isolated microbial species/strains under controlled conditions have greatly advanced our understanding of plant-pathogen interactions at the cellular level and has allowed to identify genomic regions, genes and transcriptional processes that are involved in the crosstalk between microbes and the host plant (Jones & Dangl, 2006; Desgroux *et al.*, 2018; Stewart *et al.*, 2018; Baetsen-Young *et al.*, 2019). In parallel, it has been suggested to study and understand multipartite interactions between microbes and plants in order to generate field relevant knowledge that can serve as a basis for durable crop protection (Lamichhane & Venturi, 2015). Today, many plant scientists recognize plants as a holobiont

formed by the plant and its associated microbes (Vandenkoornhuyse *et al.*, 2015). With the holobiont concept at hand, fundamental functions such as nutrient acquisition and response to abiotic and biotic stresses are not solely steered by the plant, but by its interaction with the associated microbiome.

The recent development of microbiome research in plant science, certainly also fuelled by the preceding trend in human medicine, advances our understanding of how complex interactions between pathogens and their host shape the expression of plant diseases – that is, the understanding of plant *pathobiomes* (Bass *et al.*, 2019). This concept can be illustrated by a recent study by Pauvert *et al.* (2020) who assessed the interactions among fungal microbes on powdery mildew infected grape leaves. Powdery mildew, caused by the fungus *Erysiphe necator*, is traditionally perceived as a solitary pathogen. However, the authors of this study showed that this pathogen is embedded in a complex of promoting and inhibiting interactions with other members of the pathobiome, using a combination of microbiome network analysis and co-culturing experiments.

It is continually pointed out that legumes have the vocation of building up pathogenic microbes in the soil (Lawes & Gilber, 1895; Papavizas & Ayers, 1974; Oyarzun *et al.*, 1993). This holds especially true for pea cultivation, where crop rotations of as short as five years with increased pea frequency lead to marked shifts in the plant associated microbiome towards pathogenic members (Niu *et al.*, 2018). The phenomenon associated with such detrimental shifts has been termed *soil sickness, soil fatigue*[5], *legume fatigue*, *legume yield depression syndrome*, *pea fatigue* or *pea yield depression syndrome.* Soil sickness is a phenomenon that has been repeatedly investigated and extensively described in the literature, and, on its turn, can have various underlying causes, such as nutrient depletion, accumulation of noxious substances in the soil or, as mentioned above, an accumulation of certain pathogens (Schreiner & Sullivan, 1909; Börner, 1960; Mazzola & Manici, 2012; Fuchs *et al.*, 2014; Cesarano *et al.*, 2017). Soil sickness is a generic term; therefore the term *Pea Root Rot*

[5] The expression *Bödenmüdigkeit*, literally translated "soil tiredness", is usually used in German. These phenomena and underlying causes are also tightly linked to the so-called *replant diseases* (Mazzola & Manici, 2012).

Complex (PRRC) has been established by various researchers in the field (Kerr, 1963; Xue, 2003b; Taheri *et al.*, 2017; Zitnick-Anderson *et al.*, 2018). Several examples of multipartite interactions among pathogens and the host plant are given in *Chapter 2*. A better understanding of the interactions of key microbial players within the pathobiome of legumes will allow to manage diseases through adjusted agricultural practices (e.g. suitable crop rotations) and might support the development of resistant cultivars.

1.3 IMPROVING PEA FOR FUTURE AGRICULTURE: RECENT MOLECULAR BIOLOGICAL ADVANCEMENTS

1.3.1 Pea genomic tools

Pea has been domesticated since ancient times. The area of origin of pea is the Middle East, from where cultivation spread to Europe and to Persia, India and China as far back as 8000 BC. Nearly 100,000 accessions, comprising wild relatives, landraces, breeding lines and commercial cultivars are stored in gene banks worldwide. (Smýkal *et al.*, 2013; Smykal *et al.*, 2016). The *Pisum* genus is diploid (2n = 14), with *P. sativum* having an estimated haploid genome size of 4.5 Giga bases (Gb). A large proportion (> 75%) of the DNA consists of repetitive DNA sequences (Smýkal *et al.*, 2012).

Multiple quantitative trait locus (QTL) mapping studies based on bi-parental crosses have allowed to generate genetic maps and molecular markers for agronomically important traits of pea, including growth-, yield- or pathogen resistance-related traits. Several reviews have thoroughly summarised these genomic resources (Varshney *et al.*, 2014; Rubiales *et al.*, 2015; Tayeh *et al.*, 2015b).

The recent publication of the first reference genome for pea (total length of the assembly = 3.9 Gb; N50 contigs = 38 kb; 44,756 genes) is a cornerstone in the history of Mendel's model plant (Kreplak *et al.*, 2019). The availability of a pea reference assembly will significantly enhance our understanding of the genomic organisation, genetic diversity and evolution of this crop plant. The genome assembly will facilitate future re-sequencing of additional genomes, in turn allowing for the identification of genome-wide single-nucleotide-polymorphisms (SNPs) and structural variants as it has been the case for other legumes since the release of their reference genomes (e.g. soybean in 2010; common bean in 2014; Varshney *et al.*, 2014). Furthermore, the reference assembly will promote the generation of restriction site associated DNA markers obtained by genotyping-by-sequencing (GBS; Elshire *et al.*, 2011).

The first genome-wide association study after the release of the pea reference genome was based on 16,877 high quality SNPs and allowed to identify genomic loci associated with important agronomic traits, such as

plant height, lodging resistance, grain yield and seed protein and starch content (Gali *et al.*, 2019). Earlier GBS efforts in pea have been done without a reference assembly, but they relied on non-reference SNP-calling pipelines; commonly the TASSEL UNEAK[6]. This approach has been applied, for example, for the genotyping of 431 accessions from the USDA ARS-GRIN *Pea Single Plant Collection Plus* (Holdsworth *et al.*, 2017), or for GBS-based genomic prediction (Annicchiarico *et al.*, 2017).

The availability of genome sequence information will further expand in the near future. For plant breeding, the aim of the genomic research is to optimise genetic selection gain over multiple environments in shorter time and with lower costs. Several successful examples of molecular resistance breeding are known for legumes, such as phytophthora root rot resistance in soy (Cahill & Schmidt, 2004), anthracnose and angular leave spot resistance in common bean (*Phaseolus vulgaris* L.; Garzon *et al.*, 2008; Nay, 2019) or ascochyta blight resistance in chickpea (*Cicer arietinum* L.; Bouhadida *et al.*, 2013) using marker-assisted selection. The implementation of genomic selection in legumes is still in its infancy, especially for pea (Varshney *et al.*, 2019). However, first promising studies on genomic selection used to predict terminal drought resistance and yield have been published for pea (Annicchiarico *et al.*, 2017; Annicchiarico *et al.*, 2019). Genome sequence information and molecular tools have the potential to become a future mainstay of selection and breeding together with precise and large-scale phenotyping. Key roles in this process will be held by the identification of traits important for resilience under various stresses, allowing to produce stable yields and sustainable production, the screening of large numbers of genetic resources and the integration of these phenotypes into breeding programmes (Duc *et al.*, 2015).

1.3.2 Assessing key microbial players of the PRRC by quantitative real-time PCR

Root rot diseases in pea are the result of the interactions among various pathogens, as elaborated in section 1.2. This complex of pathogens is

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furthermore embedded in the diversity of the whole root-associated microbiome. Therefore, understanding how the plant determines the microbial composition around and in their roots, how microbial key players in the rhizosphere interact and how plant-associated microbes influence the plant phenotype has the potential to support plant breeding (Wei & Jousset, 2017; Oyserman *et al.*, 2018). This is also further developed in *Chapter 2*.

Three important techniques to investigate microbial taxa, notably bacterial and fungal, associated with pea roots are (i) culture-dependent isolation, (ii) PCR-based detection and (iii) next-generation sequencing, notably rRNA amplicon sequencing. In *Chapter 3*, ITS rRNA-amplicon sequencing was used to establish a blue print of the fungal microbiome associated with diseased roots grown in infested field soil.

A large body of the present thesis (*Chapter 4*) was based on quantitative real-time PCR (qPCR). Specificity and sensitivity are major assets of qPCR assays: Well-designed assays allow quantification in the picogram range of microbial DNA present in plant tissue and allow to distinguish between microbial species or strains (Okubara *et al.*, 2005). Furthermore, once developed and tested, published qPCR assays can be readily implemented in a molecular laboratory and adapted to a specific application. Quantitative real-time PCR has been widely used to study soil-borne plant pathogens (Okubara *et al.*, 2005). Species specific qPCR assays have been developed for major root rot pathogens of pea; for instance, *Aphanomyces euteiches* (Gangneux *et al.*, 2014), *Fusarium solani, F. avenaceum, F. redolens* and *F. graminearum* and *F. culmorum* (Zitnick-Anderson *et al.*, 2018) or *Pythium ultimum* (Lievens *et al.*, 2006). Zitnick-Anderson *et al.* (2018) developed a qPCR-set for seven *Fusarium* species allowing for the detection and quantification of these important root rot pathogens. They tested the assays in the green house and the field and showed that the qPCR allows for a greater detection frequency than culture-based isolation. This benefit is particularly important for microbial species that are difficult to isolate by traditional plating methods, as it is, for instance, the case for the oomycete pea pathogen *A. euteiches* (Chatterton *et al.*, 2018). Assays developed for the study of pathogens in host plants other than pea can be implemented. In any case, also established qPCR assays should be re-tested for specificity; i.e. test against a collection of pathogen isolates, where, in the best case,

the target microbial species has been isolated from the investigated host plant. If needed, qPCR assays can also be designed for higher level taxa: For instance, Fierer *et al.* (2005) used a set of qPCR assays to characterise bacterial and fungal community structure at the phylum/class level in the soil. In *Chapter 4*, we used a qPCR assay designed for the detection of arbuscular mycorrhizal fungi (AMF) covering a large part of the species within the phylum *Glomeromycota* (Hewins *et al.*, 2015).

For phytopathologists and plant breeders, qPCR is a valuable complement to the culture-based isolation from field samples and phenotypic descriptions of disease symptoms. In contrast to the nowadays widely employed microbiome sequencing approaches, qPCR allows to specifically target known microbial taxa that might have an important role in disease expression. Therefore, this technique can significantly contribute to elucidate the interactions among key microbes in the pea root rot pathobiome. Recently, Willsey *et al.* (2018) utilised previously published qPCR assays for major pea root rot pathogens in order to study their interaction and conjoint effect on disease expression. They conducted a greenhouse experiment with artificial inoculation of pea seedlings and showed, for example, that the combined inoculation of three *Fusarium* species and *A. euteiches* significantly reduced root weight relative to single inoculations and combined inoculations of *Fusarium* spp. without *A. euteiches*. Moreover, they were partially able to describe the dynamics among the pathogens: For instance, *F. redolens* did colonise the roots stronger when co-inoculated with *A. euteiches*, pointing at a synergism between the two species. Such insights can possibly contribute to a more complete understanding of the interplay between pathogens and the host plant and lead to the development of adapted management practises in the future.

1.4 BACKGROUND AND OUTLINE

The present work is based on the observed pea depression syndrome on a field site that was part of a large survey on the cultivation of legumes in relation to soil fertility in Germany and Switzerland (Fuchs *et al.*, 2016). The site was among several fields where farmers and extension advisors reported moderate to very low legume yields. In the year of the

initial observation in 2015, the field was sown with a pea-barley mixture; however, on half of the lot pea did almost completely fail to emerge and consequently did not grow – while barley could be harvested at the end of the growth period. Soil was collected from that field in early 2016 and tested in the greenhouse according to Fuchs *et al.* (2014) confirming the high pathogenicity towards pea (Figure 1.1). The soil was barely pathogenic to lupin (*Lupinus albus* L.) and not pathogenic to barley (data not shown).

Figure 1.1. Pea root rot. Pea cultivar G74 ('Kleopatra') grown in infested field soil (left) and X-ray sterilised field soil (right) under controlled conditions. The picture was taken 21 days after sowing.

The overall goal of the present doctoral thesis was to improve our understanding of resistance to root-rot pathogens in pea (*Pisum sativum*). The research aimed to elucidate the complex interaction between the plant phenotype, its genotype as well as the associated rhizosphere microbiome with the focus on key fungal pathogens and beneficial fungal taxa. The development and implementation of a controlled-conditions resistance screening of a panel of pea gene bank accessions, breeding lines and cultivars formed the core of the project. This soil-based approach was chosen in contrast to artificial inoculation assays in order to incorporate different, simultaneously-occuring pathogens and their interactions with the remaining plant-associated microbiota in the study system. The objectives of this thesis were:

- Development of a reproducible screening for root rot resistance in natural infested soil
- Phenotyping of 312 breeding lines and genetic resources for root rot resistance as basis for a genome-wide association study and microbiome studies
- Validation of the resistance screening in a field experiment
- Validation of the resistance screening on different soils showing distinct expressions of soil sickness
- Molecular quantification of key pathogenic and beneficial microbial taxa in diseased roots of resistant and susceptible pea genotypes in different soils

The thesis is divided in five chapters including a review published in *Plant, Cell & Environment*, a research article published in *Frontiers in Plant Science* and an advanced research manuscript to be submitted to *New Phytologist*. After the foregoing general introduction delineating some aspects about the importance of pea, the complexity of microbial interactions in the root-rot pathobiome and the most recent developments in molecular biology, it follows the main body of the thesis:

- *Chapter 2* summarises current knowledge of resistance against root-rot pathogens in major grain legumes. It depicts the importance of the host genotype for the composition of plant-associated microbial communities and the role of root exudation in disease resistance of grain legumes. Finally, it discusses the relevance of these traits for resistance breeding programmes.
- In *Chapter 3*, the implementation and the results of a controlled-conditions resistance screening of 261 pea genotypes and its transferability to the field are presented[7]. Additionally, ITS-amplicon sequencing was employed to characterise the fungal community present in the roots of diseased plants grown in the infested field soil.
- In *Chapter 4,* eight pea genotypes with contrasting levels of disease susceptibility were selected based on the results from the resistance screening and further tested on four soils with different levels of pea yield depression syndrome. In analogy to *Chapter 3*, a controlled conditions experiment was setup up in order to assess resistance among different soils, having an assumed discriminative pathogen composition. Quantitative real-time PCR was used to quantify ten selected fungal and oomycete taxa in diseased roots and to assess their respective role in root-rot development.

The findings of *Chapter 3* and *Chapter 4* are discussed in the last chapter in light of the concepts that were presented in *Chapter 2*. To conclude, I will assess the relevance of these findings and present some possible lines of research for future studies on plant resistance against pathogen complexes.

[7] Initially, 312 pea genotypes were screened. However, several included breeding lines were not yet single plant selected (< *F5* generation) and therefore excluded from the final analysis.

2 | INSIGHTS TO PLANT-MICROBE INTERACTIONS PROVIDE OPPORTUNITIES TO IMPROVE RESISTANCE BREEDING AGAINST ROOT DISEASES IN GRAIN LEGUMES

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Abstract Chapter 2

Root and foot diseases severely impede grain legume cultivation worldwide. Breeding lines with resistance against individual pathogens exist, but these resistances are often overcome by the interaction of multiple pathogens in field situations. Novel tools allow to decipher plant-microbiome interactions in unprecedented detail and provide insights into resistance mechanisms that consider both simultaneous attacks of various pathogens and the interplay with beneficial microbes. Although it has become clear that plant-associated microbes play a key role in plant health, a systematic picture of how and to what extend plants can shape their own detrimental or beneficial microbiome remains to be drawn. There is increasing evidence for the existence of genetic variation in the regulation of plant-microbe interactions that can be exploited by plant breeders. We propose to consider the entire plant holobiont in resistance breeding strategies in order to unravel hidden parts of complex defence mechanisms. This review summarises (i) the current knowledge of resistance against soil-borne pathogens in grain legumes, (ii) evidence for genetic variation for rhizosphere-related traits, (iii) the role of root exudation in microbe-mediated disease resistance and elaborates (iv) how these traits can be incorporated in resistance breeding programmes.

2.1 INTRODUCTION

Grain legumes are important protein sources for human food and animal feed, with an annual world production of 27 megatons (Mt) for dry bean (*Phaseolus vulgaris* L.), 14 Mt for dry pea (*Pisum sativum* L.), 12 Mt for chickpea (*Cicer arientinum* L.), 7 Mt for cowpea (*Vigna unguiculata* (L.) Walp.), 6 Mt for lentil (*Lens culinaris* Medikus), and 4 Mt for faba bean (*Vicia faba* L.) (FAOSTAT 2016). Besides their widely acknowledged nutritional quality, they provide important ecosystem services and improve soil fertility (Lupwayi & Kennedy, 2007; Rubiales & Mikic, 2014; Foyer *et al.*, 2016). Through the symbiotic association with nitrogen-fixing rhizobia, legumes provide nitrogen (N) to the agro-ecosystem, substantially reducing the need for external N fertilisation. Replacing this biologically fixed N by mineral fertiliser would cost up to an estimate of $10 billions per year worldwide (Graham & Vance, 2003). Leguminous crops are also valuable partners in various intercropping systems throughout the world, providing a means of diversification of cropping systems (Taschen *et al.*, 2017; Wang *et al.*, 2017b). Recently, Reckling *et al.* (2016) showed that replacing mineral fertiliser with legumes in European cropping systems substantially reduces environmental impact in terms of nitrate leaching and nitrous oxide emissions while maintaining economic profitability of the system. Despite their ecological and economic importance, legume cultivation remains below expectations due to low and unstable yields, mainly because of biotic and abiotic stresses (Graham & Vance, 2003; Karkanis *et al.*, 2016; Zander *et al.*, 2016). It has repeatedly been shown that successively growing legumes on the same field leads to the build-up of various root-infecting fungi, oomycetes and nematodes, resulting in a phenomenon called ‘soil fatigue’, also referred to as ‘legume yield depression syndrome’ or ‘soil sickness’ (Emden *et al.*, 1988; Huang *et al.*, 2013; Fuchs *et al.*, 2014; Li *et al.*, 2014a; Bainard *et al.*, 2017). Nayyar *et al.* (2009) showed that eleven years of continuous pea monocropping led to a substantial increase in root rot and a concomitant grain yield reduction of 70% compared to a pea-wheat rotation. These symptoms were associated with a decrease in overall soil microbial biomass and activity in general and a reduction of arbuscular mycorrhizal

fungi in particular. Bainard *et al.* (2017) confirmed these observations assessing different legume-wheat crop rotations and showed that the inclusion of two or more grain legumes into a four-year crop rotation caused a significant shift in the soil fungal community, a decrease in fungal diversity and an increase in fungal pathogens. Accordingly, not only a mere accumulation of pathogens is responsible for the yield reduction in continuous legume cultivations, but actual shifts in the microbial community that can lead to devastating dysbiosis in the rhizosphere. As a consequence of soil fatigue in legume cultivation, rotation breaks of up to ten years are recommended for certain legume crops (Moussart *et al.*, 2013; Wilbois *et al.*, 2013). This stands in sharp conflict with efforts to increase acreage of legumes to meet the increasing protein demand of a growing world population and to strengthen low input farming systems.

Breeding for resistance has been proposed as the most efficient, economical and sustainable approach for controlling diseases in legumes (Rubiales *et al.*, 2015). Substantial progress has been made in the development of genetic material resistant to individual pathogens and the elucidation of the underlying genetic basis of resistance traits. However, plant-pathogen interactions are embedded in complex interdependencies among all the microorganisms present in a given space around the plant, i.e., the rhizosphere in the case of soil-borne pathogens. Breeding for complex traits such as resistance against soil fatigue is a challenging endeavour, but will eventually lead to sturdier agro-ecosystems. Thanks to intensive research on soil microorganisms in the rhizosphere, it became evident that the performance of a plant is strongly dependent on the interaction with the associated microbial community (Bulgarelli *et al.*, 2015; Andreote & Pereira e Silva, 2017; Hartman *et al.*, 2017). Plants are in a constant metabolic crosstalk with the associated microbiome. They are a driving force in assembling microbial communities in their vicinity and shape the root-associated microbial community through the release of root exudates which can have stimulating or suppressive action on microbes (Bais *et al.*, 2006; Hartmann *et al.*, 2009; Lakshmanan, 2015). Plants and their associate microbiome can be recognised as the holobiont, and it has been postulated that the beneficial interplay of the host plant and its microbiome is responsible for maintaining health, while diseases,

as outlined above, are correlated with microbial dysbioses (Berg *et al.*, 2017a).

The aim of this review is to examine the interplay between plant genotype, rhizosphere microbial communities and root exudation and its implications for resistance breeding of grain legumes against fungal root diseases. A broad overview of the most important fungal pathogens and resistance capacities in legume germplasm collections is followed by the current understanding of plant-pathogen interactions in the rhizosphere. The role of root exudates in direct or microbe-mediated disease resistance is depicted. Eventually, we will discuss how plant genetic diversity for rhizosphere-related traits could be utilised in legume breeding to develop cultivars with increased and stable resistance against soil-borne pathogen complexes (Box 1).

2.1.1 Major root diseases of grain legumes

The cultivation of grain legumes is severely compromised by root and foot diseases caused by many pathogens. This paper concentrates on important soil-borne fungal and oomycotan diseases. Nematodes are not included, although they are important soil-borne pests in legume cultivation (Sharma *et al.*, 1994; Rubiales *et al.*, 2015). They are involved in a complex interplay with other soil microbes, as illustrated, for example, for chickpea where different nematode species interact with rhizobia and fungal pathogens (Castillo *et al.*, 2008). Plants affected by fungal root and foot rots show various symptoms. These include brown to black lesions, spreading from the upper part of the main root into the root system and the stem, discoloration of the root system and softening and decay of the root and lower stem cortex. The above ground parts of the plant exhibit pronounced wilting, poor growth and premature collapsing, often leading to complete crop failure.

Box 1 | Key messages of Chapter 2

- Grain legume cultivation is severely impeded by root-infecting pathogens. The control of these fungal and oomycotan pathogens is challenging, as they occur as pathogen complexes in the field. Past and ongoing efforts to develop resistant cultivars in legume breeding have only shown partial success.
- Disease resistance against soil-borne pathogen complexes is not a mere plant, but a system trait involving close interactions of the plant with the root-associated microbial community.
- Various compounds exuded by plant roots influence the composition and activity of the microbial community.
- New sequencing technologies allow to investigate plant genotype-microbiome interactions. Most importantly, they allow…
  … to identify key players in pathogen complexes and key beneficial microbes that strengthen plant health and defence,
  … to elucidate mechanisms involved in microbiome-mediated disease resistance.
- Recent insights into the genetic basis of plant-microbiome interactions provide opportunities for resistance breeding of legumes.

Among soil-borne fungal pathogens, species of the Ascochyta complex are considered to be a very important biotic constraint in legume cultivation. Ascochyta foot-rots are caused by the pathogen species *Didymella pinodes* (Syn. *Peyronella pinodes* or *Mycosphaerella pinodes*) and *Peyronella pinodella* (Syn. *Didymella pinodella* or *Phoma medicaginis* var. *pinodella*) and represent a threat to legume cultivations worldwide (Haware, 1981; Aveskamp *et al.*, 2010; Barilli *et al.*, 2016; Tran *et al.*, 2016; Baćanović-Šišić *et al.*, 2018).These phytopathogenic fungi are responsible for severe leaf and stem spots and root rots on many legume host plants including pea, chickpea, lentil and faba bean (Muehlbauer & Chen, 2007). The genus *Fusarium* comprises several species that can cause severe root rot impeding cultivation of pea and common bean worldwide (Hwang *et al.*, 2014; Coleman, 2016). For instance, *F. solani* and *F. avenaceum* were among the most frequently isolated pathogens in the most important pea production regions of North America (Feng *et al.*, 2009; Chittem *et al.*, 2015; Taheri *et al.*, 2017). Yield losses were reported to reach up to 57% and 84% for pea and common bean, respectively (Basu *et al.*, 1976) (Schneider *et al.*, 2001). Fusarium root rot is also responsible for high yield losses in white lupine cultivation and seems to play some role in chickpea and lentil root rot (Raza *et al.*, 2000; Abdel-Monaim & Abo-Elyousr, 2012; Nene *et al.*, 2012; Azevedo *et al.*, 2017). *Rhizoctonia solani* is one of the main soil-borne pathogenic fungi causing seed rot, damping-off, seedling blight and root rots on pea, chickpea, bean, lupine and lentil (Abdel-Monaim *et al.*, 2011; Abdel-Monaim & Abo-Elyousr, 2012).

The *R. solani* species complex is comprised of 14 genetically diverse anastomosis groups showing different host ranges and pathogenicities (Kraft & Pfleger, 2001; Melzer *et al.*, 2016). Chickpea production is severely affected by various isolates belonging to several anastomosis groups of this fungus (Dubey *et al.*, 2014). Despite infrequent isolations from roots affected by a root rot complex, *R. solani* could be related to stand loss of pea (Mathew *et al.*, 2012). Besides fungi, two important members of the fungus-like class Oomycota, namely *Aphanomyces euteiches* and *Pythium* spp., cause severe economically important diseases of several legume crops. *Aphanomyces euteiches* is recognised as one of the most destructive soil-borne pathogens to pea and common bean, especially in France, North America and Australia (Gaulin *et al.*,

2007; Watson *et al.*, 2013; Hagerty *et al.*, 2015). There are also reports of lentil fields in North America being affected by *A. euteiches* (Vandemark & Porter, 2010). The taxon *Pythium* spp. comprises various globally distributed pathogen species causing pre- and post-emergence damping-off of pea, lentil, chickpea, bean and lupine (Ingram & Cook, 1990; Nzungize & Lyumugabe, 2012; Li *et al.*, 2013b; Bahramisharif *et al.*, 2014; Alcala *et al.*, 2016; Li *et al.*, 2016; Mathews *et al.*, 2016; Rossman *et al.*, 2017).

In the past, much research has been focused on single pathogen species and their life cycles and infection strategies have been thoroughly summarised (Allen & Lenné, 1997; Gaulin *et al.*, 2007; Nzungize & Lyumugabe, 2012). Recent research in legume diseases has increasingly focused on the co-occurrence of various pathogens as complexes (Chittem *et al.*, 2015; Taheri *et al.*, 2017; Baćanović-Šišić *et al.*, 2018). Although many pathogens of legumes have a global distribution, regional structuring of abundance exists. For example, *A. euteiches*, a major pathogen in pea cultivation of northern France and Sweden, has not been detected in German pea cultivation systems (Gaulin *et al.*, 2007; Heymann, 2008; Pflughöft *et al.*, 2012). Furthermore, the causal pathogens and their relative significance in root rot complexes can vary by region, as shown for pea-producing areas in North America (Taheri *et al.*, 2017). In this latter study, it was shown that several pathogens are present simultaneously in diseased peas and that their relative prevalence differed from one year to the other, with *F. solani* favoured by drier conditions compared to *F. avenaceum* and *Peyronellaea* spp.. Various pathogens are also associated with foot and root rot complex of faba beans (Sillero *et al.*, 2010). Species of the genus *Fusarium* are most abundant, but other pathogenic fungi, including *R. solani*, *Pythium* spp., *Phoma* spp. and *A. euteiches*, are simultaneously present in the pathogen complex.

Root and foot rots are difficult to control, as their causal agents can survive for many years either as saprophytes on plant residues or in the soil through the formation of resting structures. Furthermore, different pathogens present in the soil complement each other with respect to their ecological niche and infection strategies and even facilitate infection, as further exemplified below. Direct control of these pathogens by chemical fungicides is generally limited and recent regulatory actions concerning pesticide use call for alternative solutions. Some control of soil-borne

diseases is usually achieved through sowing of certified seed, avoidance of infested field plots and the application of long crop rotation breaks (Katan, 2017). So far, it has been difficult to diagnose pathogen occurrences in the soil, but a soil-based bioassay has been developed that can be used to determine the disease potential of agricultural fields (Fuchs *et al.*, 2014).

2.1.2 Resistance breeding against root diseases

For the long-term, resistance breeding has been acknowledged as one of the most promising approaches to achieve sustainable and affordable success against soil-borne diseases (Russell, 1978; Rubiales *et al.*, 2015). Different international grain legume germplasm collections have been characterised for resistance and sources could be identified that show some level of resistance to particular soil-borne pathogens. Infantino *et al.* (2006) thoroughly reviewed sources of resistance to root diseases in legumes. Hence, only a few examples of resistance screenings will be mentioned below, in order to complement information on resistance screenings and detection of genomic regions associated with disease resistance, and to discuss some commonalities of these studies.

Germplasm with moderate resistance against Fusarium root rot exists for common bean, pea and lupine (Silbernagel, 1990; Raza *et al.*, 2000; Grünwald *et al.*, 2003; Hagerty *et al.*, 2015). Only intermediate levels of resistance against *D. pinodes* and *P. pinodella* exist in pea germplasm despite considerable screening efforts (Kraft *et al.*, 1998; Prioul *et al.*, 2004; Fondevilla *et al.*, 2007; Carrillo *et al.*, 2013; Khan *et al.*, 2013). Moderate resistance to Rhizoctonia root and stem rot was found in pea, chickpea and lentil germplasm (Shehata *et al.*, 1981; McCoy & Kraft, 1984; Wang *et al.*, 2006; Talekar *et al.*, 2017). Moderate to high resistance against Pythium damping-off was found for pea (Ohh *et al.*, 1978), common bean (Nzungize & Lyumugabe, 2012; Li *et al.*, 2016) and chickpea (Kumar *et al.*, 1992). For pea, partial resistance has also been found against *A. euteiches* (Malvick & Percich, 1999; Wicker *et al.*, 2003).

Commercial cultivars of grain legumes generally show low resistance levels. Resistant germplasm is regularly detected among landraces, gene

bank accessions or related species or subspecies. For instance, the Mesoamerican bean landrace Puebla 152 shows enhanced resistance against Fusarium root rot compared with the cultivar Zorro (Nakedde *et al.*, 2016). Likewise, in a screen of 304 faba bean accessions and cultivars for resistance against nine different isolates of *R. solani*, gene bank accessions showed higher levels of resistance, and against more *R. solani* isolates, compared with commercial cultivars (Rashid & Bernier, 1993). Similarly, in lupine, gene bank accessions examined in *F. avenaceum* sick soil showed higher resistance compared with non-infested control plots than commercial cultivars (Chang *et al.*, 2014). Apparently, gene banks harbour the genetic potential to breed grain legumes for resistance against different forms of root rot. In Egypt, for example, two lupine cultivars resistant to Fusarium root rot were specifically developed from resistant landraces (Raza *et al.*, 2000). However, the use of resistance sources is hampered by the complex inheritance of the resistance and by the complex resistance mechanisms involved, in particular in the presence of various pathogens in the field, as will be shown in the next section.

Resistance to root rots is a quantitative inherited trait. Biparental linkage analysis and genome-wide association studies (GWAS) have been used to map quantitative trait loci (QTL) for resistance against various root pathogens in grain legumes. Resistance to Fusarium and Aphanomyces root rot has been mapped in common bean, with a co-localisation of resistance and root morphology-related QTL, indicating that a combination of physiological mechanisms and root architecture traits is responsible for disease resistance (Schneider *et al.*, 2001; Navarro *et al.*, 2008; Hagerty *et al.*, 2015; Nakedde *et al.*, 2016). In pea, extensive mapping studies have been conducted for Fusarium and Aphanomyces root rots, and co-localisation of root-architecture and resistance QTL have been evidenced, too (Pilet-Nayel *et al.*, 2002; Feng *et al.*, 2011; Hamon *et al.*, 2011; Li *et al.*, 2012; Coyne *et al.*, 2015; Desgroux *et al.*, 2016; Desgroux *et al.*, 2018). Regarding *D. pinodes*, the most virulent pathogen of the Ascochyta complex, several QTL studies identified and reconfirmed genomic regions controlling resistance in pea (Fondevilla *et al.*, 2007; Fondevilla *et al.*, 2011; Carrillo *et al.*, 2014). These studies contribute considerably to the progress in identifying genomic regions involved in resistance against root pathogens. However, there is a need to identify the genes underlying the QTL involved in resistance or at least

molecular markers more tightly linked to them, which would allow designing marker-assisted selection (MAS) approaches in grain legume breeding. While there are promising indications to apply MAS in resistance breeding programmes of legumes, so far, these tools have rarely been adopted by legume breeders (Khan *et al.*, 2013; Rubiales *et al.*, 2015). Next-generation genotyping (e.g. single nucleotide polymorphisms; SNP) will allow to generate high-density genetic maps and to refine mapping of agronomic traits, such as resistance against pathogens. Improved phenotypic scoring of resistance among assessed accessions (e.g. digital image analysis) will further contribute to improving genetic maps and it seems likely that in the near future mapping studies will identify more genomic regions, candidate genes and markers for potential MAS. Hamon *et al.* (2013) conducted a QTL meta-analysis over four mapping-populations and found seven highly consistent genomic regions associated with resistance of pea against *A. euteiches*. This analysis integrated data of 29 field environments on two continents and twelve controlled condition-assays over several years. Similarly, Desgroux *et al.* (2016) used 13,204 SNP to genotype 175 pea accessions and performed resistance screenings in field and controlled conditions for resistance to *A. euteiches*. Using a GWA study, they found 52 QTL of small size-intervals and validated most previously defined resistance QTL. Moreover, they identified putative candidate genes with various associated functions.

Most importantly, the study by Desgroux *et al.* (2016) included various field conditions and controlled condition assays with two distinct pathogen strains, making the identified genomic regions a valuable resource for future breeding efforts in pea. Although they detected QTL consistent over the different environments, significant plant genotype x environment (G × E) and plant genotype x pathogen strain interactions were observed. Strong G × E interactions cause low heritability of the assessed traits, such as disease resistance against root rot pathogens, and are a major constrain for the identification of significant genomic regions governing resistance under field conditions (Acquaah, 2012). Besides climate and physical/chemical soil properties, an important driver of G × E involves the entire plant-associated microbial community, including the varying abundance and virulence of different pathogen species and strains. A drawback of a large part of the above cited studies is that they

were performed in controlled conditions on a sterile substrate and artificial inoculation of a pathogen strain. Although this allows to apply well-defined disease score ratings and obtain reproducible sustainability levels of tested accessions this does most probably not reflect the situation in the field where plants interact with a wide variety of different micro-organisms. Screening crops for resistance under the assumption that a wide range of possible causal agents is present in the field will be a complicated endeavour. Clearly, it will be challenging to design reliable tests for the evaluation of cultivar reactions against pathogen complexes.

2.2 COMPLEX INTERACTIONS BETWEEN GRAIN LEGUMES AND THEIR ROOT-ASSOCIATED MICROBIOTA

2.2.1 The dilemma with pathogen complexes

Plant roots are involved in a myriad of interactions with different soil microbes, ranging from beneficial alliances with mycorrhizal fungi, other fungal root endophytes and plant growth-promoting rhizobacteria to detrimental associations with pathogenic bacteria, fungi and oomycetes (Dudeja *et al.*, 2012; Müller *et al.*, 2016). In contrast, plant pathology has focused its research mainly on two-way pathogen-host interactions. The identification of resistant plant genotypes and the underlying resistance mechanisms is usually achieved by artificial inoculation of genetically different plant accessions with single pathogen strains on sterile substrate or in fields with confirmed preponderance of a single pathogen species. Today, it is acknowledged that plant diseases are often caused by multilateral interactions among different pathogens and that pathogens need to be considered as parts of microbial complexes (Lamichhane & Venturi, 2015; Abdullah *et al.*, 2017). Different pathogen species or strains of the same species can infect a plant simultaneously and lead to a different disease expression than infection by a single pathogen. Below, we draw upon several examples of co-inoculation of pathogens in legume crops to illustrate how important it is to consider multi-microbial interactions to make progress in understanding root and foot rot phenomena in grain legumes.

More than half a century ago, Abdullah *et al.* (2017) observed that *P. ultimum* and *A. euteiches* infect snap bean (*P. vulgare*) simultaneously, and that disease severity is significantly higher if both pathogens infect the host plant. *F. solani* frequently acts in complexes with other pathogens such as *R. solani, F. oxysporum, A. euteiches or P. ultimum* to infect pea (Tu, 1991; Mathew *et al.*, 2012). Peters and Grau (2002) inoculated two pea cultivars with *A. euteiches* and a non-pathogenic strain of *F. solani* separately or together and observed an increase in Aphanomyces root rot symptoms with co-inoculation of both microorganisms. Different pathogen species may also inhibit each other. For alfalfa, co-inoculation with both *A. euteiches* and *P. pinodella* resulted in significantly reduced amounts of *P. pinodella* DNA compared with the individual inoculation (Hossain *et al.* 2012). Similar results have also been obtained with co-inoculation of *A. euteiches* and *Phytophthora medicaginis* (Vandemark *et al.*, 2010). In pea, the infection with an endophytic *F. equiseti* strain reduces disease severity and biomass reduction caused by *F. avenaceum* and
P. pinodella (Šišić *et al.*, 2017). Similar results were obtained with non-pathogenic *F. oxysporum* isolates protecting pea against *F. solani* (Oyarzun *et al.*, 1994). Interestingly, other strains of *F. oxysporum* and *F. equiseti* can cause disease on a wide range of different legume hosts (Goswami *et al.*, 2008; Berg *et al.*, 2017b; Li *et al.*, 2017). The fact that the same fungal species is found to be pathogenic in some experiments and non-pathogenic in others indicate how disputable the term "(non-)pathogen species" is. This coars classification neglects that the taxonomic level "species" often includes different strains with distinctive biological features. Moreover, the pathogenicity of microbial species and their more or less detrimental interactions with the plant host has to be conceived within the framework of the whole microbiome (Berg *et al.*, 2017a). Specific host resistances against pathogens identified in a certain environment do not necessarily translate to other environments. For instance, pea breeding lines exhibited different levels of tolerance to *A. euteiches* when evaluated at two different locations in the north-western US (Weeden *et al.*, 2000). Hamon *et al.* (2011) identified certain resistance QTL (*Ae-Ps2.2* and *Ae-Ps4.1*) only in French, but not in US field experiments. The authors of the latter studies argue that diversity of *A. euteiches* at the different field sites and a possible occurrence of other

pathogens, namely *Fusarium* ssp. and *P. pinodella*, explain site-specific resistance rankings and detected QTL. Abdullah *et al.* (2017) concluded that resistance capacities of pea cultivars against single pathogens, especially when determined under gnotobiotic conditions, have limited transferability to complex field conditions. To overcome such limitations, it is necessary to consider complex plant-microbe interactions and develop screening systems that account for multiple interactions among pathogens, beneficial microbes and the host plant genotype.

2.2.2 Plant-microbe interactions and disease resistance

Certain members of the microbial community play a crucial role in the expression of disease resistance of plants. Mechanisms by which beneficial microorganism protect crop plants from diseases include (i) enhancement of overall vigour (e.g. via nutrient mobilisation) (ii) direct antagonism via parasitism or antibiosis (feeding directly on phytopathogenic microbes or producing antifungal/antibacterial metabolites) (iii) niche exclusion (e.g. competition for resources), and (iv) induction of systemic and localised resistance (Xue, 2003a; Conrath *et al.*, 2006; Shoresh *et al.*, 2010; Sindhu *et al.*, 2010; Verma *et al.*, 2016). For instance, *Trichoderma* belongs to one of the best studied genera with antagonistic activity against a wide range of root pathogens (Harman *et al.*, 2004). *Trichoderma* spp. are well known to proliferate and function in association with plant roots (Hohmann *et al.*, 2011; Hohmann *et al.*, 2012). This ability has been identified as one of the most important factors for their potential to control root pathogens. For legumes, *Trichoderma* spp. were shown to be an effective biocontrol agent against Rhizoctonia seedling mortality and foot rot in pea and common bean (Nelson *et al.*, 1988; Aziz *et al.*, 1997; Akhter *et al.*, 2015; Toghueo *et al.*, 2016).

Arbuscular mycorrhizal fungi (AMF) are also known to alleviate disease in grain legumes and other crops (Bodker *et al.*, 1998; Hilou *et al.*, 2014; Dehariya *et al.*, 2015; Ren *et al.*, 2015). Other fungi do not necessarily need to colonise plant roots to confer protection against pathogens, as shown for the biocontrol fungus *Clonostachys rosea*. This mycoparasite was shown to effectively protect pea seedlings against different pathogenic microbes (Xue, 2003a). Along with fungi, bacteria

endophytically associate with plant roots and confer protection against pathogens
(Dudeja *et al.*, 2012; Rybakova *et al.*, 2016). In the first place, rhizobia strains protect legumes against root diseases, such as Pythium damping-off in pea and lentil (Bardin *et al.*, 2004; Huang & Erickson, 2007). Antagonistic activity of rhizobia against pathogens is attributed to production of antibiotics and anti-fungal compounds or to the induction of systemic resistance and enhanced expression of plant defence-related genes (Desalegn *et al.*, 2016; Das *et al.*, 2017). Besides rhizobia, various naturally occurring bacteria associate with plant roots and are effective biocontrol agents against bacterial and fungal diseases. Several plant growth-promoting rhizobacteria were shown to have such biocontrol properties in pea (Singh *et al.*, 2000), chickpea (Akhtar & Siddiqui, 2009; Misk & Franco, 2011; Egamberdieva *et al.*, 2017), pigeon pea (Dutta *et al.*, 2014) and common bean (Hsieh *et al.*, 2005; Lopes *et al.*, 2015).

Different beneficial microorganisms interact and this microbial crosstalk has important consequences for plant health (Shtark *et al.*, 2012; Cameron *et al.*, 2013). Palmieri *et al.* (2017) showed that a microbial consortium of four beneficial rhizobacteria controls *F. solani* and *F. oxysporum* of chickpea more efficiently than each bacterial isolate on its own. The control of red crown rot in soybean is more efficient when co-inoculating AMF and rhizobia compared with single inoculations with either symbiont (Gao *et al.*, 2012). The co-application of various pathogen and antagonistic microbial strains is a promising approach to identify microbial key players that are active in more complex systems.

Controlled experiments and co-inoculations depict the tripartite interaction between host plant, pathogens and beneficial microbes in a useful, though simplistic way. The situation in the field is more complex and disease severity or suppression of soil-borne diseases are the result of a complex interplay within the microbial community present in a given soil (Lareen *et al.*, 2016). New sequencing technologies enable rapid and cost-effective whole microbiome surveys of crop plants. Microbiome comparisons between cultivars with contrasting susceptibility to diseases and between different agricultural management practises are of particular interest in this regard. For instance, amplicon sequencing of root-associated fungal communities of pea with different disease expression show clear shifts in community composition between healthy and

diseased pea (Xu *et al.*, 2012; Yu *et al.*, 2012a). Notably, the health status of pea positively correlates with the abundance of AMF. Similar observations were made for long-term peanut monocultures, where plant pathogenic fungi accumulated in the soil at the expense of beneficial fungi (Li *et al.*, 2014b). Zhang *et al.* (2017b) took microbiome analysis a step further in their study on the sick soil phenomenon in monocropped tobacco fields. Combining amplicon sequencing with a functional gene analysis, the authors showed a shift in the microbial community composition to be accompanied by changes in the metabolic potential of genes involved in stress, virulence and plant cell wall degradation in the sick soil. Poor soil properties lead to a decrease of beneficial microorganisms and a build-up of soil-borne wilt-causing bacterial. Research in this direction complements our understanding about the involvement of individual microbes in crop health. This will allow to progressively disentangle functional plant-microbe and microbe-microbe interactions.

2.2.3 Plant genotype drives the microbial rhizosphere composition

It is well known that different plant species have distinct root-associated microbiomes (Garbeva *et al.*, 2007; Doornbos *et al.*, 2011; Toju *et al.*, 2013). The selective effects of crop species on microbial communities can be very specific. For instance, it was shown that chickpea, lentil and pea have different root-associated fungal communities in general, but that AMF communities do not differ between the three crops (Borrell *et al.*, 2017). Besides inter-species variation, microbial composition in the rhizosphere also differs between genotypes of the same species and (Coleman-Derr & Tringe, 2014) highlighted the role of microbial communities to confer stress tolerance to their host plants. In 1904, Lorenz Hiltner postulated the pioneering idea that the resistance of pea against soil fatigue depends on the composition of the microbial community in the rhizosphere (Hartmann *et al.*, 2008). Only with the advent of next-generation sequencing technologies, has it been possible to elucidate the composition of soil microbial community in recent years. Several studies investigated the effect of the host genotype

on the microbial community composition. For instance, Zancarini *et al.* (2013) reported significant differences in the genetic structure and diversity of the entire bacterial rhizosphere community between seven genetically diverse *Medicago truncatula* lines at an early growth stage. The rice genotype explained 30% of the variation in the microbial rhizosphere composition (Edwards *et al.*, 2015). In an extensive survey of the potato-associated microbiome, Weinert *et al.* (2011) showed that 9% of all detected operational taxonomic units revealed a cultivar-dependent abundance. Different maize cultivars were shown to differentially stimulate rhizobacteria and AMF populations (Picard *et al.*, 2008), and significant genotypic effects among 10 maize inbred lines accounted for 26% of the variation in the bacterial rhizosphere composition (Emmett *et al.*, 2017). Peiffer *et al.* (2013) showed that plant genotype explains a small but significant part of the total observed bacterial diversity in the rhizosphere among 27 modern maize inbred lines evaluated in different field environments. Ellouze *et al.* (2013) detected genotypic effects of chickpea on soil microbial diversity (using cultural methods) and their impact on the subsequent durum wheat crop. However, this effect was diminished under severe drought. A genotypic effect on the root and rhizosphere microbiome was also detected in barley (Bulgarelli *et al.*, 2015).

The plant genotype also determines the interaction with individual members of the microbial community in the rhizosphere and evidence for host genotype-dependent interaction exists, e.g. for rhizobia (Roskothen, 1989; Yang *et al.*, 2017), AMF (Hetrick *et al.*, 1996; An *et al.*, 2009) and rhizobacteria (Smith *et al.*, 1999). To date, only few studies have assessed the role of plant genotypic variation in microbe-mediated disease resistance. Mark and Cassells (1996) demonstrated a genotype-dependent interaction between the AMF *Glomus fistulosum* and *Phytophthora fragariae*, the causal agent of red stele disease in wild strawberry. *Steinkellner et al.* (2012) revealed a cultivar-dependent bioprotection effect of the AMF Glomus mosseae when different tomato cultivars were infected with *Fusarium oxysporum* f. sp. *lycopersici*. Cultivar-specific root-associated bacterial communities have been found in wheat and maize that differ in their ability to attract naturally-occurring DAPG-producing *Pseudomonas* spp. and the amount of antibiotics produced by the biocontrol strains differed between the cultivars' rhizospheres (Gu &

Mazzola, 2003; Meyer *et al.*, 2010; Wen *et al.*, 2017). Different cotton cultivars showed significant varietal responses to cyanobacteria and a *Trichoderma* sp. applied as a biocontrol formulation against a root rot complex in the field (Babu *et al.*, 2015). In lentil, significant plant genotype effects were shown for the interaction with *Trichoderma* spp. in the presence of the pathogen *A. euteiches* (Prashar & Vandenberg, 2017). Although the *Trichoderma* spp. did not hinder the pathogen from infecting the host, they significantly promoted plant growth under pathogen stressed conditions.

The cross-talk between the plant and the microbial rhizosphere community as a whole is still barely understood. Knowledge of the function and the underlying genetics is still restricted to particular well-studied plant-microbe models, such as the mapping of symbiotic loci related to rhizobial and mycorrhizal symbiosis in model species (Sandal *et al.*, 2006). However, the few cited examples of the relationship between plant genotype, the rhizosphere microbiota and plant health clearly highlight the vital role of plant-associated microbes in disease resistance. The microbiome composition and specific interactions with fungal and bacterial strains have direct implications for phytopathology and biocontrol of plant pathogens. Plant breeding is the means by which genotypic differences for rhizosphere-related traits can be exploited and a favourable rhizosphere microbiota with specific microbial antagonists will lead to improved indirect plant defences against root pathogens.

2.3 THE ROLE OF ROOT EXUDATES IN DISEASE RESISTANCE OF LEGUMES

Plants release considerable amounts of photosynthetically fixed carbon from their roots in form of carbohydrates, amino acids, organic acids, growth factors, lipids and enzymes, and, thus, provide considerable portions of carbohydrates and nitrogen to the soil microbiota (Curl & Truelove, 1986). Various organic acids are predominant in root exudates of axenically-grown crop plants and the composition of organic acids is markedly changed in the presence of pathogenic fungi (Kamilova *et al.*, 2006a; Kamilova *et al.*, 2006b). Certain organic acids, e.g. butanoic acid, ferulic acid, 3-indolepropanoic acid and rosmarinic acid, were shown to

be involved in defence responses of the plant (Bais *et al.*, 2002; Walker *et al.*, 2003). Owing to the hall mark feature of legumes to form an intimate mutualistic symbiosis with rhizobia, most attention has been paid to root exudates (e.g. flavonoids) involved in the establishment of this symbiotic association (Garg & Geetanjali, 2007; Sugiyama & Yazaki, 2012). However, root exudates have manifold direct and indirect effects on the root-associated microbiota with relevance to pathogen regulation and disease susceptibility (Figure 2.1).

2.3.1 Root exudates stimulate or suppress soil-borne pathogens

Root exudates contain compounds that directly stimulate or suppress the growth of soil-borne plant pathogens. They provide an appropriate carbon source for many soil microbes (Bais *et al.*, 2006). For instance, germination of oospores of two strains of *A. euteiches* isolated from pea roots is significantly stimulated by pea root exudates compared to exudates of other crop plants (Shang *et al.*, 2000). Different sugars and also amino acids present in root exudates stimulate chemotaxis, encystement and cyst germination of the soy pathogen *Phytophtora sojae* (Suo *et al.*, 2016). It was shown that artificially amending soils with root exudates of legume plants leads to an increase of fungal biomass in the rhizosphere (Broeckling *et al.*, 2008; Li *et al.*, 2014a). Besides many compounds commonly exuded by plants, there are several ones that are specifically released from the roots of legumes such as the strigolactone-like metabolites peagol and peagoldione with germinative activity on broomrape seeds (Evidente *et al.*, 2009). Root exudates of pea cultivars were shown to stimulate the germination of microconidia and chlamydospores of pathogenic *F. oxysporum*, macroconidia of *F. solani* and oospores of *A. euteiches* (Whalley & Taylor, 1973). Intriguingly, macroconidia germination of *F. solani* is also highly stimulated by other flavonoids known to induce *nod* genes in *R. leguminosarum* bv. *viciae*, the rhizobial symbiont of pea (Ruan *et al.*, 1995). This suggests that *F. solani* is capitalising on molecules that are released by pea roots for the purpose of the molecular crosstalk with their bacterial root symbionts. The stimulatory effect on macroconidia was reduced for *F. solani* strains that are associated with beans or absent in the case of strains associated

with non-legume plants, pointing at the specificity of the pathogen-host interaction.

Root exudates also comprise a large number of defence-related compounds with manifold effects on various rhizosphere-colonising microbes, as reviewed by Baetz and Martinoia (2014). For instance, pisatin was the first phytoalexin identified and, since then, its interaction with fungal pathogens has served as a prime example to understand plant pathogenicity and non-host resistance (Cruickshank & Perrin, 1960; Hadwiger, 2008; 2015). Pisatin is produced by peas and acts as an important defence molecule against fungal pathogens (Preisig *et al.*, 1990). Detoxification of pisatin by demethylation and subsequent metabolizing allows pathogens to evade plant defence and is associated with pathogenicity in pea, chickpea and alfalfa (Hirschi & Van Etten, 1996; Soby *et al.*, 1996; Enkerli *et al.*, 1998; Milani *et al.*, 2012). Other plant defensins are known, but explicit experimental reports on their occurrence and mode of action in root exudation of legume species is scarce (Thomma *et al.*, 2002; Hanks *et al.*, 2005). Certainly, more research is needed to address the importance of defensins in root exudates of legumes and their role in plant defence against soil-borne pathogens and to elucidate the genetic basis of defensins exudation and variability. Other anti-microbial compounds found in root exudates include chitinases, glucanases and lipid transfer proteins that showed inhibitory effects on conidia germination and hyphal growth of *F. oxysporum in vitro* (Nóbrega *et al.*, 2005). In this latter experiment, as in many other studies, root exudates were recovered from plants growing in an axenic, pathogen-free hydroponic system, indicating that legumes constitutively exude defence-related compounds into the rhizosphere. Roots may exude important antifungal compounds; however, the susceptibility of plants to fungal pathogens can also depend on entire exudation profiles. For instance, the anthocyanin delphinidin present in seed coats of peas is exuded during germination and has a fungistatic activity against conidial germination of *F. solani*, but this activity is nulled by a sufficient exudation of carbohydrates at the same time (Kraft, 1977). Li *et al.* (2013a) assessed the effect of root exudates of peanut cultivars on different pathogenic fungi and generally observed a stimulation of fungal growth at intermediate concentrations of exudates. However, the stimulation decreased with higher concentrations of exudates, suggesting

that the root exudates contained antimicrobial substances along sugars and amino acids. To assess the effect of root exudation on microbiome-related processes and on plant health, it is therefore important to not only identify key root exudate compounds, but also determine exudate composition on a quantitative level.

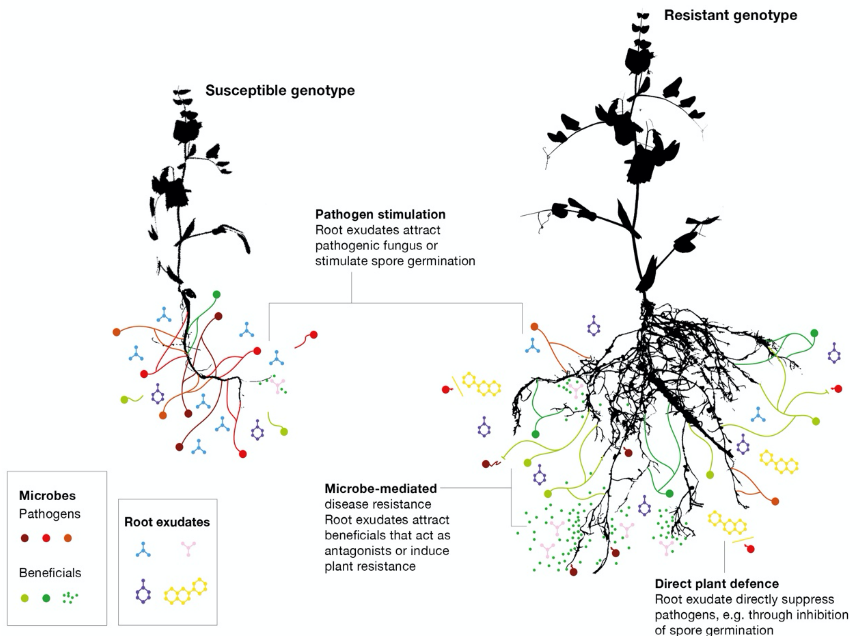

Figure 2.1. Schematic representation of plant genotype-dependent interactions in the rhizosphere. Left: Plant genotype susceptible to a complex of soil-borne pathogens. Right: Resistant plant genotype. Four hypothetical root exuded compounds (mock molecules), three pathogenic microbial species (reddish colours) and three beneficial species (greenish colours) are represented. Note: All microbial species are present in the rhizosphere of both plant genotypes but their relative abundance is different in the two cases. Mainly fungal pathogens are attracted by the susceptible genotype, and the plant is heavily infected, consequently, plant growth is stunted. The resistant genotype exudes either compounds that suppress pathogens directly (yellow) or compounds that attract beneficial microbes which

in turn mediate defence against pathogens, e.g. through direct antagonism, niche exclusions or localised or induced systemic resistance.

2.3.2 The interplay between root exudates and the microbial community

Beside direct antimicrobial effects, root exudates also influence plant health indirectly by attracting beneficial microorganisms. Rudrappa *et al.* (2008) showed that the secretion of malic acid in *Arabidopsis thaliana* was induced by pathogenic *Pseudomonas syringae* that, in turn, led to the recruitment of an antagonistic strain of *Bacillus subtilis*. Plant growth-promoting rhizobacteria and *Trichoderma* spp. are readily attracted by organic acids released from roots (Zhang *et al.*, 2014a; Zhang *et al.*, 2014b).

The regulation and the composition of root exudates is highly dynamic and changes with the physiological state of the plant (Yuan *et al.*, 2015). Root exudation is also affected by the soil microbial community. For instance, defensin genes are generally upregulated in legumes upon pathogen attack, as shown for the interaction between pea and *F. solani* (Chiang & Hadwiger, 1991). Interestingly, the same genes are induced in *Medicago truncatula* in response to the infection by an AMF, pointing at a possible mechanism of mycorrhiza-mediated disease resistance (Hanks *et al.*, 2005). Besides direct induction of plant defence-related metabolic responses, beneficial microorganisms can also prime the plant, a state of increased alertness (Conrath *et al.*, 2006; Pieterse *et al.*, 2014). Seeds of parasite weeds (e.g. broomrape; *Orobanche* sp.) conceive root exudates of their host plants as a signal to germinate and subsequently infect the host. Mabrouk *et al.* (2007) assessed the interaction of rhizobia (*Rhizobium leguminosarum*), pea root exudates and broomrape and found that the germination rate of broomrape seeds significantly decreases in the presence of root exudates collected from rhizobia inoculated peas. They identified marked changes in root exudate composition following inoculation with rhizobia, notably with significantly higher exudation of various phenolic compounds and flavonoids. Root exudation patterns also change upon contact and colonisation by mycorrhizal fungi, with much of the change related to the regulation of the symbiosis itself

(Jones *et al.*, 2004). Strigolactones are involved in the establishment of the mycorrhizal symbiosis. These phytohormones stimulate the spore germination and hyphal branching of AMF, but also trigger seed germination of parasitic plants (Gomez-Roldan *et al.*, 2008; Foo & Reid, 2012). The chemistry and mode of action of various individual root exudates involved in plant defence are known today. As illustrated, certain compounds have very specific effects on individual microorganisms, while others attract or suppress both beneficial and detrimental microorganisms. In turn, the microbial rhizosphere community greatly influenced root exudation of the plant. Consequently, the overall effect of the interplay between root exudates and root-associated microbes is the result of very complex reciprocal processes.

2.3.3 Genotypic differences in root exudation

Several studies on different legume species revealed genotypic differences in root exudate composition and their effect on rhizosphere processes. For instance, chickpea cultivars with different levels of susceptibility to Fusarium wilt vary in their production of chitinase, protease and glucanase in germinating seeds and in roots leading to distinct effects on *F. oxysporum* spore germination and hyphal growth (Haware *et al.*, 1984; Stevenson *et al.*, 1995). The expression of these anti-fungal compounds was shown to be induced in the resistant cultivars upon pathogen attack (Giri *et al.*, 1998). For pea, root exudates of eight different genotypes showed variable effects on quiescence levels in entomopathogenic nematodes (Hiltpold *et al.*, 2015). It was further shown that varying root exudate concentrations had contrasting effects on activity and infectiousness of beneficial as well as parasitic nematodes. For pathogenic fungi, similar contrasting effects of certain root exudate compound levels have been observed. For instance, root exudates of two peanut cultivars significantly differed in their stimulating effect on spore germination, hyphal growth and sporulation of the fungal pathogens *F. oxysporum* and *F. solani* (Li *et al.*, 2013a). Pavan *et al.* (2016) reported on a pea landrace highly resistant to a parasitic weed (*Orobanche crenata*), and that the resistance mechanism is likely due to the reduced secretion of strigolactones. Similar results were obtained with radiation-

mutagenised chickpea, where a decrease in stimulatory activity of root exudates towards broomrape seed germination was responsible for strong resistance of some mutants (Brahmi *et al.*, 2016). Genotypic differences for root exudation patterns also exist for other rhizosphere-related processes in legumes (Subbarao *et al.*, 1997; Wouterlood *et al.*, 2004; Kato & Arima, 2006; Rose *et al.*, 2010). A noteworthy comparable phenomenon to soil fatigue in legumes is the so called "apple replant disease" which is responsible for severe yield reduction in apple production. Recently, Leisso *et al.* (2017) evidenced differences in root exudation composition among four apple rootstock genotypes that show different levels of resistance to apple replant disease. The authors of this study deployed an elaborate experimental setup to control for rootstock- and root-associated bacterial origin of exudates and for developmental stage of the plant. Although it was not possible to draw a clear partitioning between disease resistant and susceptible genotypes based on patterns of root exudates, particular molecular compounds were significantly more present in individual genotypes. For example, levels of benzoic acid were significantly more produced by a resistant than a susceptible genotype. Benzoic acid is a preferred substrate of the bacterium *Burkholderia capacia* which was previously shown to be abundant in the rhizosphere of replant disease resistant root stocks and to act as a biological control against soil-borne pathogens (Pumphrey & Madsen, 2008).

Genotypic variation in microbial rhizosphere composition can be attributed to a differential exudation of compounds (Micallef *et al.*, 2009; Aira *et al.*, 2010; Peiffer *et al.*, 2013). For instance, differences in root exudation between one wild-type and two mutants of *Arabidopsis thaliana* are significantly correlated with differences in the bacterial and archaeal community composition in the rhizosphere (Carvalhais *et al.*, 2015). Variation in two plant genes (*su1* or *sh2*) responsible for plant carbon allocation strategy in maize greatly modified the structure and activity of the microbial community in the maize rhizosphere (Aira *et al.*, 2010). These findings demonstrate a genetic basis of the plant to stimulate or inhibit individual microbial strains or entire consortia through regulatory actions such as root exudation. The presented interplay between roots and their associated microbiome through exudates is a key determinant for plant health.

2.4 INTEGRATING THE MICROBIOME TO IMPROVE RESISTANCE AGAINST BIOTIC STRESSES IN LEGUME BREEDING

As described in the previous sections, pathogenic and beneficial micro-organisms in the rhizosphere interact with each other and the plant host. Despite the complexity of microbe-microbe and plant-microbe interactions, a solid scientific basis for directed manipulations of these interactions in agro-ecosystems is emerging. Actions targeting plant-microbe interactions will most likely improve plant health and productivity, and thus, lead to more sustainable agriculture (Turner *et al.*, 2013). Plant-microbe interactions can be harnessed either through the direct manipulation in form of an external supply of specific microbes or through plant breeding. We will briefly summarise some recent publications of successful applications of bio-inoculants, before discussing plant breeding for microbiome-mediated disease resistance in the last sections.

2.4.1 Increased crop productivity with microbial amendments

Bio-inoculations are a relatively simple but effective means to increase crop productivity through the amendment of certain micro-organisms. Seed coating of grain legumes has been especially successful for N-fixing bacteria and mycorrhizal fungi (Patil & Alagawadi, 2010; O'Callaghan, 2016; Oliveira *et al.*, 2017). A yield increase of 52% was obtained through co-inoculation of chickpea with an N-fixing (*Mesorhizobium ciceri*) and a P-solubilising (*Pseudomonas jessenii*) bacteria (Valverde *et al.*, 2006). Similarly, for mung bean (*Vigna radiata*), 23% yield increase was reported after co-inoculation with two AMF-species (Tarafdar & Rao, 1997). Yield increases of 20% and 39% were obtained with lentil after single or co-inoculation with three complementary bacterial strains, respectively (Chandra & Kumar, 2008). Shcherbakova *et al.* (2017) identified improved N-fixation, a change in the exudation activity and the rhizosphere microbiome of chickpea after a combined inoculation with *Mesorhizobium* sp., *Bacillus subtilis* and physiologically active

molybdenum. Significantly, these studies moved beyond applications of individual bio-inoculants and showed improved performances with co-application compared to the individual inoculation of microbes.

Major constraints for a successful establishment of the introduced microbes in the rhizosphere are limited viability and storage time, unfavourable abiotic soil properties or environmental conditions, insufficient competition or incompatibility of the inoculant with the indigenous microbial community (Fließbach *et al.*, 2009; Chaparro *et al.*, 2014; Abujabhah *et al.*, 2016; Hu *et al.*, 2017; Mnasri *et al.*, 2017). In the future, it will be possible to introduce more complex microbial associations as synthetic community inoculants (Niu *et al.*, 2017). Cole *et al.* (2017) proposed a starting point for a targeted improvement of the competitiveness of introduced microbes by identifying 115 genes relevant for the successful colonisation of *Pseudomonas simiae* of plant roots. Such approaches seem very promising and develop into fast-emerging business advances focussing on microbial seed coating (Waltz, 2017).

2.4.2 Engineering the microbial community through plant breeding

The potential of breeding for microbe-mediated disease resistance in legume crops is based on the hypothesis that plant functions, including resistance to biotic and tolerance to abiotic stresses, are the result of the plant's metagenome that includes the associated internal and external microbes (Berendsen *et al.*, 2012; Mendes *et al.*, 2013; Coleman-Derr & Tringe, 2014; Pieterse *et al.*, 2016; Berg *et al.*, 2017a; de Boer, 2017). This line of thought is additionally fuelled by the new advent in human health research related to the gut microbiome (Gilbert *et al.*, 2016). Vandenkoornhuyse *et al.* (2015) stressed the holobiont concept as a unit of selection and adaptation, considering the holobiont as one entity or megaorganism including functions and interactions of the plant and all associated internal and external microorganisms (i.e. the plant microbiome). The genetic information of this holobiont is defined as the hologenome. However, Douglas and Werren (2016) argue that selection might not necessarily happen always at the holobiont level, but that both mutualistic and antagonistic evolution (including fitness conflicts) can occur among constituent members of the host-microbiome communities.

2.4.3 Selecting against beneficial microbes?

It has been postulated that plant domestication and breeding, under optimal supply of input factors such as fertilisers, has unintendedly led to selection against a genetically diverse plant-associated microbial community (Morgan *et al.*, 2005; Bennett *et al.*, 2013; Bulgarelli *et al.*, 2015; Pérez-Jaramillo *et al.*, 2016; Hohmann & Messmer, 2017; Wang *et al.*, 2017a; Wen *et al.*, 2017). However, although Bulgarelli *et al.* (2015) could show small but significant differences in root-associated bacterial communities between a wild barley genotype, a barley landrace and the elite cultivar 'Morex', a general reduction in diversity was not found. Similar results were obtained when comparing ancient and modern maize (Johnston-Monje *et al.*, 2014). In contrast, wild beet plants harboured distinct operational taxonomic units and a more diverse bacterial community than domesticated sugar beet plants (Zachow *et al.*, 2014). A

more recent study further elaborated on the link between common bean domestication, specific root morphological traits and rhizobacterial communities and found a higher abundance of Bacteroidetes and less Actinobacteria and Proteobaceria in wild beans compared to modern bean cultivars (Pérez-Jaramillo *et al.*, 2017b). A common drawback of most of these studies is the limited number (<10) of genotypes analysed. However, comparing 33 wild populations, landraces and modern cultivars of sunflower (*Helianthus annus*), a significant shift in the fungal rhizosphere community was observed, while there was no change in bacterial rhizosphere and root community (Leff *et al.*, 2017). At the current taxonomic resolution, there seems to be a shift rather than presence-absence of operational taxonomic units along the footprint of domestication. Nevertheless, *in situ* conservation of crop wild relatives in their centre of origin offer a great potential to rediscover microbial associations that have co-evolved with the crop and to support modern breeding programmes aiming at enhanced plant immunity (Hale *et al.*, 2014).

2.4.4 Breed where the microbes are

Future breeding strategies to promote plant health should focus on multiple aspects of a plant in its given environment, including phenotypic, genotypic and metabolomic data but also plant microbial communities and the potential of the plant genotypes to steer their microbial communities (Box 2) (Smith & Goodman, 1999; Hartmann *et al.*, 2009; Wissuwa *et al.*, 2009; Bakker *et al.*, 2012; Lakshmanan, 2015; Pérez-Jaramillo *et al.*, 2016; Hohmann & Messmer, 2017). Although much more research is needed to close major knowledge gaps and link microbial diversity with function and ecosystem services (Finkel *et al.*, 2017; Hartman *et al.*, 2017; Oyserman *et al.*, 2018), there are already certain strategies and tools breeders can consider to integrate microbiome functions in breeding programmes.

Plant selection should occur in its target environment in living soil, allowing to account for plant-microbe and microbe-microbe interactions, as whole microbial communities rather than a few pathogen species might be different in that soil. Gaue (1998) stated that breeding of red clover

(*Trifolium pratense*) against soil-borne fungal diseases is only successful under field conditions. He argued that plant selection needs to be performed in fields where a naturally occurring complex of root rot pathogens is present. The soil fungal community of lotus (*Nelumbo nucifera*) in healthy and Fusarium wilt-infected fields showed significant differences, indicating reduction of beneficial microorganism and accumulation of fungal pathogens under continuous lotus cultivation (Cui *et al.*, 2016). These results are in line with the above cited observations on microbial dysbiosis in pea (Xu *et al.*, 2012; Yu *et al.*, 2012b), peanut (Li *et al.*, 2014a) and tobacco (Zhang *et al.*, 2017a). Such an approach will allow to select plant genotypes that associate with a disease-suppressive or "healthy" microbiome that can restrict the virulence of predominant pathogens. Plant breeding typically aims to develop cultivars with best performance over a range of different environments. This makes sense for the breeder from an economic point of view, but does not lead to varieties that are best adapted to local conditions, including efficient interactions with the local microbial community. For instance, Chang *et al.* (2014) evaluated lupine accessions and cultivars for resistance against *F. avenaceum* in field trials in Canada. Two cultivars, included in the screen, successfully developed for *F. avenaceum* resistance in Denmark and Germany were not resistant in the Canadian field trial. The authors reasoned that this observation might reflect differences in pathogen strains between Europe and Canada or differences in the environmental factors, including the resident microbial community (see also section *2.1.2 Resistance breeding against root diseases*). Such results show, that, in the case of breeding against root rot complexes, only selection under target environment conditions will produce improved cultivars.

Multi-location, multi-year testing is also crucial for the detection of plant genes or genetic loci with influence on the microbiome composition, because experiments under controlled conditions alone, by reducing environmental variability, might overestimate the importance of certain genes (Anderson *et al.*, 2013; Wagner *et al.*, 2016). Using naturally-infested agricultural soils that harbour multiple pathogens will allow to identify resistances to several interacting pathogens and, thus, better reflect the situation in the field. Experimental fields should be characterised not only for soil type, soil structure, nutrient content and

pH, but also for the microbial bulk soil and rhizosphere community. Chang *et al.* (2017) were able to identify groups of microbes associated with productivity of soybean based on a metagenome-wide association study assessing bulk soil from different field sites. Subsequently, they used a machine learning algorithm to successfully predict soybean productivity based on microbiome data. Characterisation of the resident field microbiome through metagenomic approaches could allow plant breeders to make well informed choices of field sites for selection and variety testing.

Besides field screenings, meaningful high-throughput selection can be achieved by standardised growth-chamber experiments that use agricultural field soils instead of sterile substrate. It was shown that disease assessments under controlled conditions can strongly correlate with field ratings (Wang *et al.*, 2006; Hamon *et al.*, 2011), indicating that these screening systems can serve as a valuable tool to identify possible sources of resistance in legumes. In contrast, selection steps conducted in sterile conditions ignore important plant-microbe interactions and might even lead to an overestimation of genotypic effects due to vertically transmitted microbes via seeds (Coleman-Derr & Tringe, 2014; Johnston-Monje *et al.*, 2014).

Another approach is to identify and quantify key players from target environments and use a synthetic community (including key pathogen species within a simplified microbial community) as inoculant under controlled conditions (Gopal *et al.*, 2013; Oyserman *et al.*, 2018). Niu *et al.* (2017) used a simplified representative bacterial community to study the community assembly dynamics on axenic maize seedlings under presence of root pathogens. Bazghaleh *et al.* (2015) were able to identify significant differences in root fungal community compositions between 13 chickpea cultivars and verified positive and negative effects of certain isolated strains on plant performance in a greenhouse.

Box 2 | Microbiome-supported resistance breeding

- The plant genotype significantly effects the composition of the rhizosphere microbiome.
- Selection of plant genotypes needs to be conducted in environments that reflect the pathogen situation in the field and that are favourable for plant-microbe interactions (i.e. in the absence of pesticides and excessive fertilisers).
- Individual pathogenic or beneficial key players (via real-time quantitative PCR) or whole microbiome profiles (via next-generation sequencing) can be determined to support the selection process in target environments.
- Plant breeders can screen for specific root exudate compounds that are involved in microbiome-mediated disease resistance.
- The heritability of plant resistance traits can be increased through the inclusion of plant genotype x environment x microbiome interactions.
- The identification of genomic regions associated with microbiome-mediated disease suppression allows to design marker-assisted selection (MAS) approaches.
- Microbiome-wide association studies can be used to predict plant health-associated capacities of microbial communities. Metagenomic characterisation of soil microbiomes allow plant breeders to make well-informed choices of field sites for selection and variety testing.

2.4.5 The plant-microbiome as a plant trait

Functional plant-microbiome interactions should be incorporated into breeding processes as a heritable trait. In order to disentangle direct and microbe-mediated resistance and prove the concept of holobiont selection, multi-factorial trials are needed to test the genotypes in sterile and non-sterile soils with different level of pathogen pressure. In one of the first studies, Panke-Buisse *et al.* (2014) showed that *Arabidopsis*-associated root microbiomes that were selected for the plant trait "flowering time" over ten generations would determine early or late flowering in axenic *Arabidopsis* microcosms. Indeed, the plant-associated microbiome of plants is a heritable plant trait as evidenced in different plant-microbe systems. The efficiency of selection for microbiome-mediated resistance depends on the heritability of the trait (i.e. genotypic variance divided by phenotypic variance). The host plant phenotype (P) is determined by the genotype (G), the environment (E), the genotype × environment interaction (G × E) and random effects (e). Integrating the soil microbiome into the formula results in P = G + E (including microbiome) + G × E (including plant-microbe interaction and plant × microbe × E interaction) + e. While breeders can utilise the variation based on G, the microbiome is mainly determined by the environment (soil type and structure, pH, climatic conditions, management practices), and the plant driven shift of microbial community is part of the G × E interaction. Part of G × E interactions that is linked to specific environmental factors like soil type, climatic conditions, crop management, or microbial communities can be utilised in breeding for local adaptation (Annicchiarico *et al.*, 2005; Busby *et al.*, 2017). Likewise, breeding more diverse cropping systems (e.g. mixed cropping, intercropping, undersowing) which harbour and maintain greater microbial diversity (Chave *et al.*, 2014; Granzow *et al.*, 2017; Lori *et al.*, 2017; Wang *et al.*, 2017b) will foster selection for beneficial plant microbe interaction.

Wagner *et al.* (2016) reported significant plant genotype effects and genotype x environment interaction of wild perennial mustard *Boechera stricta* on the microbiome community of leaves as well as effect of the plant age. The authors stress the importance of replicating microbiome

experiments across sites and time points, in order to reveal genes controlling microbiome variation that are of actual relevance for given farming environments. However, direct selection of plants promoting a beneficial soil microbiome community is very challenging, because few studies are available where microbial diversity is linked with improved plant health and because fields are not yet characterised according to their microbiome profile. In the future, further research of the soil microbiome and employment of additional tools like metagenome-wide association studies will allow to predict traits such as disease resistance based on the rhizosphere community composition (Nogales *et al.*, 2016; Chang *et al.*, 2017).

2.4.6 Genetic markers for beneficial microbial communities

As discussed in earlier sections, the plant genotype determines microbial community composition in the rhizosphere and this microbial community has direct implications for plant health. To take advantage of this knowledge and to integrate it into plant breeding, future research needs to explore which plant loci govern the interaction with the microbiome. In addition to illuminating the microbiome of different crop species and cultivars, the vast amount of microbiome sequencing data will allow to perform promising genome-wide association studies (GWAS) to identify plant loci responsible for specific plant-microbe interactions. In this regard, the work of Smith *et al.* (1999) has pioneer character. They evaluated the efficacy of the biocontrol agent *Bacillus cereus* against the tomato seed pathogen *Pythium torulosum* and detected significant phenotypic variation among recombinant inbred tomato lines for *B. cereus*-mediated disease resistance. Genetic analysis revealed that three major QTL, associated with disease suppression by *B. cereus*, explained 38% of the observed phenotypic variation among the recombinant inbred lines. Horton *et al.* (2014b) revealed that the microbial community of *Arabidopsis* is genotype-dependent and used a GWAS to identify plant loci responsible for the host genotype-dependent structuring of the microbial community. Intriguingly, gene sets involved in defence reaction and signal transduction in the plant were identified as being most important in the structuring of the microbial composition. With more and

more genomic information available beyond model species (e.g. reference genome for common bean (Schmutz *et al.*, 2014)), the identification of genomic loci and candidate genes involved in plant-microbiome interactions will be possible for legume crop species as well. For instance, a publicly available single nucleotide polymorphism (SNP) marker data set has recently been released for pea (Holdsworth *et al.*, 2017) and chickpea (Parween *et al.*, 2015). Association mapping has furthermore been successfully applied to identify markers for various agronomic important traits in legumes (Cheng *et al.*, 2015; Zuiderveen *et al.*, 2016). Leveraging plant genomic resources together with metagenomic information of the associated microbiota would enable the identification of markers for resistance against soil-borne pathogen complexes and the application of marker-assisted selection in breeding programmes in the future (Kroll *et al.*, 2017). This would ultimately lead to an enhancement of a favourable interplay between crop plants and their associated soil microbiota.

2.4.7 Plant selection based on key root exudates

Root exudates are central elements in the dialogue between plants and the microbial community in the rhizosphere. Genetic variation in root exudate composition could be exploited in breeding programmes if certain key exudates can be linked to microbial-mediated disease resistance. Breeding lines can be screened for resistance on pathogen-infested soil. Subsequent profiling of root exudates using untargeted metabolomics (e.g. gas chromatography-mass spectrometry; Zhang *et al.*, 2012) will enable the identification of key metabolites or exudate profiles significantly associated with disease resistance. Pavan *et al.* (2016) found a pea cultivar highly resistant to broomrape (*O. crenata*) infection and evidenced a reduced exudation of strigolactones as the explanation for resistance. However, strigolactones are also of great importance for AM symbiosis and care must be taken before such a selection target is chosen to avoid trade-offs (Bakker *et al.*, 2012). Parallel analysis of the microbiome composition allows to link plant resistance, root exudation profile and the occurrence of key microbes responsible for plant health. For instance, Wu *et al.* (2017) showed that organic acids exuded by the

herb *Radix pseudostellariae* have negative effects on biocontrol microbes but favour pathogenic fungi and bacteria. Association mapping can be applied to identify genes involved in the synthesis and regulation of such key root exudates. Thereby, plant breeders have an intermediary trait at their command that allows them to select for microbe-mediated disease resistance. Moreover, recent advances in –omics technologies propel the ambition to engineer the plant rhizosphere for healthier and more productive crop plants (Ramalingam *et al.*, 2015; Zhang *et al.*, 2015). This knowledge will be a valuable source of information for the design of molecular plant breeding strategies.

2.5 CONCLUDING REMARKS

The importance of grain legumes for feed and food production is likely to increase in the near future. Rich in high quality proteins, minerals and vitamins, they represent a healthy food component in human diet. In many developing countries, they are already an irreplaceable part of the daily dishes, and, in the lifestyle societies of industrialised countries, they contribute to a reduced meet consumption. Through the symbiotic association with nitrogen-fixing rhizobia, legumes are able to significantly improve soil fertility and hence, represent an ecologically important crop in low-input farming systems. Moreover, cool-season grain legumes provide an important alternative to soy-based protein imports. In the past decade, many reviews summarised the importance of microbial communities for plant health. Associations between roots and beneficial microorganisms, including the well-studied examples of symbiotic associations of legumes with rhizobia or AM symbiosis, form the basis of our current understanding of plant-microbe interactions. We can expect that we will be able to go beyond these reductionist approaches in the near future and that our knowledge on complex plant-microbiome interactions will grow. More and more experiments assess complex plant-microbe interactions in soil-based systems and we begin to elucidate how plants protect themselves by shaping the microbial complexity of the rhizosphere. The understanding of the chemical dialogue between plants and microbes along the genomic deciphering of microbiome compositions will reveal leveraging points for resilient crop production

systems. Plant breeding is the means by which plant-microbiome interactions can be harnessed to shape healthy and beneficial microbial communities in the rhizosphere. Integrating the knowledge on multi-functional interactions between crop plants and microbes in future agricultural systems and plant breeding will eventually lead to sustainable solutions to reduce the threat imposed by soil-borne pathogens.

3 | HERITABLE VARIATION IN PEA FOR RESISTANCE AGAINST A ROOT ROT COMPLEX AND ITS CHARACTERISATION BY AMPLICON SEQUENCING

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Abstract Chapter 3

Soil-borne pathogens cause severe root rot of pea (*Pisum sativum* L.) and are a major constraint to pea cultivation worldwide. Resistance against individual pathogen species is often ineffective in the field where multiple pathogens form a pea root rot complex (PRRC) and conjointly infect pea plants. On the other hand, various beneficial plant-microbe interactions are known that offer opportunities to strengthen plant health. To account for the whole rhizosphere microbiome in the assessment of root rot resistance in pea, an infested soil-based resistance screening assay was established. The infested soil originated from a field that showed severe pea root rot in the past. Initially, amplicon sequencing was employed to characterise the fungal microbiome of diseased pea roots grown in the infested soil. The amplicon sequencing evidenced a diverse fungal community in the roots including pea pathogens *Fusarium oxysporum, F. solani*, *Didymella* sp. and *Rhizoctonia solani* and antagonists such as *Clonostachys rosea* and several mycorrhizal species. The screening system allowed for a reproducible assessment of disease parameters among 261 pea cultivars, breeding lines, and land races grown for 21 days under controlled conditions. A sterile soil control treatment was used to calculate relative shoot and root biomass in order to compare growth performance of pea lines with highly different growth morphologies. Broad sense heritability was calculated from linear mixed model estimated variance components for all traits. Emergence on the infested soil showed high (H^2 = 0.89), root rot index (RRI_{CC}; H^2 = 0.43) and relative shoot dry weight ($SDW_{Rel.}$; H^2 = 0.51) medium heritability. The resistance screening allowed for a reproducible distinction between PRRC susceptible and resistant pea lines. Subsequently, the resistance ranking was verified in an on-farm experiment. We found a significant correlation (r_s = 0.73, p = .03) between the controlled conditions and the resistance ranking in a field with high PRRC infestation. The screening system allows to predict PRRC resistance and offers a tool for selection at the seedling stage in breeding nurseries. Using the complexity of the infested field soil, the screening system provides opportunities to study plant resistance in the light of diverse plant-microbe interactions occurring in the rhizosphere.

3.1 INTRODUCTION

Pea (*Pisum sativum* L.) is an important protein sources for human consumption and animal feed. It has an annual worldwide production of 36 mega tonnes, making it the second most important pulse after common bean (FAOSTAT, 2019a). Pea represents a valuable crop for sustainable cropping systems: Through the symbiosis with nitrogen-fixing rhizobacteria, pea cultivation improves soil fertility and reduces the demand for external nitrogen fertilizers (Foyer *et al.*, 2016). In Europe, an increase in pea acreage is expected due to current incentives to promote regionally produced plant-based protein as an alternative to overseas soybean imports (Reckling *et al.*, 2016).

Pea cultivation is challenged by various abiotic and biotic stresses (Rubiales & Mikic, 2014). Most importantly, several soil-borne diseases threaten pea cultivation, especially in the temperate zones, where the most important pea cultivation areas are. The most devastating diseases are caused by fungal pathogens, including various species of the genus *Fusarium* (most notably, *F. solani*, *F. avenaceum*, and *F. oxysporum*), *Didymella pinodes* (formerly known as *Mycosphaerella pinodes*), *D. pinodella* (formerly known as *Phoma medicaginis* var. *pinodella*), *Rhizoctonia solani, Sclerotinia sclerotiorum*, and the oomycetes *Aphanomyces euteiches* and *Pythium* spp. (Wille *et al.*, 2019). These pathogens are responsible for severe seed rot, damping-off, seedling blight, and root and foot rot.

Multiple pea pathogen species co-occur in the field, leading to the adoption of the term pea root rot complex (PRRC). It has repeatedly been shown that root-infecting pathogens interact and aggravate disease in pea (Kerr, 1963; Muehlbauer & Kraft, 1973; Shehata *et al.*, 1983). However, research has only recently readopted this line of work in order to understand the complexity, distribution and interplay among multiple pathogens in PRRC (Šišić *et al.*, 2017; Taheri *et al.*, 2017; Willsey *et al.*, 2018). Moreover, the rhizosphere harbours a vast diversity of micro-organisms involved in plant-microbe and microbe-microbe interactions, ranging from plant pathogenic to plant beneficial and antagonistic to synergistic, respectively (Müller *et al.*, 2016).

Microbial dysbiosis caused by inappropriate culturing practices such as narrow crop rotations are often at the origin of outbreaks of soil-borne diseases. For instance, Bainard *et al.* (2017) showed that increasing the frequency of pea in a four-year crop rotation causes the build-up of fungal pathogens in the soil, and Xu *et al.* (2012) showed that pathogenic species displace beneficial fungi in diseased pea roots. Respecting long rotation breaks is the most constructive strategy in this regard. However, this is in conflict with efforts to increase the cultivation area of legume crops in general and pea in particular. Control by chemical fungicides is only available through the application of seed treatments, but shows only moderate effects on emergence and disease severity of pea in infested fields (Xue, 2003b; Wu *et al.*, 2019). Biological control agents are a possible alternative to chemicals; however, they still need to demonstrate their efficacy to confer protection under field conditions (Alabouvette *et al.*, 2009).

Breeding resistant varieties is considered the most promising approach for sustainable pea cultivation, especially with the increasing necessity to shift from large-scale applications of chemical pesticides and seed treatments towards more integrative solutions (Rubiales *et al.*, 2015). However, multipartite interactions among pathogens and other microbes in the PRRC are rarely considered in resistance studies. Resistance screenings are commonly performed under controlled conditions, where seedlings, grown on sterile substrate, are inoculated with single pathogen isolates. This practice allows for reproducible mono-factorial disease scorings and has led to the identification of resistance sources for major pathogens in various pea germplasm collections over the last decades (Infantino *et al.*, 2006; Rubiales *et al.*, 2015). However, resistance against individual pathogen species or strains assessed under controlled conditions is frequently ineffective when moved to the field as different pathogens are present in the PRRC (Hamon *et al.*, 2011). The work of Abdullah *et al.* (2017) suggests that plant-pathogen interactions and resistance should be studied in field representative systems to achieve progress in disease management.

The observation that multiple soil-borne pathogens interact to shape the development of root rot is underlined by results from field resistance trials. For instance, pea breeding lines exhibited different levels of resistance to Aphanomyces root rot when evaluated at two different

locations in the north-west U.S. (Weeden *et al.*, 2000). Further, Hamon *et al.* (2011) reported significant genotype-by-environment interactions in a quantitative trait loci (QTL) study for Aphanomyces root rot resistance carried out in different French and U.S. field sites. The authors of these studies concluded that pathogen diversity together with the co-occurrence of other pathogens at the different field sites explain site-specific resistance rankings and thereof identified resistance loci. These results highlight the importance of respecting the soil microbial community as an integral part of the environment.

Plant breeders rely on reproducible screening systems that allow the screening of large numbers of lines. These screening systems need to include major factors of the target environment – including the soil type and the microbiome composition of that particular soil – to provide reliable and field-relevant data for subsequent breeding efforts (Duc *et al.*, 2015; Wei & Jousset, 2017). It is widely accepted that plant health also depends on the plant-associated microbial community (Berg *et al.*, 2017a). Thus, incorporating microbiome-associated phenotypes in resistance breeding will provide a more solid basis to breed crops for enhanced disease resistance (Oyserman *et al.*, 2018; Wille *et al.*, 2019).

In order to account for the interactions between the plant genotype and the pathogen complex embedded in the entire rhizosphere microbiome, we designed a resistance screen based on infested soil. The overall aim of this study was to develop an infested soil-based resistance screening at seedling stage under controlled conditions to allow for a reproducible assessment of resistance against PRRC and to assess its relation to resistance in the field. Specifically, we aimed at (i) establishing a screening system that allows to differentiate between susceptible and resistant pea lines; (ii) assessing broad-sense heritability of various disease-related traits; (iii) examining the relationship among these traits in order to better understand the disease expression and identify most suited parameters to assess PRRC resistance; and (iv) relating the controlled conditions resistance ranking with field performance in order to evaluate the relevance of the proposed screening tool for resistance breeding. In addition, we applied amplicon sequencing to characterise fungal diversity present in the rhizosphere of PRRC diseased pea to identify potential pathogens and beneficials.

3.2 MATERIAL AND METHODS

3.2.1 Plant material

This study is based on a set of 261 pea (*Pisum sativum* L.) lines, including 177 genebank accessions from the USDA-ARS GRIN Pea Core Collection (https://www.ars-grin.gov), 47 advanced breeding lines provided by a private organic breeder organisation (Getreidezüchtung Peter Kunz, Switzerland, https://www.gzpk.ch/) and 34 registered cultivars from Europe (Supplementary Table 1). Additionally, two cultivars were included as reference lines, namely 'EFB.33' (experimental identifier: "C1"), a cultivar with known resistance capacities against root rot (Baćanović-Šišić *et al.*, 2018), and 'Respect' ("C2"), a standard registered variety susceptible to root rot. The set contained full-leaf and semi leafless type pea lines.

3.2.2 Infested field soil used for controlled conditions screening

Naturally infested soil was collected from a field under certified organic production located in Kirchlindach, Canton Bern, Switzerland (47°00'14.5"N 7°24'37.7"E) in March 2016. Physico-chemical soil characteristics are given in Table 3.1. Soil from this field site was previously assessed in a study on PRRC in Swiss and German fields and showed strong signs of PRRC (Fuchs *et al.*, 2014). Sieved soil was stored in polypropylene boxes at 4°C in the dark until further use.

3.2.3 Protocol of controlled conditions resistance screening based on infested soil

The set of pea lines was evaluated for resistance against PRRC in the naturally infested soil. Seeds were surface-sterilized in 70% ethanol for 30 seconds followed by a 1:1 (v:v) ddH_2O-bleach solution (M-Classic Javel Wasser, Migros, Switzerland; final concentration approx. 2.5%) for ten minutes. Finally, seeds were thoroughly rinsed in ddH_2O and soaked for 2 h. Four seeds per line were planted in a 2:1 (v:v) mixture of infested

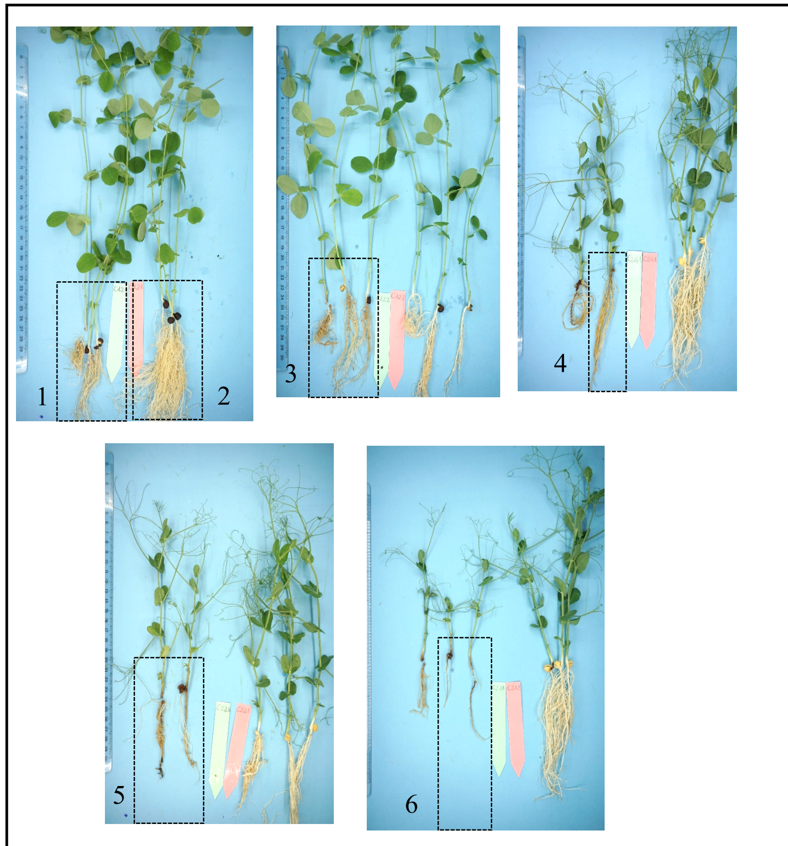


Figure 3.2 Root rot index (RRI_{CC}) on a 1 to 6 scoring scale (1 = no symptoms, 2 = small localised lesions on lower stem or upper root, covering less than 50% of circumference, 3 = light brown discoloration and moderate disintegration (< 30% compared to uninoculated control) of the root system, 4 = dark brown discoloration and strong disintegration (> 30% compared to uninoculated control) of the root system, 5 = only tap root left attached to the plant, 6 = complete disintegration of the root system). Plants grown on infested field soil are on the left side in the picture, plants grown on sterilized field soil on the right. The levels 1 – 6 are displayed in the pictures. Pictures (A) and (B) display pea genotype C1 (cv. 'EFB.33'). Pictures (C), (D) and (E) display pea genotype C2 (cv. 'Respect'). A 30 cm ruler is included in each picture.

soil and sterilised sand (Quartz d'Alsace, 0.2 – 0.63 mm grain) in plastic pots (200 ml, Migros, Switzerland). For the control treatment, soil and sand were sterilised (X-Ray irradiation 30-100 kGy, Synergy Health Däniken AG, Switzerland) and kept vacuum packed at 4°C in the dark until use.

Pots were arranged in a randomised complete block design with four replications. The replications were run in a series over four months. Complete blocks were further divided into five incomplete blocks of 52 or 53 pea lines augmented with two entries of the two reference cultivars C1 and C2. Each experimental unit was set up as a pair of two pots, containing either infested soil or sterilised soil. The five incomplete blocks of one replication were sown on five consecutive days and harvested over five days in the same order. Plants were grown under controlled conditions in the growth chamber for 21 days. A 16/8 light/dark cycle was applied, providing a photosynthetically active photon flux density of 200 µmol m^{-2} s^{-1} over the waveband 400 – 700 nm. Plants were watered every 72 h by flooding the pots 4 cm high for 30 min. Growth chamber mean (± s.e.), minimum and maximum temperature over the course of the experiment was 20.1 ± 0.3 °C, 17.7 ± 0.7 °C and 26.9 ± 2.9 °C, respectively, and mean, minimum and maximum relative humidity was 85.3 ± 14.9%, 40.4 ± 6.9% and 94.6 ± 12.2%, respectively. Pots were inspected on a daily basis for seedling emergence and plants were thinned out to reach a maximum of three plants per pot. Plants emerging after 14 days were removed and not considered in any analysis.

3.2.4 Phenotypic assessments in the controlled conditions resistance screening

Seedling emergence was recorded 14 days after sowing and a plant emergence rate (n/4; 0 – 1) was calculated on a per pot basis. Pea lines with a seed germination rate below 0.85 in the control treatment (seven lines; Figure 3.1) were excluded from the analysis of emergence. Twenty-one days after sowing, the plants were removed from the pots and roots were washed under running tap water. Plants were visually inspected and the following disease scores and vitality parameters were assigned to individual plants: 1) *Controlled Conditions Root Rot Index* (RRI_{CC}) using

a 1 to 6 scoring scale (Figure 3.2; 1 = no symptoms, 2 = small localised lesions on lower stem or upper root, covering less than 50% of circumference, 3 = light brown discoloration and moderate disintegration (< 30% compared to uninoculated control) of the root system, 4 = dark brown discoloration and strong disintegration (> 30% compared to uninoculated control) of the root system, 5 = only tap root left attached to the plant, 6 = complete disintegration of the root system). This RRI_{CC} was developed as the observed disease picture did not fit previously described disease score indexes for major pea root rot pathogens such as *Fusarium solani* (Grünwald *et al.*, 2003; Bodah *et al.*, 2016), *Fusarium* ssp. and *Didymella pinodella* (Pflughöft *et al.*, 2012) or *Aphanomyces euteiches* (Moussart *et al.*, 2008); 2) *Cortex Decay Index* (CDI) on root and epicotyl using a 1 to 5 scoring scale (1 = no symptoms, 2 = cortex locally cracked, 3 = local disintegration of the cortex (< 5mm), 4 = strong disintegration of the cortex (>5mm) and vascular tissue visible, 5 = total disintegration of cortex, roots hanging attached to vascular tissue); 3) *Shoot Lesion Index* (SLI) on the epicotyl and stem base using a 1 to 6 scoring scale (1 = no symptoms, 2 = small localised discolorations, 3 = spread of discoloration up to max. second lowest shoot node, 4 = spread of discoloration above second node, 5 = discoloration and drying-out or soft, water-soaked stem base, 6 = discoloration and disintegration of stem base); 4) *Nodulation Index* (NOD) using a 1 to 7 scale (1 = no nodules, 2 = 1-5 nodules, 3 = 6-10 nodules, 4 = 11-20 nodules, 5 = 21-40 nodules, 6 = 41-60 nodules, 7 > 60 nodules). Pot medians were calculated from scores of individual plants for these four traits. Furthermore, 5) *Plant height* (from the cotyledons to youngest node); and 6) *Disease Progress* (DIS; length of lesion on the stem above the cotyledons) were measured in [cm]; and 7) *Wilted Nodes Ratio* (WIL;$(N_{Wilted\ nodes} / N_{Total\ nodes})_{Infested\ soil} - (N_{wilted\ nodes}/N_{Total\ nodes})_{Sterile\ soil}$) calculated. Pot means were calculated for these three traits. Finally, fresh shoot and root biomass was recorded. Subsequently plants were dried at 105°C until constant weight before recording dry weight. Biomass measurements per pot were standardised with the number of plants per pot at harvest. *Relative Shoot and Root Fresh and Dry weights* were calculated by dividing the biomass of the infested soil treatment by the biomass of the corresponding sterile control treatment of the same genotype in each replication ($SFW_{Rel.}$, $RFW_{Rel.}$, $SDW_{Rel.}$ and $RDW_{Rel.}$, respectively).

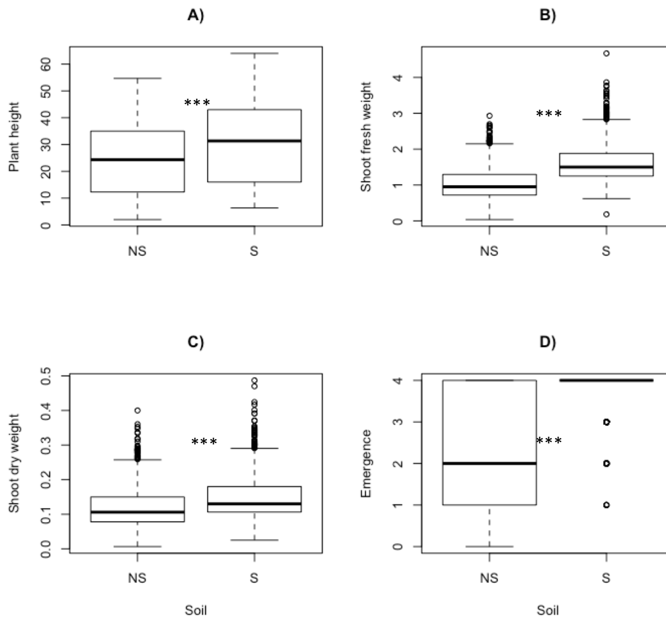


Figure 3.1. Growth characteristics of 261 pea lines in naturally infested field soil (NS) and in sterilised soil (S). Paired t-tests were used to calculate the significance of the difference between the treatment means. For emergence a Wilcoxon signed rank test was applied. A) Overall mean plant height after 21 days ($t = 29.15$, $df = 982$, $p < .001$); B) overall mean shoot fresh weight after 21 days ($t = 39.7$, $df = 980$, $p < .001$); C) overall mean shoot dry weight after 21 days ($t = 18.5$, $df = 975$, $p < .001$); D) overall mean plant emergence after 14 days ($V = 313710$, $p < .001$).

3.2.5 On-farm verification of the controlled conditions resistance screening

Seven pea lines were selected from the controlled conditions experiment and evaluated together with the two reference cultivars on two different on-farm sites in 2018. The pea lines were selected based on contrasting root rot resistance (RRI_{CC}) and to represent gene bank accessions as well as breeding lines. One experimental site was located in the field where the naturally infested soil for the pot trial was obtained ('heavily infested site'). The second site was located within 50 m to the first site ('moderately infested site'), with similar soil characteristics compared to the heavily infested site (Table 3.1). According to the personal communication of the farmer, this second site was less affected by pea root rot in 2014. The crop rotation for both field sites from 2014 to 2017 was: Pea/barley – winter wheat – oat/vetch – potato – winter wheat. Field sites were on-land ploughed and the seed bed preparation was carried out with a spring-tooth harrow. Both sites were arranged in a randomized complete block design with three replications per pea line. The plot size was 1.7 m x 1.5 m with three single rows of pea flanked by a row of spring barley ('Atrika') on each side. Pea and barley seeds were sown on April 10, 2018 at a density of 94 and 200 seeds per m^2, respectively. Sowing depth was 5 cm. The trial was operated under certified organic farming conditions. Weeding was done manually as needed. Twenty-four days after sowing 'Kaolin' (Surround, Stähler) was applied according to manufacturer instruction (32kg/ha) to combat pea leaf weevil (*Sitona lineatus*). Cumulative rainfall March – April – Mai 2018 in the region (*Agrometeo* station '*Oeschberg*') was 143.3 mm (2008-2017 long-term mean ± s.e. for the same period: 312.0 ± 44.3 mm). Fifty-five days after sowing, 15 plants per plot were randomly selected, carefully dug out and roots were washed with tap water. Root rot was assessed using a 1 to 8 scoring scale adapted from Pflughöft (2008) (1 = no symptoms, 2 = small localised lesions at hypo-/epicotyl, 3 = light-brown discoloration/lesion, with <50% circumference of the tap root, 4 = dark discoloration/lesion, with >50% circumference of the tap root, 5 = progress of the discoloration up to first lower leaf and/or <3cm in the tap root, possibly localised drying and bursting, 6 = progress of the

discoloration further than first lower leaf and/or >3cm in the tap root, possibly localised drying and bursting, 7 = decay of root and/or lower stem cortex, possibly visible vascular tissues, 8 = total disintegration of the root system or the stem, plant dying. A field root rot index (RRI_{Field}) per plot was calculated as the median of 15 plants.

3.2.6 Statistical analysis of phenotypic data

All calculations were performed with R 3.5.2 (R Core Team, 2018). Mixed model analyses were performed on the controlled conditions screening data with the following model: $y_{ijk} = \mu + g_i + r_j + s_k + \varepsilon_{ijk}$. Here, y_{ijk} represents the pot observation for trait y, μ denotes the overall mean, g_i the effect of pea line i, r_j the effect of replicate j, s_k the effect of the incomplete block k and ε_{ijk} the residual. For the estimation of genotypic means, the effects μ, g_i and r_j were considered fixed, the others as random. In order to meet the assumptions of the model plant height and biomass weights were $\log_{10}$-transformed before analysis. Relative biomass, wilted nodes ratio, disease progress, root rot, cortex decay, shoot lesion, and nodulation indices were transformed using an inverse Lambert W × F_X function before analysis (*LambertW* package) (Goerg, 2015). Data on seedling emergence was analysed as probabilities using a generalized linear mixed model fitting a binomial distribution of the errors using maximum likelihood estimation. A Wald χ^2 test with type II sums of squares (*Anova* function in the *car* package) was applied on each model to calculate the p-values for the fixed effects of pea line and replicate. Marginal R^2 were calculated according to Nakagawa *et al.* (2013).

For the estimation of variance components due to genotypic effects of pea lines (σ^2_g) and residual factors ($\sigma^2\varepsilon$), g_i was considered as random. Variance components were computed by restricted maximum likelihood, except for seedling emergence, where a maximum likelihood approach was used. Broad sense heritability (H^2) on entry mean basis were calculated as: $H^2 = \sigma^2_g/(\sigma^2_g+\sigma^2_\varepsilon/R)$, where σ^2_g is the genetic variance component, σ^2_ε the residual variance component and R the number of replicates. Bootstrapping (bootMer function in the lme4 package) was used to estimate standard errors of variance components and H2. Mixed

Table 3.1. Soil characteristics of two field sites with strong signs of pea root rot complex (Fuchs *et al.*, 2014). Soil from site ‘heavy infestation’ was used for the controlled conditions resistance screening of 261 pea lines. In 2018, a selection of nine lines was evaluated in a field trial on both sites. Soil samples were taken on 14 March 2018 with a soil auger (3 cm diameter) to 20 cm depth in each plot. Twenty-four samples per site were pooled and homogenised before analysis. Grain composition, organic matter content (OM), pH and soil nutrient analysis (except N_{min}) were performed by the *Labor für Boden- und Umweltanalytik* (lbu), Switzerland. N and C analysis were performed by the *Research Institute for Organic Agriculture* (FiBL), Switzerland according to Agroscope (1996).

Site	Grain composition			pH	Soil nutrient content [%]			Soil nutrient content [mg kg^{-1}]							
	Clay	Silt	Sand		N_{tot}	C_{tot}	C_{org}	N_{min}	P	K	Mg	B	Mn	Cu	Fe
‘Heavy infestation’	16.5	37.4	46.1	7.1	0.19	1.95	1.90	2.6	123.4	115.9	90.3	0.6	179	12.2	253
‘Moderate infestation’	16.5	28.9	54.6	6.8	0.19	1.91	1.89	3.9	65.1	106.6	120.9	0.4	263	13.3	270

model calculations were done using the packages *lme4* 1.1-20 (Bates *et al.*, 2015) and *emmeans* (Lenth, 2019). Compliance of the model assumptions was controlled by visual inspection of the residual plots.

A Principal Component Analysis (PCA) was performed on a subset of eleven phenotypic variables, using the estimated means from the mixed model analysis (PCA function in the FactoMineR package (Le *et al.*, 2008)). Pairwise relationships between the estimates of plant emergence, root rot index and relative shoot dry weight were explored by calculating Spearman's correlation coefficients (*cor.test* function).

A linear fixed effect model was used to analyse the root rot data from the on-farm trial (RRI_{Field}): $y_{ijk} = \mu + g_i + f_j + g_{fij} + b_k + \varepsilon_{ijk}$, where y_{ijk} represents the plot observation for RRI_{Field}, μ denotes the overall mean, g_i the effect of pea genotype i, f_j the effect of field site j, g_{fij} the effect of the interaction between genotype i and field site j, b_k the effect of block k and ε_{ijk} the residual. As for the controlled conditions experiment, root rot data was transformed using an inverse Lambert W x F_X function before analysis. Spearman's correlation coefficients were calculated for the analysis of pairwise relationships between $SDW_{Rel.}$, RRI_{CC} and RRI_{Field}. All data is presented as the back-transformed means.

3.2.7 Assessment of root fungal community composition

In order to describe the fungal community composition present in the infested field soil, an additional experiment was set up with the pea lines S12 and S164, each grown as described above with our seeds per line and replicated four times. Diseased roots and rhizosphere soil were sampled after 21 days according to Lundberg *et al.* (2013). Briefly, roots were shaken to remove loosely attached soil and washed in sterile 50 ml tubes in 25ml sterile water by vortexing. Washed roots were stored at -20°C until further processing. The rhizosphere soil suspension was centrifuged for 15 min at 3,200 g. The pellet was resuspended, transferred to 1.5 ml tubes and centrifuged again at 10,000 g for 5 min to form a pellet. DNA was extracted from lyophilised rhizosphere soil and roots using the NucleoSpin Soil Kit (Macherey-Nagel) and the Quick-DNA Plant/Seed Miniprep Kit (Zymo Research), respectively, according to manufacturer's instructions. Primers ITS1f (Gardes and Bruns, 1993) and ITS2 (White *et*

al., 1990) were used to amplify the ITS1 region. The reaction volume was 20 µl and contained 1x 5 Prime Hot Master mix and 200 nM of each primer. The PCR program consisted of an initial denaturation step of 2 min at 95°C, followed by 32 cycles (based on qPCR pre-test) of denaturation at 94°C for 20 sec, annealing at 52°C (based on gradient PCR pre-test) for 10 sec, elongation at 65°C for 50 sec followed by a final elongation step at 65°C for 10 min. PCR reactions were run in triplicates with a negative control. Triplicate PCR products were pooled and purified with home-made solid-phase reversible immobilization (SPRI) beads (https://openwetware.org/wiki/SPRI_bead_mix). Pooled PCR products were indexed using the Nextera XT Index kit v2 (Illumina) with 1x KAPA HiFi HotStart ReadyMix according to manufacturer's instructions, verified on 1.5% agarose gel, purified using SPRI beads, quantified using QUBIT DNA BR assay (Thermofisher Scientific) with SPARK 10M Platereader (Tecan) and combined in equimolar fashion. The library was quantified and quality was validated with Tapestation (Agilent Technologies). The library was sequenced at the Genomic Diversity Center (Zurich, Switzerland) on the Illumina MiSeq Personal Sequencer (Illumina) using a 600 cycle v3 Sequencing kit (Cartdridge. no. MS-565-2828), in paired-end 2 x 300 bp mode.

The MiSeq data was processed similar to the workflow described in Bodenhausen *et al.* (2019). Briefly, read ends were trimmed using *usearch* v10.0.240 (Edgar, 2013) and subsequently merged into amplicons using *FLASH* v1.2.11 (Magoc and Salzberg, 2011). In a next step, *CUTADAPT* v1.12 was used to trim off primer sequence (Martin, 2011). Subsequently, reads were quality filtered using *prinseq-lite* v0.20.4 (Schmieder and Edwards, 2011). The quality filtered sequences were clustered into operational taxonomic units (UPARSE – OTUs, ≥ 97% sequence similarity) and amplicon sequence variants (UNOISE) using *usearch* v10.0.240 (Edgar, 2013). *SINTAX* (Edgar, 2016) was used for taxonomic assignments using *UNITE* v7.2 (Abarenkov et al., 2010) database for the fungal community. Taxonomic information of unassigned sequences (below family rank) were further explored using BLAST analysis of the Nucleotide collection database. BLAST taxonomic information was considered at query cover >92% and sequence identity of 100%.

Statistical analyses were performed using R 3.5.2 (R Core Team, 2018). Permutational multivariate analysis of variance (PERMANOVA) was performed to determine the effects of the factor levels bulk soil, rhizosphere soil and roots. PERMANOVA analyses of the fungal community compositions revealed no differences between the two pea genotypes for the rhizosphere and the root. Therefore, relative OTU abundances in the rhizosphere and root of pea are analysed based on the mean of both genotypes. Root-enriched OTUs were identified using *EdgeR* (Robinson *et al.,* 2010). The data was filtered to remove low-abundant OTUs (OTUs with less than four sequences in less than four samples) and normalised by trimmed mean of M-values normalisation (Robinson and Oshlack, 2010). Dispersion was estimated with the *estimateGLMRobustDisp* function (Zhou *et al.,* 2014). A negative binomial model was fitted to the data with the *glmFit* function and the coefficient of interest was tested with the *glmLRT* function. To calculate the mean relative abundance, the data were first transformed by dividing each count by the total sum and transformed with $\log_2(x+1)$. Data is presented as the back-transformed means.

3.3 RESULTS

3.3.1 Assessment of the controlled conditions resistance screening

Clear pathogenesis was observed over all lines and replications 21 days after sowing, with significant lower plant emergence, plant height and shoot biomass in the infested soil compared to the sterile control (Table 3.2, Figure 3.1). Disease development ranged from single localised lesions on the root and the lower stem to heavily decayed root systems. Most plants showed an intermediate infection with light to dark brown discoloration and reduced volume of the root system (Figure 3.2). Reproducible differentiation was achieved and the two reference cultivars fit the expected response, with C1 showing significantly higher emergence rate and relative shoot dry weight ($SDW_{Rel.}$) and lower root rot RRI_{CC} than C2 (Figure 3.3, Figure 3.4). High heritability was found for plant height, shoot biomass and plant emergence in both the infested

Table 3.2. Means, ranges, genotypic (σ^2_g) and residual (σ^2_ε) variance components and broad-sense heritability (H^2) with standard errors (s.e.) for the phenotypic traits evaluated in 261 pea lines in the controlled conditions resistance screening. Plants were grown in naturally infested and sterilised soil under controlled conditions in four replications and evaluated 21 days after sowing.

Trait		Mean	Range	Variance components		$H^2 \pm$ s.e.[a]
				σ^2_g	σ^2_ε	
Infested soil						
Plant height [cm]		24.0	2.0 – 54.7	0.314	0.059	0.96 ± 0.005
Shoot fresh weight [g]		1.0	0.1 – 2.9	0.020	0.025	0.76 ± 0.025
Shoot dry weight [g]		0.12	0.01 – 0.40	0.027	0.030	0.78 ± 0.022
Relative shoot fresh weight	$SFW_{Rel.}$	0.65	0.06 – 1.81	0.008	0.040	0.46 ± 0.057
Relative shoot dry weight	$SDW_{Rel.}$	0.82	0.03 – 2.14	0.014	0.053	0.51 ± 0.052
Relative root fresh weight	$RDW_{Rel.}$	0.29	0.0002 – 0.94	0.005	0.014	0.58 ± 0.053
Relative root dry weight	$RDW_{Rel.}$	0.42	0.0019 – 1.25	0.009	0.034	0.51 ± 0.066
Root rot index	RRI_{CC}	3.7	2 – 6	0.067	0.355	0.43 ± 0.059
Cortex decay index	CDI	2.4	1 – 5	0.114	1.425	0.24 ± 0.081
Shoot lesion index	SLI	3.7	1 – 6	0.250	1.500	0.40 ± 0.065
Disease height [cm]	DIS	2.2	0 – 6	0.379	1.014	0.60 ± 0.043
Wilted nodes ratio[b]	WIL	0.34	-0.38 – 1	0.011	0.056	0.43 ± 0.060
Nodulation index	NOD	2.5	1 – 5	0.547	0.923	0.70 ± 0.031
Emergence rate		0.6	0 – 1	1.99	1.000	0.89 ± 0.013
Sterilised soil						
Plant height [cm]		29.7	6.3 – 64.0	0.299	0.009	0.99 ± 0.001
Shoot fresh weight [g]		1.6	0.2 – 3.8	0.015	0.004	0.94 ± 0.006
Shoot dry weight [g]		0.15	0.03– 0.49	0.022	0.007	0.93 ± 0.007
Emergence rate		0.95	0.25 – 1	NA[c]	NA	NA

[a] SE of variance components and H^2 were estimated by bootstrap (1000 iterations) using the *bootMer* function in R.
[b] The wilted nodes ratio was corrected by subtraction of the wilted nodes ratio in the corresponding control pot.
[c] Calculation of variance components with the R function *lmer* is not possible on this data due to missing variation.

soil and the sterilised soil, ranging from 0.76 to 0.96 and from 0.93 to 0.99, respectively (Table 3.2).

In 67 out of 1092 pots of the sterile control treatment, at least one plant showed nodule formation. No significant correlation was found between nodulation index and shoot fresh weight (r_s = 0.21, p = .09) or shoot dry weight (r_s = 0.18, p = .15). In a preliminary experiment we compared the growth of rhizobia (ProGrow-PRX 753, Progress, Germany) inoculated and non-inoculated on sterilised soil. No significant differences were found for biomass measurements 28 days after sowing (data not shown). Hence, data from nodulated control plants were not excluded from the analysis.

3.3.2 Resistance screening of 261 pea landraces, breeding lines and cultivars

The number of emerged plants per pot 14 days after sowing in the infested field soil differed significantly among pea lines ($p < .0001$). Relative shoot and root biomass, i.e. the ratio between the infested and the sterilised soil treatment, were calculated to assess disease-related growth performance of the pea lines in the infested field soil (Table 3.2). Relative shoot fresh weight ($SFW_{Rel.}$) and $SDW_{Rel.}$ showed significant differences ($p < .0001$) between lines, ranging from 0.06 to 1.81 (mean = 0.65) and from 0.03 to 2.14 (mean = 0.82), respectively. Relative root fresh ($RFW_{Rel.}$) and dry ($RDW_{Rel.}$) weights also showed significant differences ($p < .0001$) between lines, ranging from 0.0002 to 0.94 (mean = 0.29) and 0.0019 to 1.25 (mean = 0.42), respectively. Significant pea line effects were also found for RRI_{CC}, cortex decay (CDI), shoot lesion (SLI), disease progress (DIS), wilted nodes ratio (WIL) and nodulation (NOD) ($p < .0001$). The estimate of heritability for plant emergence in the infested soil was high ($H^2 = 0.89$, Table 1). Very high heritability values were obtained for plant height ($H^2 = 0.96$ in the infested soil and 0.99 in the sterilised soil) as well as for fresh and dry shoot weight in sterilised soil ($H^2 = 0.94$ and 0.93, respectively). Moderate to high heritabilities ($H^2 = 0.4 – 0.7$) were found for relative biomasses, RRI_{CC}, SLI, DIS, WIL and NOD. Only CDI showed a low heritability of $H^2 = 0.24$.

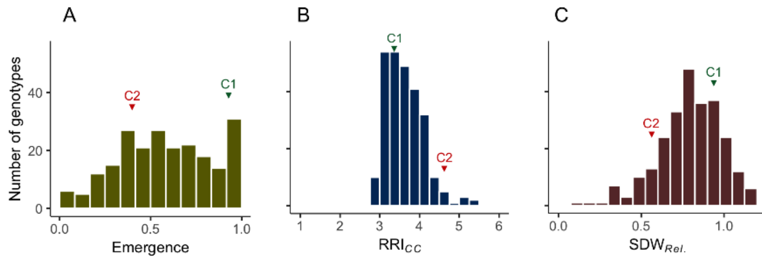


Figure 3.3. Frequency distributions of the estimated means of (A) plant emergence rate (Emergence), (B) root rot index (RRI$_{CC}$; levels 1-6) and (C) relative shoot dry weight (SDW$_{Rel.}$) assessed on 261 pea lines after 14 days (Emergence) or 21 days (RRICC and SDW$_{Rel.}$) under controlled conditions on infested soil. The means of reference cultivars C1 (tolerant) and C2 (susceptible) are indicated in green and red, respectively.

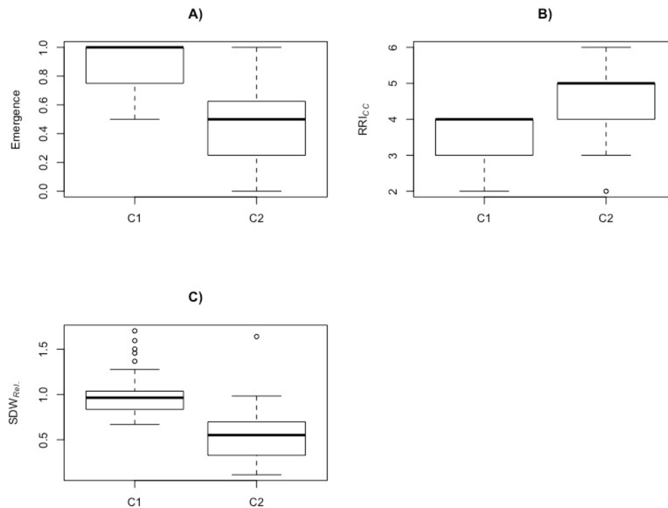


Figure 3.4. Growth characteristics of reference cultivars C1 (cv. 'EFB.33') and C2 (cv. 'Respect') on naturally infested soil. Wilcoxon rank sum test was used to calculate the significance of the difference between the two cultivars (n = 4; means of each replication). A) Emergence rate after 14 days (W= 16, p = .028); B) Root rot index (RRI$_{CC}$) after 21 days (W= 16, p = .027); C) Relative shoot dry weight (SDW$_{Rel.}$) after 21 days (W= 16, p = .029).

3.3.3 Relation between disease parameters

A PCA was performed to explore the relationship among eleven traits assessed in the controlled conditions resistance screen for 261 pea lines. The two first principal components explained 57.9% of the total variance (PC1: 35.2% and PC2: 22.7%; Figure 3.5). The eleven traits resulted in four distinct groups: (i) relative biomass measurements and NOD (upper right quadrant); (ii) RRI_{CC} (lower left quadrant); (iii) WIL, CDI, SLI and DIS (upper left quadrant); and (iv) plant emergence between group (i) and (iii) (Figure 3.5 B). In the first group, relative shoot fresh and dry biomass are well represented on the first axis ($cos^2 > 0.49$). RRI_{CC} is pointing in opposite direction and is also well represented on the first axis ($cos^2 = 0.51$). On the second axis, emergence ($cos^2 = 0.34$), SLI ($cos^2 = 0.55$) and DIS ($cos^2 = 0.58$) are well represented. CDI and WIL are equally well represented on axis 1 and 2. Pea lines with extreme positive (upper right) or negative coordinates (lower left) were considered as the most resistant or susceptible lines, respectively (Figure 3.5 A). Generally, the dispersion of the pea lines in the two first dimensions showed that the frequency of resistant and susceptible lines was homogeneous among the evaluated collection. The position of the two reference cultivars are according to the expectations, with C1 emerging well and being more resistant (lower RRI_{CC} and higher relative shoot weight) and C2 poorly emerging and being highly susceptible (high RRI_{CC} and low relative shoot weight). No grouping of the evaluated pea lines according to leaf type was detected with respect to the eleven traits assessed.

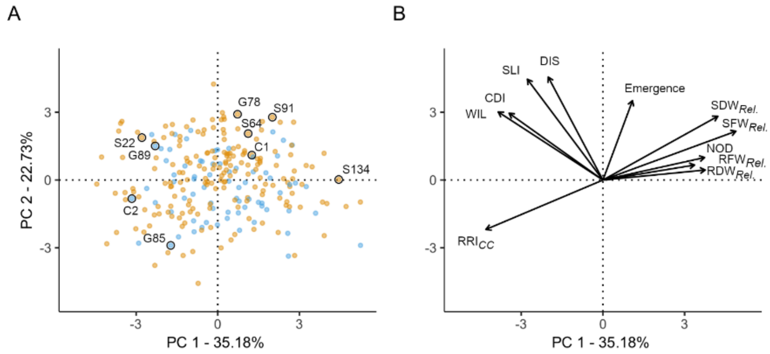


Figure 3.5. Principal component analysis of 261 pea genotypes and eleven phenotypic traits assessed under controlled conditions in infested soil. The first two principal components are shown, accounting for 57.91% of the variance in the data set. (A) Individuals factor map; nine pea lines evaluated in the field experiment are explicitly labelled. Blue and orange dots represent semi leafless and full genotypes, respectively. (B) Factor map of the eleven variables; the coordinates of the variables were multiplied by seven to produce clear visual display. Emergence = emergence rate; $RFW_{Rel.}$, $RDW_{Rel.}$, $SFW_{Rel.}$, and $SDW_{Rel.}$ = relative root and shoot fresh and dry weight, respectively; NOD = nodulation index; RRI = root rot index; WIL = wilted nodes; CDI = cortex decay index; SLI = shoot lesion index; DIS = disease progress.

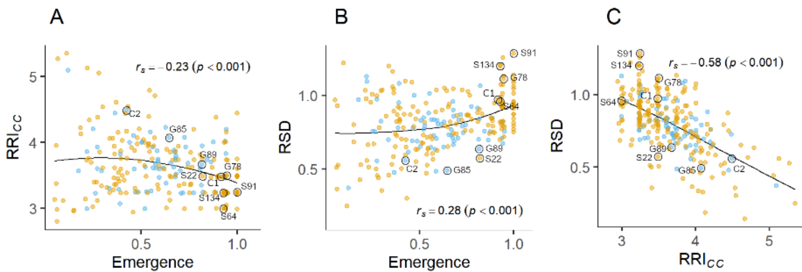


Figure 3.6. Pairwise relations between linear-mixed model estimated means of plant emergence rate, root rot index (RRI_{CC}) and relative shoot dry weight ($SDW_{Rel.}$) assessed on 261 pea lines after 14 days (Emergence) or 21 days (RRI_{CC} and $SDW_{Rel.}$) under controlled conditions in infested soil. Rank correlation coefficients (Spearman's *rho*) and associated *p*-values are displayed in the plots. A LOESS regression line is included in each panel. Nine pea lines evaluated in the field experiment are labelled. Blue and orange dots represent semi leafless and full genotypes, respectively.

Plant emergence rate, RRI_{CC} and $SDW_{Rel.}$ were selected for further examination (Figure 3.3, Figure 3.5, Figure 3.6). Genotypic means of emergence and $SDW_{Rel.}$ showed considerable variation among the evaluated 261 pea lines. Emergence rate in the infested soil showed a bimodal distribution, with 22% of the pea lines having an emergence rate ≥ 0.9 in the infested soil. In contrast, RRI_{CC} showed less variation and a strong truncated distribution with a positive skew towards susceptibility. Most pea lines got an average score between 3 and 4 and no line got the score 1 (healthy, no symptoms) or 2 (single, localised lesions). Relative shoot dry weight ($SDW_{Rel.}$) showed a close to normal distribution with negative skew towards susceptibility and good differentiation between the pea lines. All three variables were significantly correlated with each other (Figure 3.6). Emergence showed a weak negative rank correlation with RRI_{CC} ($r_s = -0.23$, $p < .0001$) and a weak positive correlation with $SDW_{Rel.}$ ($r_s = 0.28$, $p < .0001$). $SDW_{Rel.}$ showed a medium negative correlation with RRI_{CC} ($r_s = -0.58$, $p < .0001$).

Relative root and relative shoot biomass were moderately correlated with each other ($r_s = 0.32 - 0.44$, $p < .001$). Shoot lesion index was positively correlated with cortex decay ($r_s = 0.61$, $p < .001$), disease progress in stem base ($r_s = 0.67$, $p < .001$) and wilted nodes ratio ($r_s = 0.55$, $p < .001$). NOD was positively correlated with $SFW_{Rel.}$ ($r_s = 0.50$, $p < .001$) and negatively correlated with RRI_{CC} ($r_s = -0.58$, $p < .001$; Figure 3.6).

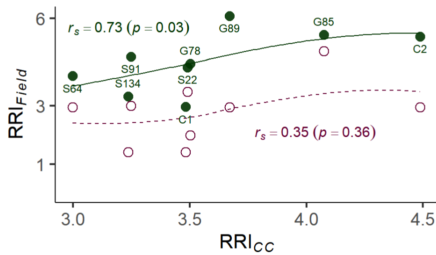
Table 3.3. Means of relative shoot dry weight ($SDW_{Rel.}$), root rot index (RRI_{CC} and RRI_{Field}) and emergence rate for nine pea genotypes evaluated under controlled conditions and in on-farm field trails. Estimated means, followed by the upper and lower 95% confidence interval limits are presented for controlled conditions and field data. Lines are sorted by $SDW_{Rel.}$ in descending order.

	Controlled conditions			Field sites	
				Heavy infestation	Moderate infestation
Genotype	Emergence	RRI_{CC}	$SDW_{Rel.}$	RRI_{Field}	RRI_{Field}
Reference cultivars					
C1	0.92 [0.86, 0.95]	3.48 [3.3, 3.67]	0.97 [0.89, 1.04]	2.96 [1.52, 4.4]	1.09 [-0.68, 2.85]
C2	0.42 [0.34, 0.51]	4.43 [4.21, 4.64]	0.57 [0.48, 0.65]	5.59 [3.77, 7.41]	2.94 [1.18, 4.71]
Field tested lines					
S91	1 [0, 1]	3.24 [2.65, 3.83]	1.23 [1, 1.45]	4.73 [3.28, 6.17]	2.99 [1.23, 4.76]
S134	0.93 [0.62, 0.99]	3.23 [2.64, 3.82]	1.16 [0.9, 1.43]	3.32 [1.88, 4.77]	1.09 [-0.68, 2.85]
G78	0.95 [0.69, 0.99]	3.50 [2.91, 4.09]	1.09 [0.87, 1.32]	4.47 [2.66, 6.28]	1.89 [0.12, 3.66]
S64	0.93 [0.62, 0.99]	2.98 [2.39, 3.56]	0.96 [0.73, 1.18]	4.03 [2.58, 5.47]	2.94 [1.18, 4.71]
G89	0.82 [0.5, 0.94]	3.67 [3.08, 4.26]	0.64 [0.41, 0.86]	7.41 [5.59, 9.22]	2.94 [1.18, 4.71]
S22	0.82 [0.56, 0.94]	3.49 [2.9, 4.08]	0.58 [0.35, 0.81]	4.34 [2.52, 6.15]	2.94 [1.18, 4.71]
G85	0.65 [0.39, 0.84]	4.07 [3.48, 4.65]	0.51 [0.28, 0.73]	5.69 [4.25, 7.14]	4.95 [3.18, 6.71]

3.3.4 On farm verification of the controlled conditions resistance screening

A subset of nine pea lines, including the two reference cultivars, was evaluated for PRRC resistance in the field in order to verify the results of the resistance screen under controlled conditions (Table 3.3). Significant genotype (p = .009) and field site effects (p = .0001) were found for RRI_{Field}. The genotype x field site interaction was not significant (p = .49). Genotypic means ± s.e. of root rot index ranged from 3.0 ± 0.6 to 6.0 ± 0.0 in the heavily infested field site. Root rot was lower in the adjacent field site with moderate root rot potential, ranging from 1.5 ± 0.5 to 4.5 ± 1.5. The two check cultivars fit the expected response, with C1 showing lower RRI_{Field} than C2 in both field sites. A significant rank correlation was found between RRI_{CC} and RRI_{Field} in the heavily infested field site (r_s = -0.73, p = .03; Figure 3.7 A). No significant correlation was found between RRI_{CC} and RRI_{Field} in the moderately infested field site or between RRI_{CC} and $SDW_{Rel.}$ (Figure 3.7).

A



B

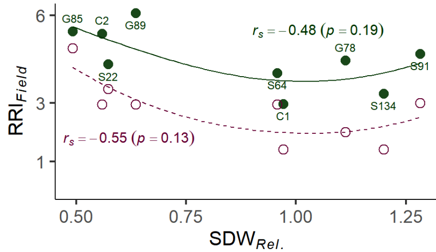


Figure 3.7. (A) Correlation between root rot assessed under controlled conditions on infested soil 21 days after sowing (RRI_{CC}; 1: no symptoms – 6: plant dead) and root rot assessed in the on-farm experiment (RRI_{Field}; 1: no symptoms – 8: plant dead). (B) Correlation between relative shoot dry weight assessed under controlled conditions ($SDW_{Rel.}$) and RRI_{Field} of nine pea lines. The nine pea lines with contrasting resistance phenotypes were evaluated on a field site with heavy pea root rot complex (PRRC) infestation (closed dots, solid LOESS line), and on a field site with moderate PRRC infestation (open dots, dashed LOESS line). Estimated means are presented for the nine field evaluated lines. Rank correlation coefficients (Spearman's *rho*) and associated *p*-values are indicated for both field sites.

3.3.5 Fungal community in pea roots grown in infested soil under controlled conditions

Sequencing of the ITS1 region from total DNA extracted from bulk soil, rhizosphere soil and root revealed a total of 1,190,412 high-quality sequences with a median of 55,670 sequences per sample. The rarefaction analysis showed that samples reached an asymptote, maximising the number of distinguishable operational taxonomic units (OTUs), with decreasing OTU richness from bulk soil to rhizosphere soil to root samples (Figure 3.8). There was no significant differentiation between the two pea lines; therefore, the sequencing data was pooled for further analysis. Among the most abundant OTUs present in pea roots, sequences could be assigned to several putative pea pathogens including several *Fusarium* spp., *Rhizoctonia solani*, *Didymella* sp. and other putative plant pathogens (Table 3.4). Putative plant beneficial fungi included *Clonostachys rosea* (5th most abundant taxa in roots), *Coprinellus* sp. and several members of arbuscular mycorrhizal fungi, e.g. *Funneliformis* spp., *Entrophspora* sp. and *Diversispora* spp..

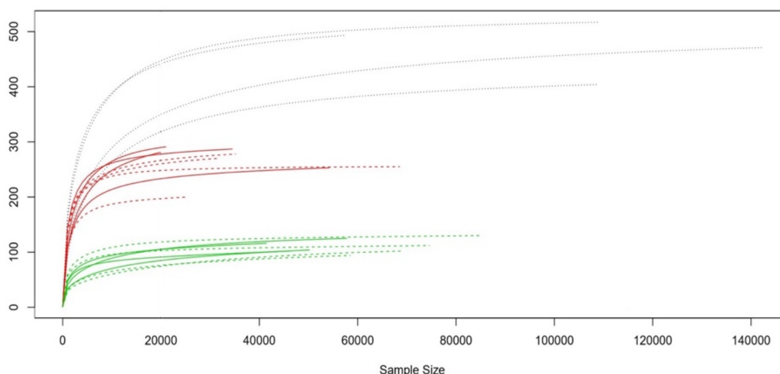


Figure 3.8. Rarefaction curves of the ITS amplicon sequencing data of bulk soil (n = 4; grey), rhizosphere soil (n = 8; red) and root (n = 8; green) samples. For the rhizosphere and root samples dashed and solid lines represent pea lines S164 and S12, respectively. OTUs were defined at a 97% similarity threshold.

Table 3.4. Taxonomic information and mean relative abundance of the 20 most abundant operational taxonomic units (OTUs) and further selected OTUs in bulk soil (n = 4), rhizosphere soil (n = 8), and root (n = 8) of 21 days old pea grown in infested soil under controlled conditions. Bold values highlight significantly enriched OTUs compared with bulk soil (FDR-adjusted; $p < .05$).

Taxon[a]	Taxonomic rank[b]	ID	Relative abundance [‰]		
			bulk soil	rhizosphere	root
Top 20 taxa in the root					
Ilyonectria	g	ZOTU4	1.79	**9.06**	**241.62**
Fusarium oxysporum	s	ZOTU10	8.97	14.22	**95.25**
Polyphilus	g	ZOTU3	0.10	**0.66**	**60.60**
Olpidium brassicae	s	ZOTU5	0.32	2.19	**48.62**
Clonostachys rosea	s	ZOTU11	0.73	**7.07**	**33.46**
Fusarium solani	s	ZOTU396	1.23	2.89	**27.69**
Fungi	k	ZOTU31	0.00	**1.29**	**9.81**
Fusarium solani	s	ZOTU34	0.72	1.55	**8.39**
Tetracladium	g	ZOTU19	8.91	4.45	7.21
Cylindrocarpon	s	ZOTU25	2.74	4.25	6.84
Microdochium bolleyi	s	ZOTU38	0.80	0.22	**6.46**
Rhizoctonia solani	s	ZOTU111	0.00	**0.81**	**5.87**
Sordariomycetes	c	ZOTU29	1.87	0.32	5.81
Dendryphion nanum	s	ZOTU46	0.98	1.11	**5.24**
Didymella	g	ZOTU7	1.09	2.16	4.95
Stephanosporaceae	f	ZOTU13	0.01	0.52	**3.96**
Fusarium	g	ZOTU20	2.03	1.81	3.54
Plectosphaerella	g	ZOTU114	1.10	0.37	3.37
Exophiala equina	s	ZOTU1671	8.55	6.18	3.19
Orbiliaceae	f	ZOTU100	0.01	**2.21**	**3.13**
Further known pea pathogens					
Fusarium solani	s	ZOTU185	0.02	0.30	0.68
Fusarium solani	s	ZOTU122	0.07	0.01	0.67
Fusarium	s	ZOTU80	1.52	2.21	0.36
Didymellaceae	f	ZOTU33	11.01	5.22	0.08
Further putative beneficials					
Corprinellus	g	ZOTU28	0.00	**0.97**	**2.38**
Arthrobotrys oligospora	s	ZOTU37	0.15	3.15	1.29
Funneliformis mosseae	s	ZOTU355	0.18	0.13	0.98
Entrophspora	s	ZOTU489	0.20	0.01	0.55
Funneliformis mosseae	s	ZOTU470	0.23	0.01	0.52
Funneliformis mosseae	s	ZOTU408	0.17	0.11	0.19
Diversispora	g	ZOTU780	0.05	0.05	0.15
Diversispora	g	ZOTU1390	0.11	0.08	0.11
Arthrobotrys musiformis	s	ZOTU222	0.01	0.65	0.09

[a] *dark orange = putative pea pathogen; light orange = putative plant pathogen; purple = putative plant beneficial.*
[b] *s = species; g = genus; f = family; c = class; k = kingdom.*

3.4 DISCUSSION

There is increasing evidence that plants should be recognized as a holobiont with their health status largely depending on well-balanced networks within their microbial community (Berg *et al.*, 2017a). It has been shown that the plant genotype influences the microbiome composition and that the microbial community can be shaped by plant breeding (Horton *et al.*, 2014a; Pérez-Jaramillo *et al.*, 2017a). From this point of view, we designed a resistance screening where 261 pea landraces, breeding lines and cultivars were grown under controlled conditions in naturally infested field soil harbouring a complex of pea root rot pathogens and non-pathogenic, potentially beneficial microbes.

3.4.1 Evaluation of the PRRC resistance screening

Plant emergence in the infested soil showed ample genotypic variation and high heritability. Poor emergence and damping-off is a constant threat to pea production, especially in the temperate regions, where peas are spring-sown under cool and wet conditions. Damping-off can be a major yield limiting factor and cause severe economic loss (Lamichhane *et al.*, 2017). Screening for resistance against damping-off based on artificial inoculation was shown to be poorly correlated with resistance scorings obtained in the field (Muehlbauer & Kraft, 1973). By contrast, we show that plant emergence can be easily and reproducibly assessed in a complex system and represents a valuable trait for assessing resistance of pea lines against damping-off and early stages of root rot.

The root rot index assessed under controlled conditions (RRI_{CC}) allowed to differentiate between highly susceptible and partially resistant pea lines. Heritability of RRI_{CC} was in the range of previous studies on root rot pathogens in pea: While Desgroux *et al.* (2016) reported heritabilities as high as 0.9 for artificial inoculations with single isolates of *A. euteiches*, heritability was as low as 0.28 in their field experiments. Muehlbauer and Kraft (1973) screened pea lines for resistance on field soil heavily infested with *F. solani* and *P. ultimum* and found a heritability of 0.44, similar to our experiment. Thus, the assessment of a root rot index is useful for breeding nurseries and field trials as it allows to quantify

complex disease expressions and provides a direct estimation of resistance capacities.

The assessment of relative biomass allowed to compare biomass loss among morphologically highly differentiated pea lines of different leaf type and agronomic end-use. Relative biomass measurements showed abundant variation and allowed to differentiate between highly susceptible and partially resistant pea lines. We found similar pea shoot biomass reductions and associated heritabilities as previously reported from artificial inoculation assays with isolates of *Fusarium avenaceum*, *F. oxysporum* and *F. solani* (Šišić *et al.*, 2018) and *Aphanomyces euteiches* (Pilet-Nayel *et al.*, 2005). However, measuring relative biomass requires that each genotype is tested twice on sterilised and on infested soil, requiring more resources. Comparing the growth performance of pea lines in infested field sites to non-infested field sites stands for a practical alternative to sterile soil control. This would further benefit from higher biological significance, as the non-infested soil harbours a resident microbial community. However, multi-site experiments need to be planned carefully, as possible interactions between the plant genotype, different properties of the soil (e.g. physico-chemical or microbial), agricultural management and climatic conditions can significantly influence the results and the conclusions drawn upon them (Busby *et al.*, 2017).

Relative biomass measurements represent valuable surrogate traits for plant health and their relation to other disease symptoms of the plant allows for further exploration of genotypic differences. Several pea lines showed below-average RRI_{CC} and above-average $SDW_{Rel.}$, indicating exploitable enhanced disease resistance. None of the evaluated lines showed full root rot resistance, but considerable variation in $SDW_{Rel.}$ was evidenced for lines with RRI_{CC} values between 3 and 4. Few pea lines even showed high RRI_{CC} while still growing above average, indicating enhanced levels of disease tolerance for these lines, where tolerance is the ability of plants to perform well when infected with a pathogen. Conner *et al.* (2013) found significant differences in tolerance against Aphanomyces root rot infection in a collection of pea breeding lines in the field. They concluded that above-ground plant growth traits could be useful in the selection of breeding lines with tolerance to Aphanomyces root rot, still producing high yield despite the infection. Based on the

proposed screening system, we suggest to select lines with high relative biomass and low RRI_{CC} for further field assessments and as possible resistance sources against the root rot complex, as breeders will ultimately be interested in both, disease resistance (low levels of disease symptoms) and disease tolerance (low yield depression when symptoms occur in the target environment).

Estimates of heritability for disease-related traits calculated from single-isolate inoculations and controlled conditions screenings are typically higher compared with field experiments or infested soil-based systems because random experimental variance can be minimised. Single-isolate experiments are highly valuable for linkage-mapping and genome-wide association studies (GWAS). Various published studies employed artificial inoculation resistance screenings and successfully identified loci controlling partial resistance against individual root rot pathogens of pea (Hamon *et al.*, 2013; Pilet-Nayel *et al.*, 2017; Desgroux *et al.*, 2018; Coyne *et al.*, 2019b). However, they assume that the different pathogens have additive effects and ignore the complex interactions between pathogenic and other microorganisms under farming conditions.

Here we show that considerable heritability for resistance traits can be found in systems based on infested field soil. Such screening systems are valuable tools for early resistance breeding efforts without having to know the presence and compositions of pathogens in the soil. In our on-farm experiment, we were able to confirm two distinct infestation levels in two close-by field sites and found a significant genotype effect for RRI_{Field}. Our field results confirm the applicability of the controlled conditions screening system, with a significant correlation between RRI_{CC} and RRI_{Field} in the highly infested site. On the other hand, in the close-by moderately infested site, disease development was low and no significant correlation with the controlled conditions screening could be found. Future pot and field experiments should include different naturally infested soil types to evaluate potential deviations in identified resistance levels due to different compositions of relevant pathogens or other microbial species.

The experimental design of this soil-based screening system requires replication and, thus, cannot be used for single plant selection in early generations but only for advanced generations when sufficient homogeneity is given (e.g., F_4, F_5 lines). For selection in earlier

generations molecular tools need to be developed. The moderate to high heritability values obtained for the different resistance traits under controlled conditions would allow for GWAS or genomic prediction. These molecular resources would not only allow to identify possible PRRC resistance loci but also to disentangle innate and microbiome-mediated resistance mechanisms when paired with root microbiome data. Then, these genomic tools would represent a powerful tool to study the role of the plant-associated microbial community in PRRC resistance.

3.4.2 Plant resistance in the light of pathogen complexes

The ITS-amplicon sequencing allowed to detect several pathogenic fungi in the soil and root of diseased peas. Taxa including known members of pea pathogens *F. oxysporum*, *F. solani*, and *R. solani* were enriched in the roots compared to the bulk soil. These three pathogens belong to the most important root rot pathogens of pea in the temperate zones (Mc Phee *et al.*, 2012; Chittem *et al.*, 2015; Coyne *et al.*, 2015; Melzer *et al.*, 2016). *Fusarium solani* and various isolates of *Rhizoctonia solani* are also causal agents of damping-off in pea (Melzer *et al.*, 2016; Lamichhane *et al.*, 2017). The high abundance of these pathogens in diseased roots indicates their probable role in the reduction of plant emergence and later disease expression. The sequencing data revealed the presence of two taxa, *Didymellaceae* and *Didymella* sp. which indicates the role of any of the two major pea pathogens *Didymella pinodella* and *Didymella pinodes* in PRRC in the studied soil. Both pathogens have been frequently isolated from diseased peas in German fields (Pflughöft *et al.*, 2012; Baćanović-Šišić *et al.*, 2018). Furthermore, the two taxa *Ilyonectria* and *Olpidium brassicae* were enriched in the roots. Both taxa are plant pathogens with a wide host range and have been detected in earlier microbiome studies of field pea (Yu *et al.*, 2012a; b). However, their status in relation to pea root rot remains elusive, as they are not known pea pathogens. Lay *et al.* (2018) stated that the unresolved taxonomy of the *Olpidium* complex could lead to an overrepresentation in amplicon sequencing studies. The demonstrated co-occurrence of pea root rot pathogens emphasises the PRRC concept, as already stated in

other studies (Chittem *et al.*, 2015; Taheri *et al.*, 2017; Willsey *et al.*, 2018).

Co-inoculation of fungal and oomycotan pathogens (e.g. *P. ultimum* – *F. oxysporum* or *R. solani* – *F. solani* – *A. euteiches* in pea and *P. ultimum* – *A. euteiches* in bean) have been show to significantly increase disease development (Kerr, 1963; Pfender & Hagedorn, 1982; Shehata *et al.*, 1983). The utilised primer pair specifically targets members of the kingdom Fungus, underrepresenting oomycetes. Therefore, our sequencing data does not allow for clear statements about the presence of *Aphanomyces euteiches*, *Pythium* sp. or *Phytophtora* sp. These oomycetes are also important pea pathogens causing damping-off and root rot (Gaulin *et al.*, 2007; Heyman *et al.*, 2013; Alcala *et al.*, 2016). Symptoms of not emerged or died-off seedlings included water-soaked, decaying seeds and rotting of the hypocotyl, possible indications of the presence of *Pythium* spp.; at later stages roots were honey-brown and water-soaked, indicating *A. euteiches* to be involved in the PRRC. Additional molecular analyses with species-specific primers are required to analyse these oomycotan pathogens.

In addition to the pathogenic taxa, the ITS-amplicon sequencing allowed to detect plant beneficial fungal taxa. The taxa *Clonostachys rosea* and *Corprinellus* were significantly enriched in the roots. *Clonostachys rosea* is able to colonize seeds and young pea plantlets and is a known antagonist of fungal pathogens. It has been shown to significantly limit the growth of major PRRC pathogens in vitro and to reduce disease in the field (Xue, 2003a). Members of the taxon *Corprinellus* produce anti-bacterial and anti-fungal compounds (Zahid *et al.*, 2006) and an isolate of *C. curtus* was shown to reduce growth of *Rhizoctonia solani* in the rhizosphere of cabbage (Nakasaki *et al.*, 2007). Various sequences were also attributed to three taxa belonging to arbuscular mycorrhizal fungi (AMF), well-known plant symbionts gaining recent attention for AMF-mediated disease resistance (Hohmann & Messmer, 2017). Bioprotection of pea by AMF against the pathogen *A. euteiches* has been repeatedly reported (Bodker *et al.*, 1998; Slezack *et al.*, 2000; Li *et al.*, 2019b). In contrast to the before-mentioned direct fungal antagonists, AMF's action is indirect: they induce plant resistance and regulate the plant defence mechanisms (Jung *et al.*, 2012).

Our sequencing data revealed a diverse collection of fungal pathogens and putative beneficials present in the roots of infected pea plants. This finding confirms the study of Xu *et al.* (2012), where the health status of pea (infected or healthy) only affected the fungal community composition in the roots, but not in rhizosphere or bulk soil. The data suggests that the analysis of bulk soil alone does not allow to assess the occurrence of specific pathogens because pea pathogens and fungal antagonists were specifically enriched in the roots, in comparison to the bulk soil. Future research is necessary to compare groups of resistant and susceptible plant genotypes in different infested soils in order to validate causal agents of PRRC and identify diversity indices or key taxa involved in microbiome-mediated disease resistance.

This study demonstrates the value of controlled conditions screenings in predicting the performance of pea lines in PRRC-infested field sites. The resistance screening assay reproducibly identified partially resistant and highly tolerant pea lines despite the complexity of the fungal community in the used substrate. Heritabilities of the assessed resistance traits show promise to use the screening system in molecular and conventional pea breeding, and therefore to strengthen resistance breeding of this ecologically and economically invaluable crop. The use of agricultural soils allows to screen for plant resistance mechanisms of the entire ecological unit consisting of the plant and its associated microbial community. This is assumed to be one of the main reasons for the strong correlation between controlled and field conditions performance. For future lines of research, it will be revealing to link plant performance, host genetics and microbiome diversity and functions to assess plant health at the holobiont level. This holistic approach will broadly support breeding of pea and other major crops and promote sustainable food production.

4 | COMPOSITION OF KEY MICROBIAL TAXA OF THE PEA ROOT ROT COMPLEX IS DETERMINED BY SOIL × GENOTYPE INTERACTIONS

This chapter is currently in preparation for submission to *New Phytologist.*

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Abstract Chapter 4

A complex of root rot pathogens challenges pea (*Pisum sativum* L.) cultivation and impedes resistance breeding. Little is known about the interaction among root rot pathogens and the role beneficial microbes could play in disease resistance. Eight pea genotypes that showed distinct root rot resistance in a previous study were evaluated under controlled conditions in four agricultural soils showing different disease pressure. Despite significant soil (ANOVA: $p < .001$) and genotype ($p < .001$) effects, no significant soil × genotype interaction ($p < .342$) was found for plant growth reduction and the resistance ranking of the eight pea genotypes could be validated in all infested soils. Molecular quantification of eight putative root rot pathogens and two potentially beneficial taxa revealed a diverse microbial complex in diseased roots, with *Aphanomyces euteiches*, *Fusarium solani* and *F. oxysporum* being the most abundant pathogens. Besides confirming the importance of these pathogens in the pea root rot complex, this is the first evidence for the presence of *A. euteiches* in German and Swiss pea fields. Different pathogens correlated significantly with disease in the different soils. Arbuscular mycorrhizal fungi were negatively correlated with disease in all infested soils. Permutational multivariate analysis of variance revealed significant effects of the factors soil, genotype and their interaction ($p < .0001$) for the microbial community composition in the roots. In particular, resistant pea genotypes showed significantly lower F. solani and A. euteiches, and higher arbuscular mycorrhizal fungi abundance in the roots (Wilcoxon rank-sum test; $p < .05$). The identification of key microbial players in the root rot complex and their relation to disease resistance provides plant breeders with additional information for selection.

4.1 INTRODUCTION

Pea (*Pisum sativum* L.) is the most widely cultivated pulse in the temperate zones with a harvested area of 3, 2.6 and 1.4 million hectares in Europe, China and Canada, respectively (FAOSTAT, 2019b). It is a healthy, protein-rich food and feed, with the potential to significantly contribute to shifting towards a plant protein-based diet and to reduce the dependency on overseas import of soy derived feed. Its capacity to fix atmospheric nitrogen through the symbiosis with rhizobacteria has made it a valuable asset in sustainable cropping systems (Reckling *et al.*, 2016).

Soil-borne root and foot diseases caused by several fungal and oomycete pathogens severely threaten pea cultivation around the globe. *Aphanomyces euteiches*, *Didymella pinodes*, *D. pinodella*, *Fusarium avenaceum*, *F. oxysporum*, *F. redolens*, *F. solani*, *Pythium* sp., *Rhizoctonia solani* and *Sclerotinia sclerotiorum* are among the most important causal agents of pea root rot worldwide (Kraft & Pfleger, 2001; Gaulin *et al.*, 2007; Pflughöft *et al.*, 2012; Alcala *et al.*, 2016; Taheri *et al.*, 2017). Control of these pathogens is difficult as they survive on plant debris or form resting structures in the soil. It has been shown that increasing the frequency of pea or other legumes in the crop rotation provoke the build-up of root rot pathogens (Li *et al.*, 2014b; Bainard *et al.*, 2017). Currently, rotation breaks of six to ten years are recommended for pea cultivation areas where above mentioned pathogens have established in order to reduce the inoculum potential of the soil.

Together with adapted management practises, breeding for resistance has been proposed to be the most effective and economical control method. Major efforts have been made for breeding pea for resistance against root rots (Infantino *et al.*, 2006; Rubiales *et al.*, 2015). For instance, intensive research, screening and variety testing have shown important progress in Aphanomyces and Fusarium root rot resistance, (Davis *et al.*, 1995; Pilet-Nayel *et al.*, 2005; Desgroux *et al.*, 2018) (Muehlbauer & Kraft, 1973; McPhee *et al.*, 1999; Coyne *et al.*, 2019a). Despite considerable advances in understanding the pathogens as well as the plant genetic basis for resistance against some of them, root-rots remain major constrains to pea cultivation. The fact that various strains with different pathogenicity are known for all these pathogen species is a

common challenge to all resistance breeding programmes. Furthermore, various pathogens interact in the soil, forming a pea root rot complex (PRRC) and conjointly infect the plant (Xue, 2003b; Baćanović-Šišić *et al.*, 2018). Co-infection of two or more microbial species can break down resistance against single pathogens and aggravate disease as shown for the pathosystems *Pythium* sp. – *F. solani* - *F. oxysporum* (Kerr, 1963), *R. solani* – *F. solani* – *P. ultimum* – *A. euteiches* (Shehata *et al.*, 1983), *A. euteiches* – *F. solani* (Peters & Grau, 2002), *Fusarium* spp. – *A. euteiches* (Willsey *et al.*, 2018) or among various *Fusarium* species (Zitnick-Anderson *et al.*, 2018). It has been postulated to take this complexity into account early in the resistance breeding process (Oyserman *et al.*, 2018; Wille *et al.*, 2019). In consequence, we established a naturally infected field soil-based resistance screening assay that allows to reproducibly assess resistance against PRRC (Wille *et al.*, 2019; Wille *et al.*, 2020).

Screening pea germplasm for resistance on naturally infested soil in contrast to controlled conditions inoculation assays allows for complex plant-microbe interactions, including multiple interactions among pathogens, beneficial microbes, and the plant genotype. Plant associated microbiota are key drivers of plant health, and the plant genotype in turn determines the composition of these microbes (Berendsen *et al.*, 2012). Plant beneficial microbes were shown to be involved in the suppression of pathogens of the PRRC: For instance, the mycoparasite *Chlonostachys roseae* has the ability to increase germination and reduce root rot in pea infected with *F. oxysporum, F. solani, D. pinodes, R. solani,* or *S. sclerotiorum* (Xue, 2003a). Similarly, it has been shown that arbuscular mycorrhizal fungi (AMF) increase resistance of pea against Aphanomyces root rot (Thygesen *et al.*, 2004). *C. roseae* and various sequences assigned to AMF genera and species were detected in diseased pea roots grown in PRRC infested soil using ITS-amplicon sequencing (Wille *et al.*, 2020).

PCR assays targeting various pathogens of the PRRC have been established and employed to study the abundance of different pathogens in the field or the synergistic/antagonistic effects of selected pathogens under controlled conditions. Chatterton *et al.* (2018) surveyed the occurrence of major PRRC pathogens present in the roots sampled on Canadian pea fields from 2014 – 2017. They used an end-point PCR approach andd revealed *A. euteiches*, *F. avenaceum*, *F. oxysporum*, *F.*

redolens and *F. solani* as the major causal agents of root rot. Remarkably, they showd that *A. euteiches* was not detectable by traditional culturing methods, despite its highly frequent detection by PCR. Zitnick-Anderson *et al.* (2018) and Willsey *et al.* (2018) have developped quantitative real-time PCR (qPCR) assays to quantify major pathogens in diseased pea roots. Using this method, the latter authors showed that *A. euteiches* facilitates root colonization by *Fusarium* species eventually leading to increased root decay in comparison to the single inoculations.

Recently, a qPCR assay has been developped to quantify the biocontrol agent *C. rosea* in plant debris and grains (Gimeno *et al.*, 2019). An AMF qPCR assay is also available that has been used to monitor AMF colonisation in wild leek roots from the field (Hewins *et al.*, 2015).

The main objective of this study was to assess the soil and genotype dependent composition of selected pathogenic and beneficial microbes in diseased pea roots using qPCR. With a set of resistant and susceptible pea lines and four agricultural soils showing different levels of PRRC infestation, we aimed at (i) comparing levels of root rot resistance among different pea genotypes and soils, (ii) relateing resistance levels to the quantification of selected pathogens and beneficials detected in the roots. Finally, we wanted to (iii) test the hypothesis that resistant and susceptible pea genotypes show distinct compositions of key microbial taxa.

4.2 MATERIAL AND METHODS

4.2.1 Plant growth and phenotyping

Eight pea lines were selected based on a previous study on root rot resistance: These lines showed contrasting levels of resistance to a PRRC present in a naturally infested field soil (Wille *et al.*, 2020). The present selection includes four varieties and four genebank accessions from the USDA-ARS GRIN Pea Core Collection (Table 4.1).

Soil was collected from four agricultural field sites showing different levels of PRRC infestation: Feldbach (F; healthy), Kirchlindach (K; sick), Puch (P; sick), and Neu-Eichenberg (N; sick) (Table 4.2). Sieved soil was stored in polypropylene boxes at 4°C in the dark until further use. For the

control treatment, soils were sterilised (X-Ray irradiation 30-100 kGy, Synergy Health Däniken AG, Switzerland) and stored vacuum packed.

Pea seeds were surface-sterilized in 70% ethanol for 30 seconds followed by a 1:1 (v:v) ddH_2O-bleach solution (M-Classic Javel Wasser, Migros, Switzerland; final concentration approx. 2.5%) for ten minutes. Finally, seeds were thoroughly rinsed in ddH_2O and soaked for 2 h. Seven seeds per line were planted in a 2:1 (v:v) mixture of soil and sterilised sand (Quartz d'Alsace, 0.2 – 0.63 mm grain) in plastic pots (600ml).

Pots were arranged in a randomised complete block design with the factors 'soil' (four levels) and 'genotype' (eight levels) in four replications. Each experimental unit was set up as a pair of two pots, containing either infested soil or sterilised soil. The four replications were sown on four consecutive days and harvested over four days in the same order. Plants were grown under controlled conditions in the growth chamber for 29 days. A 16/8 light/dark cycle was applied, providing a photosynthetically active photon flux density of 200 µmol m-2 s-1 over the waveband 400 – 700 nm. Plants were watered with tap every 72 h by flooding the pots 4 cm high for 30 min. Growth chamber mean temperature over the course of the experiment was 20°C, relative humidity 85%. Pots were inspected on a daily basis for seedling emergence and plants were thinned out to reach a maximum of five plants per pot. Plants emerging after 14 days were removed and not considered in any analysis.

Seedling emergence was recorded seven days after sowing. Twenty-nine days after sowing, the plants were removed from the pots and roots were washed under running tap water. Plants were visually inspected. The Root Rot Index (RRI: 1 = healthy; 6 = complete root rot, plant dead) described by Wille et al. (submitted) was attributed to individual plants and medians were calculated from scores of individual plants for each pot. Roots were separated from shoots with clean scissors and kept on ice before transfer to -80°C. Fresh shoot biomass was recorded on a pot basis. Subsequently plants were dried at 105°C until constant weight before recording dry weight. Biomass measurements per pot were standardised with the number of plants per pot at harvest. Relative Shoot Dry Weight ($SDW_{Rel.}$) was calculated by dividing the biomass of the infested soil treatment by the biomass of the corresponding sterile control treatment of the same genotype in each replication.

Table 4.1. Genebank accessions and cultivars of *P. sativum* evaluated for root rot resistance and microbial composition in diseased roots.

Line ID	cultivar name/ ARS-GRIN no.	Morphology: Leaf type, flower color, seed color, TGW [g]	Country of origin (Breeding company)[a]	PRRC Resistance level [b]
C1	E.F.B. 33	Full leaf, pigmented, brown, 100	DE (DSV)	R
S91	PI 269777	Full leaf, pigmented, 140	Unilever, UK	R
S134	PI 86430	Full leaf, pigmented, green, 111	Nepal	R
G78	Roch	Full leaf, pigmented, brown, 180	PL (PHR)	R
S64	PI 241593	Full leaf, pigmented, green, 200	Taiwan	R
C2	Respect	Semi leafless, white, yellow, 220	FR (Agri Obtentions)	S
S22	PI 164612	Full leaf, white, green, 180	India	S
G89	Volt	Semi leafless, white, yellow, 250	DE (NPZ)	S

[a] DSV: Deutsche Saatveredelung; PHR: Poznaska Hodowla Roslin; NPZ: Norddeutsche Pflanzenzucht
[b] Based on Wille et al. (2020).

4.2.3 Quantification of microbial taxa in diseased pea roots

Previously published qPCR assays were used to quantify ten microbial taxa in the roots of plants grown in the non-sterile treatment (Table 4.3). As a control, roots of pea lines C1 and C2 grown in the sterilised soil were also analysed. Frozen roots were lyophilised and grinded to a fine powder for 10 sec at 25 Hz in a Mixer Mill (Retsch, Haan, Germany) using a 20 mm steel bead. DNA was extracted from 20 ± 1 mg root powder using the Mag-Bind® Plant DNA DS 96 Kit (Omega Bio-tek, Norcross, United States) according to manufacturer instructions. DNA concentration was measured photospectrometrically using a NanoDrop™ 2000 (Thermo Fisher Scientific, Waltham, United States) and samples were normalized to a DNA concentration of 50 ng μL^{-1}. DNA extractions were done on a per pot basis (roots of all plants in one pot pooled). DNA extractions and subsequent qPCR analysis were each run in two technical replications.

Table 4.2. Soil characteristics of four field soils used to assess the composition of root rot associated microbes in diseased pea roots. For each soil 150 l were collected from the field, sieved using a 20 mm mesh and stored at 4°C in polypropylene boxes in the dark until further use. Representative samples were taken from the collected soil prior to the experiment. Grain composition, pH and soil nutrient analysis were performed by the *Labor für Boden- und Umweltanalytik* (lbu), Switzerland. N and C analysis were performed by the *Research Institute for Organic Agriculture* (FiBL), Switzerland according to Agroscope (1996).

Field site (country)	Pea growth in the field	Coordinates	Grain composition			pH	Soil nutrient content [%]			Soil nutrient content [mg kg^{-1}]						
			Clay	Silt	Sand		N_{tot}	C_{tot}	C_{org}	P	K	Mg	B	Mn	Cu	Fe
Feldbach (CH)	healthy	47.238882, 8.788594	28.3	34.0	37.7	7.1	0.27	2.57	2.41	56.7	110.7	520.6	0.3	578	19.8	436
Kirchlindach (CH)	sick	47.004760, 7.410004	16.5	37.4	46.1	7.1	0.19	1.95	1.90	123.4	115.9	90.3	0.6	179	12.2	253
Puch (DE)	sick	48.189986, 11.215125	17.9	51.0	31.1	6.8	0.34	7.77	7.65	80.2	129.2	685.2	0.6	319	6.7	456
Neu-Eihenberg (DE)	sick	51.379118, 9.910005	19.0	59.2	21.8	7.3	0.14	1.45	1.44	63.1	130.5	148.1	0.6	383	6.8	316

Four standard curves per target region were obtained using 10-fold serial dilutions (10^3 to 10^0 pg μl^{-1} DNA) of target DNA. In order to approximate the ratio between target DNA and plant DNA, the serial dilutions were established in diluted plant DNA. To this end, DNA was extracted from axenically grown (X-ray sterilised sand, ultra-pure water), 14 days old pea seedlings and diluted in ultra-pure water [50 ng μL^{-1}]. For each target taxon, genomic DNA was isolated from a patch of mycelium (approx. 9 cm^2) of 5- to 10-day-old cultures. Mycelium was scraped of the agar with a sterile scalpel, freeze dried and extracted in the same way as the plant material. Oomycete and fungal isolates used in this study are listed in Table 4.3. The isolates were grown on potato dextrose agar in the dark at room temperature. For the AMF assay five standard curves were obtained using 10-fold serial dilutions of transformed plasmids containing an AMF 18S rDNA sequence and serial dilution ranging from 10^6 to 10^2 copies μl^{-1}. Two replicate qPCR reactions were run for each sample on a Rotor-Gene Q real-time PCR detection system (QIAGEN, Hilden, Germany). The 13 µL qPCR reactions contained 1.5 µL of template DNA, 6.5 µL of KAPA FAST qPCR master mix (SYBR or PROBE, depending on the assay), forward and revers primers and hydrolysis probe, where necessary. PCR programs consisted of an initial denaturation step for 5 min at 95°C, followed by 40 cycles of denaturation for 10 s at 95°C, annealing for 30 s at the assay specific temperature (Table 4.3) and extension for 10 s at 72°C including signal detection.

4.2.4 Statistical analysis

Statistical analyses were performed with R 3.6.1 (R Core Team, 2018). R Markdown files for all analyses are provided on: *https://github.com/dendrologicus*.

Plant emergence after seven days, $SDW_{Rel.}$ and RRI, were analysed using linear regression according to the models:

Y ~ soil + genotype + soil:genotype + replication, and:

Y ~ soil + resistance level + soil:resistance level + replication,

where the factor 'soil' has four levels (F, K, P, N), 'genotype' eight levels (eight pea lines), 'resistance level' two levels (R: resistant pea line; S: susceptible) and replication four levels. $SDW_{Rel.}$ was transformed using

an inverse Lambert W × F_X function before analysis (*LambertW* package) (Goerg, 2015). Emergence and RRI data was rank-transformed and analysed with a reduced model without the factor replication using *ARTool* (Kay & Wobbrock, 2019). In a first step, the regression analysis was performed over all four soils; in a second step, for $SDW_{Rel.}$ and RRI, the analysis was performed for the three sick soils only, using the factors 'replication' and 'genotype' or 'resistance level'. Compliance of the model assumptions was controlled by visual inspection of the residual plots. Where applicable the genotypic means over the three soils (i.e. when the interaction of soil × genotype was not significant) were compared. Marginal means for the factors soil and genotype were calculated based on the linear models using *emmeans* (Lenth, 2019). Pairwise differences were calculated and tested for significance using Tukey's honestly significant difference (HSD) at a 5% level of significance using the latter package.

Non-metric multidimensional scaling (NMDS; two dimensions; function *metaMDS()*) of the Bray-Curtis dissimilarities between samples was used to explore structural similarities between the microbial composition of the four soils and eight genotypes. Permutational multivariate analysis of variance (PERMANOVA; *adonis()*) was used to test differences in the microbial composition among the different factor levels in two steps (all four soils; three sick soils) according to the models stated above. The associations between the ten qPCR variables and the phenotypic variables $SDW_{Rel.}$ and RRI and each ordination was determined by calculating the-goodness-of-fit statistic r^2 with *envfit()*. NMDS, PERMANOVA and goodness-of-fit were performed with *vegan* (Oksanen *et al.*, 2019).

Pairwise correlations between the abundance of microbial taxa, as well as between $SDW_{Rel.}$ and RRI with the microbial abundances were explored by calculating Spearman's *rho*.

Multiple linear regressions of $SDW_{Rel.}$ and RRI on the centered and scaled abundances of the ten microbial taxa were calculated and model reduction was performed using stepwise backward variable selection (*stepAIC()*).

Table 4.3. Ten qPCR assays employed to detect fungal taxa in diseased pea roots. Indicated reference strains were used to prepare standard curves.

Organism	Target	Reference strain	Primer/probe names	Primer/probe conc. [nM]	Annealing temp.	Reference
AMF	18s rRNA	Transformed plasmid	AMG1F/AM1	250/250	67°C	Hewins *et al.* (2015)
Aphanomyces euteiches	ITS I	AeRB84 (M.-L. Pilet; INRA Rennes, FR)	Ae_ITS1_39F/Ae_ITS1_167R	250/250	61°C	Gangneux *et al.* (2014)
Clonostachys rosea	Actin gene	CRP 1104 (A. El Hassan, Hohenheim University, DE)	VTTact-f/VTTact-r/VTTact-pr	300/300/100	62°C	Gimeno *et al.* (2019)
Fusarium avenaceum	*EF-1α*	F.ave_ku1 (A. Šišić, Kassel University, DE)	AveF/AveR/AvePr	900/900/200	60°C	Zitnick-Anderson *et al.* (2018)
Fusarium oxysporum	ITS II	F.ox_ku1 (A. Šišić, Kassel University, DE)	FOF1/FOR1	300/300	65°C	Mishra *et al.* (2003)
Fusarium redolens	*EF-1α*	Isolate 1425 (A. Šišić, Kassel University, DE)	RedF/RedR/RedPR	900/900/200	60°C	Zitnick-Anderson *et al.* (2018)
Fusarium solani	*EF-1α*	F.sol_ku1 (A. Šišić, Kassel University, DE)	SolF/SolR/SolPr	900/900/200	62°C	Zitnick-Anderson *et al.* (2018)
Didymella pinodella	*EF-1α*	D. pin ku1 (A. Šišić, Kassel University, DE)	Tef1F/Tef1R/Tef1Pr	900/900/250	61°C	Šišić et al., in prep.
Rhizoctonia solani	ITS II	F122 (Geisenheim, DE)	ST-RS1/ITS4	300/300	60°C	Lievens *et al.* (2006)
Pythium ultimum	ITS II	5808 (Syngenta, CH)	AFP276/ITS4	500/500	58°C	Lievens *et al.* (2006)

4.3 RESULTS

4.3.1 Plant phenotypic assessments

In the three sick soils, K, P, and N, overall shoot fresh and dry weight in the non-sterile soil was significantly reduced compared with the sterilised soils. No reduction in plant growth was observed in the healthy control soil F (Figure 4.1). Soil and genotype had a significant effect on $SDW_{Rel.}$ ($F_{3,86} = 16.7$, $p < .001$ and $F_{7,86} = 4.2$, $p < .001$, respectively), as well as 'resistance level' ($F_{1,110} = 19.5$, $p < .001$). The interaction between 'soil' and 'genotype' was not significant ($F_{21,86} = 1.11$, $p < .356$), however the interaction between 'soil and 'resistance level' was ($F_{3,110} = 5.1$, $p = .003$). Mean (SD) relative shoot dry weight ($SDW_{Rel.}$) was 1.06 (0.43) in the F soil. $SDW_{Rel.}$ was significantly lower in the three sick soils with 0.69 (0.26), 0.60 (0.18), and 0.80 (0.24) for K, P, and N soils, respectively (Tukey HSD: F-K: $p < .0001$; F-P: $p < .0001$; F-N: $p = .006$). Furthermore, $SDW_{Rel.}$ was significantly lower in P soil than in N soil (Tukey HSD: $p = .017$; Figure 4.2). In the three sick soils, susceptible lines had significantly lower $SDW_{Rel.}$ than resistant lines (K: estimated difference = 0.36, $p < .001$; P: 0.20, $p = .012$, N: 0.23, $p = .007$). In the F soil, the two groups did not have significantly different $SDW_{Rel.}$. To analyse growth performance of the pea lines on sick soil further, ANOVA was performed for the three sick soils only showing significant effects of 'soil' ($F_{2,65} = 11.2$, $p < .001$) and 'genotype' ($F_{7,65} = 7.4$, $p < .001$). The interaction between these two factors was not significant ($F_{14,65} = 1.1$, $p < .342$). Post-hoc analysis revealed that pea line S91 grew best over all sick soils, followed by C1 and G78. C2 showed the worst growth (Figure 4.2). Over the three sick soils, SDW_{Rel} was significantly higher for resistant than for susceptible pea lines: Mean (SD) = 0.84 (0.23) versus 0.68 (0.45) (Wilcoxon rank-sum test: $p < .001$; Figure 4.6).

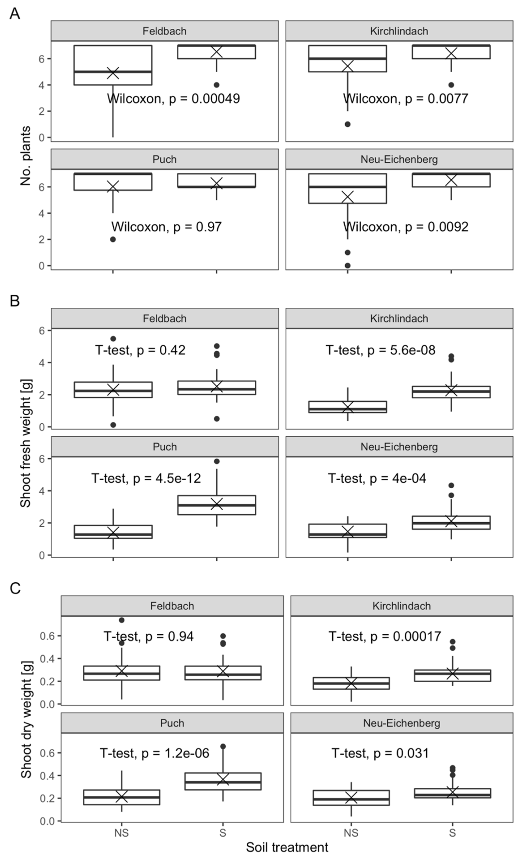

Figure 4.1. Growth of pea plants in four different soils, either sterilised (S) or non-sterilised (NS). A) Plant emergence seven days after sowing with initially seven seeds sowed. B) shoot fresh weight and C) shoot dry weight, the latter two both normalised to one plant per pot. ‘‘Feldbach’’ soil was classified as healthy soil, the three others as sick. Boxplots show the median and the interquartile range; the ends of the whiskers represent 1.5 times the interquartile range; the mean is indicated by a cross. Wilcoxon rank sum test and t-tests were performed to test significant differences between the means of the treatment NS and S (n = 32).

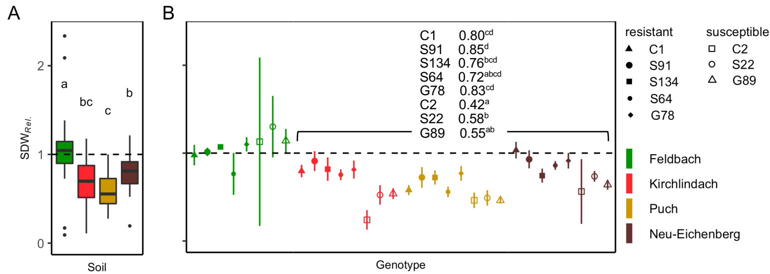

Figure 4.2. Relative shoot dry weight ($SDW_{Rel.}$) of eight pea lines grown for 29 days under controlled conditions on four soils. A) Boxplots for each soil over all genotypes and replicates (n = 32) showing the median and the interquartile range; the ends of the whiskers represent 1.5 times the interquartile range. Soil means followed by a common letter are not significantly different ($p < .05$, Tukey HSD). B) Mean $SDW_{Rel.}$ for eight pea lines (symbols) in each soil (colour): Solid symbols represent pea lines categorised as resistant; open symbols represent susceptible pea lines. Bars represent the standard error of the means. Genotypic means are presented over the three sick soils; means followed by a common letter are not significantly different ($p < .05$, Tukey HSD).

Factors 'soil', 'genotype' and their interaction had a significant effect on root rot index (RRI) ($F_{3,96} = 38.8$, $p < .001$, $F_{7,96} = 9.6$, $p < .001$, $F_{21,96} = 2.8$, $p < .001$, respectively). 'Resistance level' also had a significant effect on RRI ($F_{1,120} = 37.3$, $p < .001$). The interaction between 'soil' and 'resistance level' was not significant ($F_{3,120} = 1.1$, $p < .363$). Mean (SD) root rot indexes (RRI) of K, P and N soils were 3.22 (0.52), 3.50 (0.76), 3.08 (0.81), respectively, significantly higher compared with 2.05 (0.95) in the F soil (Tukey HSD: F-K: $p < .0001$; F-P: $p < .0001$; F-N: $p < .0001$; Figure 4.3). Among the three sick soils, P soil showed significantly higher RRI compared with N soil (Tukey HSD: $p = .008$). In the analysis of the three sick soils only, significant effects of the factors 'soil' ($F_{2,72} = 4.4$, $p < .015$) and 'genotype' ($F_{7,72} = 3.3$, $p < .004$) were detected, with no significant interaction ($F_{14,72} = 0.88$, $p < .574$) between the two factors. Post-hoc analysis showed that only genotype S91 had a significantly lower RRI than C2 and G89. Over the three sick soils, RRI was significantly lower for resistant than for susceptible pea lines: Mean (SD) = 2.7 (0.8) versus 3.3 (1.0) (Wilcoxon rank-sum test: $p < .001$; Figure 4.6).

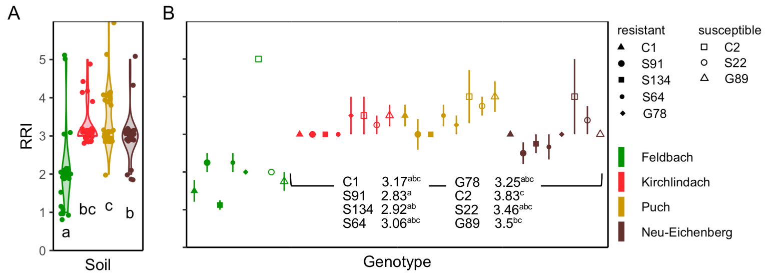

Figure 4.3. Root rot index (RRI) assessed on eight pea lines grown for 29 days under controlled conditions on four soils. A) Violin plots for each soil over all genotypes and replicates (n = 32). Soil means followed by a common letter are not significantly different ($p < .05$, Tukey HSD). B) Mean RRI for eight pea lines (symbols) in each soil (colour): Solid symbols represent pea lines categorised as resistant; open symbols represent susceptible pea lines. Bars represent the standard error of the means. Genotypic means are presented over the three sick soils; means followed by a common letter are not significantly different ($p < .05$, Tukey HSD).

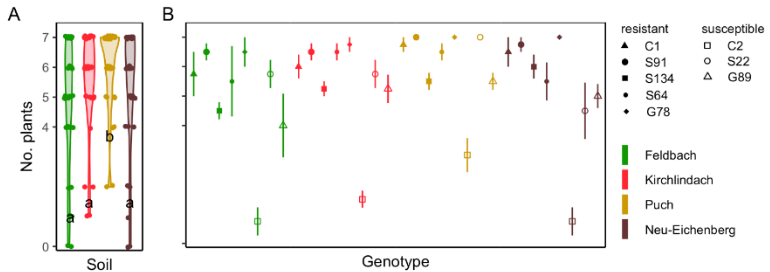

Figure 4.4. Plant emergence seven days after sowing assessed on eight pea lines grown under controlled conditions on four soils. A) Violin plot for each soil over all genotypes and replicates (n = 32). Soil means followed by a common letter are not significantly different ($p < .05$, Tukey HSD). B) Mean emergence for eight pea lines (symbols) in each soil (colour): Solid symbols represent pea lines categorised as resistant; open symbols represent susceptible pea lines. Bars represent the standard error of the means.

Plant emergence after seven days was significantly lower in the non-sterile soils compared to the sterilised soils, except for Puch (P) soil (Figure 4.4). There were significant effects of the factors 'soil' ($F_{3,96}$ = 37.6, $p < .001$) and 'genotype' ($F_{7,96}$ = 10.1, $p < .001$) on emergence in the non-sterile soil. The factor 'resistance level' was also significant ($F_{2,120}$ = 49.4, $p < .001$). Interactions of 'soil' with 'genotype' ($F_{21,96}$ = 1.2, $p < .277$) or 'resistance level' ($F_{3,120}$ = 1.6, $p < .186$) were not significant. (Figure 4.4). Mean (SD) emergence in P soil was 6.03 (1.38), significantly higher compared with 4.91 (1.89), 5.44 (1.81) and 5.25 (2.01) in the F, K and N soils (Tukey HSD: F-P: $p < .0001$; K-P: $p = .0005$; N-P: $p = .0001$; Figure 4.4).

4.3.2 Quantification and composition of key microbial taxa

Previously published qPCR assays were successfully implemented and allowed for the DNA quantification of ten microbial taxa in diseased pea roots. Average PCR efficiencies and R^2 of the standard curves were 1.00 (min. 0.97 / max. 1.00) and 0.91 (0.71 / 1.17), respectively (Table 4.4). In the control samples of pea roots grown in the sterilised soil (genotypes C1 and C2 in all four soils) 78 out of 320 tests showed positive qPCR signals. The range of these non-zero quantifications in the control samples was 0.001 – 0.5 (median) – 111.4 pg rct^{-1} over all soils and both genotypes.

A. euteiches and *F. solani* were the most abundant pathogens in diseased roots, clearly distinguishing the healthy F soil from the three sick soils. The mean (SD) of these two pathogens over the three sick soils was 327 (316) and 410 (537) pg rct^{-1}, respectively. (Figure 4.5, Table 4.5). Diseased roots from P soil showed the highest *A. euteiches* concentrations. For *F. solani*, K and P showed similar concentrations. N was characterised by lower concentrations of *A. euteiches* and *F. solani* compared with P. There was a tendency that resistant pea genotypes have a lower total pathogen amount in the roots than susceptible, mainly accountable to *F. solani* and *A. euteiches*. Genotypes S91 and S134 consistently showed low pathogen abundance in all three sick soils. Genotypes S64 and G78 took an intermediate position in P, but showed

Table 4.4. Amplification efficiencies (E) and R^2 for the standard curves of ten qPCR assays targeting microbial taxa in diseased pea roots. Efficiencies and R^2 are shown for every independent qPCR run. Quantifications were performed on two independent DNA extractions; "a" and "b", respectively. Each sample was tested in duplicate qPCR reactions. AMF = arbuscular mycorrhizal fungi.

	Biological rep.	1		2		3		4		MEAN
	Technical rep. (DNA extraction)	a	b	a	b	a	b	a	b	
A. euteiches	R^2:	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
	E:	0.86	0.84	0.81	0.84	0.72	0.85	0.82	0.86	0.83
AMF	R^2:	1.00	0.99	0.99	0.99	0.99	1.00	0.98	0.99	0.99
	E:	0.85	0.86	0.83	0.95	0.99	0.86	0.88	0.91	0.89
C. rosea	R^2:	1.00	1.00	1.00	1.00	1.00	0.99	1.00	0.99	0.99
	E:	0.99	0.94	0.98	0.93	1.05	0.99	1	0.93	0.98
D. pinodella	R^2:	1.00	0.99	1.00	1.00	1.00	1.00	0.98	1.00	0.99
	E:	0.71	0.79	0.83	0.78	0.77	0.87	0.81	0.83	0.80
F. avenaceum	R^2:	0.99	0.99	0.98	0.99	0.97	1.00	0.99	0.99	0.99
	E:	0.86	0.91	0.79	0.93	0.90	0.96	0.85	0.8	0.88
F. oxysporum	R^2:	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
	E:	0.87	0.90	0.84	0.88	0.97	0.85	0.88	0.92	0.89
F. redolens	R^2:	1.00	1.00	1.00	1.00	1.00	1.00	1.00	0.99	1.00
	E:	0.97	0.95	1.00	0.93	0.96	0.97	0.97	0.93	0.96
F. solani	R^2:	1.00	0.99	0.99	1.00	1.00	1.00	1.00	1.00	1.00
	E:	0.98	1.11	1.17	1.13	0.95	0.99	0.98	0.89	1.03
P. ultimum	R^2:	1.00	1.00	1.00	1.00	1.00	1.00	0.99	1.00	1.00
	E:	0.95	1.01	0.94	0.95	0.99	0.91	1.06	0.90	0.96
R. solani	R^2:	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
	E:	0.86	0.86	0.88	0.87	0.85	0.85	0.88	0.79	0.86

pathogen amounts comparable to the susceptible lines in K soil. In N soil, S64 was as low as S91 and S134; G78 was as high as the susceptible genotype S22. The resistant genotype C1 showed higher total pathogen concentrations than S91 and S134, and had especially high pathogens amounts in the highly infested soil P. Over all three sick soils, resistant genotypes showed lower amounts of *F. solani* and *A. euteiches* in the roots than susceptible genotypes (Wilcoxon rank-sum test: $p < .01$; Figure 4.6). *F. oxysporum* was present in all soil-genotype combinations with an overall mean of 68 (77) pg rct^{-1}. However, the abundance was lower in P soil (22 (35) pg rct^{-1}) compared to the three other soils. It was uniformly present in all genotypes grown in N soil. In K soil, *F. oxysporum* was more present in roots from genotypes that had high overall pathogen loads; i.e. C2, S22, S64, G78 and G89. Over all three sick soils, resistant genotypes showed lower amounts of *F. oxysporum* in the roots than susceptible genotypes (Wilcoxon rank-sum test: $p = .016$). *F. avenaceum* and *F. redolens* were quantified at very low levels, with means (SD) of 1

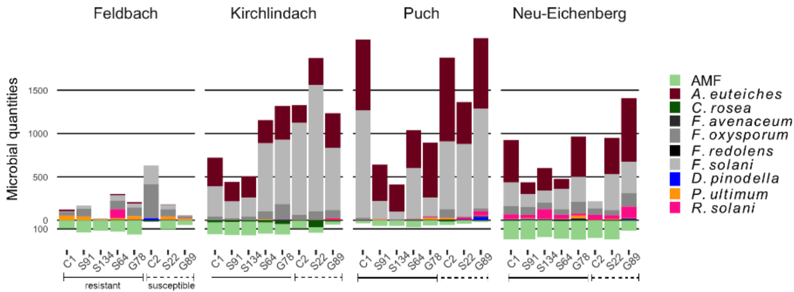

Figure 4.5. Composition of ten microbial taxa in diseased pea roots. Microbes were quantified by quantitative real-time PCR in roots of eight different pea lines (either resistant or susceptible to root rot) grown in four different soils (Feldbach, Kirchlindach, Puch, Neu-Eichenberg): Mean quantification (in pg rct^{-1}, or copies rct^{-1} for AMF; n is given in Table 4.5) of the ten microbial taxa, with pathogens extending above of the 0-scale bar, beneficial taxa below (AMF quantifications were square root transformed for the presentation).

(3) and 2 (5) pg rct^{-1}, respectively. *F. avenaceum* was almost exclusively detected in samples grown in P soil (3 (6) pg rct^{-1}), and *F. redolens* in N soil (7 (7) pg rct^{-1}). Over all three sick soils, resistant genotypes showed lower amounts of *F. avenaceum* in the roots than susceptible genotypes (Wilcoxon rank-sum test: p = .008). *R. solani*, *P. ultimum* and *D. pinodella* showed overall low levels of detected DNA, with 24 (62), 12 (23) and 4 (15) pg rct^{-1}, respectively. *R. solani* showed considerably higher levels in N soil, with 64 (77) pg rct-*1* over all genotypes. In F soil, it was almost only detected in genotype S64. *P. ultimum*, on the other hand, showed the highest concentrations in the roots from F soil (32 (33) pg rct^{-1}). *D. pinodella* was uniformly present in diseased roots from all genotypes in N soil only. The beneficial fungus *C. rosea* was detected at low levels in samples from F, P and N soils (2 (6), 5 (9), 4 (6) pg rct^{-1}, respectively) and with a mean (SD) of 29 (34) of pg rct-1 in samples from K soil, generally uniformly present over all genotypes. AMF could be detected in all soil-genotype combinations, with the highest levels in samples grown in N soil (41100 (26100) versus the overall mean of 18600 (20400) copies rct-*1*). The concentration of AMF was significantly higher in the roots of resistant genotypes than in susceptible genotypes (Wilcoxon rank-sum test: $p < .0001$).

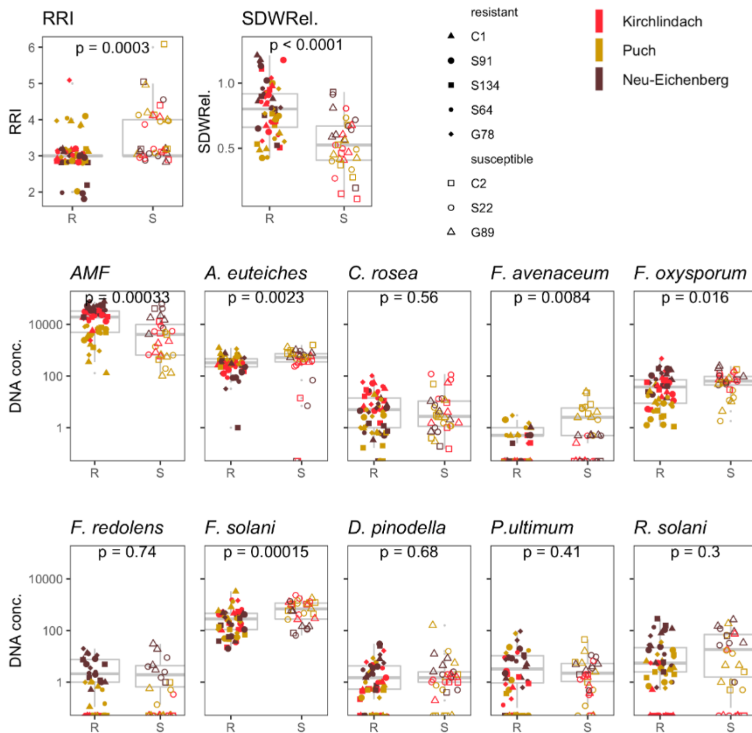

Figure 4.6. Comparison of phenotypic variables (RRI and $SDW_{Rel.}$) and abundances of ten microbial taxa in diseased pea roots. Data was assessed on eight pea lines, classified as 'resistant' (five lines; closed symbols) and 'susceptible' (three lines; open symbols), grown in three sick field soils (Kirchlindach, Puch and Neu-Eichenberg) under controlled conditions. (four replications). Microbial taxa were quantified using quantitative real-time PCR; DNA concentrations are given in pg rct^{-1}. Note: For AMF, quantities are expressed as 10^3 ITS copies rct^{-1}. Boxplots show the median and the interquartile range; the ends of the whiskers represent 1.5 times the interquartile range. Wilcoxon rank sum tests were performed to test differences between the two resistance levels across the three sick soils; *p*-values thereof are indicated in the plots.

Table 4.5. qPCR quantifications for ten microbial taxa in diseased roots of eight pea lines grown for 29 days in four different soils (F = Feldbach, K = Kirchlindach, P = Puch, N = Neu-Eichenberg). The mean ± standard deviation of quantified microbial DNA (in pg rct^{-1}) is presented along the number of analysed replicates (n; in brackets). For better readability numbers were rounded to the nearest integer. For AMF, quantities are expressed as 10^3 ITS copies rct^{-1}. *F. ave. = F. avenaceum; F. oxy. = F. oxysporum; F. red. = F. redolens; F. sol. = F. solani; D. pin. = D. pinodella; R. sol. = R. solani; P. ult. = P. ultimum;* AMF = arbuscular mycorrhizal fungi; *C. ros. = C. rosea.*

	Acc.	*F. ave.*	*F. oxy.*	*F. red*	*F. sol.*	*D. pin.*	*R. sol.*	*A. eut.*	*P. ult.*	AMF	*C. ros.*
F	S91	0±0 (4)	89±80 (4)	0±0 (4)	32±29 (4)	0±0 (4)	0±0 (4)	0±0 (4)	46±15 (4)	19.1±6.5 (4)	1±1 (4)
	S134	0±0 (4)	7±7 (4)	0±0 (4)	4±4 (4)	0±0 (4)	0±0 (4)	1±1 (4)	7±3 (4)	14.4±5 (4)	1±1 (4)
	G78	0±0 (4)	101±23 (4)	0±0 (4)	48±31 (4)	3±3 (4)	0±0 (4)	11±18 (4)	44±36 (4)	27.4±7.1 (4)	1±1 (4)
	C1	0±0 (4)	44±56 (4)	0±0 (4)	7±6 (4)	0±0 (4)	0±0 (4)	18±18 (4)	53±61 (4)	9±3.2 (4)	0±1 (4)
	S64	3±5 (4)	96±73 (4)	0±0 (4)	63±97 (4)	0±0 (4)	107±213 (4)	13±23 (4)	21±17 (4)	16.4±6.9 (4)	2±1 (4)
	G89	0±0 (3)	27±9 (3)	0±0 (3)	10±8 (3)	0±0 (3)	4±8 (3)	0±0 (3)	18±13 (3)	2.9±1.3 (3)	1±1 (3)
	S22	0±0 (4)	86±108 (4)	0±0 (4)	49±56 (4)	5±7 (4)	0±0 (4)	5±8 (4)	41±41 (4)	13.1±8.7 (4)	1±1 (4)
	C2	0±0 (2)	389±NA (1)	0±NA (1)	216±76 (2)	25±33 (2)	0±NA (1)	0±0 (2)	2±NA (1)	NA	17±21 (2)
	mean	**0±2 (29)**	**77±89 (28)**	**0±0 (28)**	**44±67 (29)**	**3±9 (29)**	**16±81 (28)**	**7±13 (29)**	**32±33 (28)**	**15.0±0.8 (25)**	**2±6 (29)**
K	S91	0±0 (4)	23±22 (4)	0±0 (4)	191±166 (4)	2±2 (4)	1±3 (4)	222±87 (4)	1±1 (4)	22.7±7.4 (4)	21±22 (4)
	S134	0±0 (4)	41±30 (4)	0±0 (4)	220±232 (4)	0±0 (4)	0±0 (4)	243±68 (4)	1±1 (4)	26.9±8.4 (4)	15±12 (4)
	G78	0±0 (4)	180±206 (4)	0±0 (4)	741±483 (4)	6±8 (4)	1±3 (4)	392±63 (4)	0±0 (4)	16.4±11.3 (4)	43±46 (4)
	C1	0±0 (4)	42±31 (4)	0±0 (4)	352±202 (4)	0±0 (4)	0±0 (4)	327±48 (4)	0±0 (4)	20.5±11.1 (4)	24±23 (4)
	S64	0±0 (4)	98±49 (4)	0±0 (4)	783±532 (4)	1±1 (4)	0±0 (4)	267±146 (4)	6±7 (4)	18.4±10.8 (4)	25±11 (4)
	G89	0±0 (4)	92±44 (4)	0±0 (4)	717±511 (4)	1±1 (4)	23±35 (4)	400±115 (4)	1±1 (4)	1.4±0.8 (4)	16±27 (4)
	S22	0±0 (4)	99±44 (4)	0±0 (4)	1459±813 (4)	1±0 (4)	0±0 (4)	310±73 (4)	3±3 (4)	5.1±3.4 (4)	79±49 (4)
	C2	0±0 (2)	64±48 (2)	0±0 (2)	1059±16 (2)	1±0 (2)	0±0 (2)	205±343 (3)	1±1 (2)	8.8±6 (2)	3±5 (3)
	mean	**0±0 (30)**	**81±88 (30)**	**0±0 (30)**	**666±578 (30)**	**2±3 (30)**	**3±14 (30)**	**299±136 (31)**	**2±3 (29)**	**15.4±11.3 (27)**	**29±34 (31)**
P	S91	1±1 (4)	3±4 (4)	0±0 (4)	206±184 (4)	0±0 (4)	6±8 (4)	418±140 (4)	6±11 (4)	4.3±1.9 (4)	1±2 (4)
	S134	0±0 (4)	3±1 (4)	0±0 (4)	91±41 (4)	1±0 (4)	4±5 (4)	315±75 (4)	0±0 (4)	4.4±2.6 (4)	0±0 (4)
	G78	1±1 (4)	8±1 (4)	1±1 (4)	216±169 (4)	3±4 (4)	12±16 (4)	629±337 (4)	23±37 (4)	4.3±4 (4)	1±1 (4)
	C1	1±0 (4)	24±17 (4)	0±0 (4)	1238±1394 (4)	0±0 (4)	5±2 (4)	818±278 (4)	0±0 (4)	1.4±1.5 (4)	5±9 (4)
	S64	0±0 (4)	11±6 (4)	0±0 (4)	580±508 (4)	2±3 (4)	4±4 (4)	439±200 (4)	4±8 (4)	5.3±2.5 (4)	7±4 (4)
	G89	9±11 (4)	36±36 (4)	0±0 (4)	1147±458 (4)	41±78 (4)	49±92 (4)	812±340 (4)	6±7 (4)	0.4±0.3 (4)	4±5 (4)
	S22	4±3 (4)	14±11 (4)	0±0 (4)	833±558 (4)	4±8 (4)	24±29 (4)	481±34 (4)	0±0 (4)	1.8±2.2 (4)	2±2 (4)
	C2	10±8 (4)	98±71 (3)	0±1 (3)	781±344 (4)	1±1 (4)	1±1 (3)	964±456 (4)	17±24 (3)	2.3±2.6 (2)	14±23 (4)
	mean	**3±6 (32)**	**22±35 (31)**	**0±0 (31)**	**637±674 (32)**	**6±28 (32)**	**14±35 (31)**	**609±324 (32)**	**7±16 (31)**	**3.1±2.7 (27)**	**5±9 (32)**
N	S91	0±0 (4)	91±79 (4)	4±3 (4)	152±181 (4)	10±14 (4)	38±47 (4)	135±67 (4)	10±7 (4)	46.6±10.3 (4)	3±3 (4)
	S134	0±0 (4)	75±80 (4)	4±3 (4)	139±119 (4)	7±13 (4)	113±137 (4)	261±184 (4)	3±6 (4)	38.2±15 (4)	1±1 (4)
	G78	0±0 (4)	137±74 (4)	12±7 (4)	285±164 (4)	5±5 (4)	24±28 (4)	464±297 (4)	36±43 (4)	53.2±36.9 (4)	5±3 (4)
	C1	0±1 (4)	93±67 (4)	6±6 (4)	273±227 (4)	3±3 (4)	57±51 (4)	490±210 (4)	3±2 (4)	48±20.8 (4)	5±6 (4)
	S64	0±0 (4)	52±25 (4)	4±6 (4)	198±133 (4)	2±2 (4)	37±66 (4)	104±47 (4)	4±4 (4)	34.3±32.3 (4)	7±14 (4)
	G89	1±0 (4)	152±84 (4)	14±13 (4)	363±254 (4)	7±4 (4)	135±100 (4)	734±216 (4)	4±1 (4)	14.7±7.5 (4)	4±4 (4)
	S22	0±0 (4)	64±29 (4)	4±3 (4)	412±627 (4)	2±1 (4)	45±51 (4)	416±451 (4)	6±5 (4)	53.3±40.3 (4)	6±6 (4)
	C2	0±NA (1)	72±NA (1)	1±NA (1)	81±NA (1)	0±NA (1)	62±NA (1)	0±NA (1)	2±NA (1)	41.1±NA (1)	0±NA (1)
	mean	**0±0 (29)**	**94±67 (29)**	**7±7 (29)**	**254±273 (29)**	**5±7 (29)**	**64±77 (29)**	**359±304 (29)**	**9±18 (29)**	**41.1±26.1 (29)**	**4±6 (29)**
	mean	**1±3 (120)**	**68±77 (118)**	**2±5 (118)**	**408±536 (120)**	**4±15 (120)**	**24±62 (118)**	**325±316 (121)**	**12±23 (118)**	**18.6±20.4 (116)**	**10±21 (121)**

Non-metric multidimensional scaling (NMDS) analysis of the composition of the ten microbial species based on Bray-Curtis distances between individual samples revealed a clear clustering according to the four soils, with the healthy soil F showing almost no overlap with the sick soils (Figure 4.7). Results from the PERMANOVA support this grouping, with 33% of the variance explained by the factor 'soil'. When the F soil is excluded from the PERMANOVA, the factor 'soil' explains 40% of the variance in the microbial composition. The factor 'genotype' explains 14% and 16%, while the interaction 'soil × genotype' explains 25% and 16% in the analyses over four soils and three sick soils, respectively. The analysis within the three sick soils separately revealed a significant effect of the factor 'genotype' for all three soils ($p < .05$). The same hold true for the factor 'resistance level' in K and P; in N this factor was only marginally significant ($p = .051$). The visualization of the NMDS supports the evidenced levels of significance, with apparent 'resistance level' grouping in K and P, but not in N soil. Over the four soils, all microbes correlated significantly with the ordination, except for *D. pinodella* (Figure 4.7). The three variables showing the best fit with the ordination were *A. euteiches* ($r^2 = 0.52$), *F. solani* ($r^2 = 0.39$) and AMF ($r^2 = 0.29$), with AMF pointing in opposite direction than *F. solani*. $SDW_{Rel.}$ and RRI both correlated significantly with the NMDS ordination ($r^2 = 0.27$ and $r^2 = 0.53$, respectively), pointing in opposite directions (Figure 4.7).

In the pairwise Spearman correlation analysis among microbial quantifications several patterns reappeared in each of the three sick soils. Generally, significant positive relationships between pathogenic taxa were found, with correlations ranging from 0.37 to 0.87 (Figure 4.8 A): *F. solani* and *A. euteiches* showed positive correlations in K and P soils, but not in N soil. *F. oxysporum* was generally correlated with different pathogens in the three soils. It was positively correlated with *F. solani* in all three sick soils and it was positively correlated with *P. ultimum* and *D. pinodella* in K and N soils. In P soil, it was positively correlated with *F. avenaceum* that was only evidenced in this soil. In P soil, *F. avenaceum* also showed a significant correlation with *F. solani*, and *R. solani* and *P. ultimum* were negatively correlated. *F. redolens*, only quantified in roots from the F soil, showed strong correlations with *F. solani*, *F. oxysporum* and *D. pinodella*. AMF showed a negative relationship with pathogenic

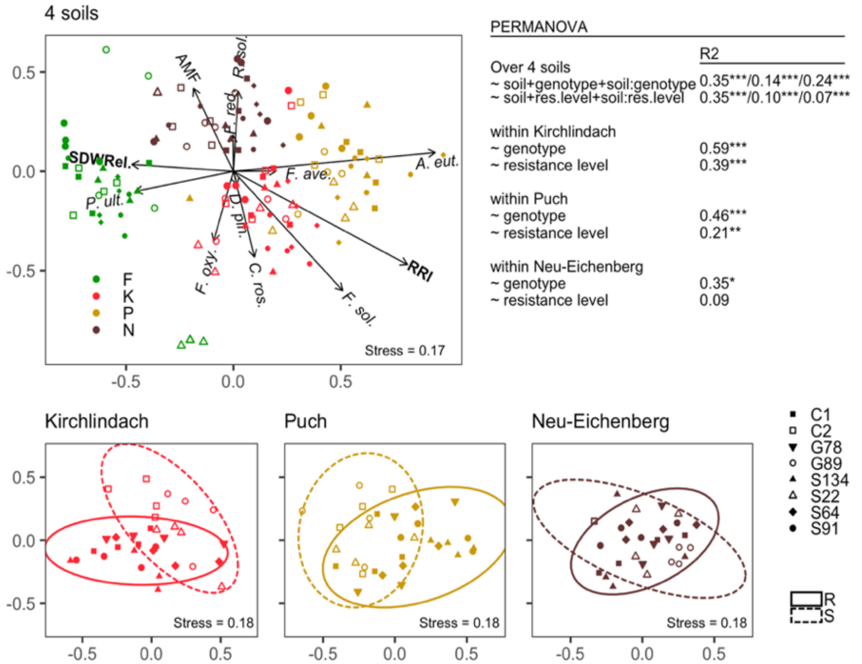

Figure 4.7. Composition of ten microbial taxa in diseased pea roots. Microbes were quantified by quantitative real-time PCR in roots of eight different pea lines grown in four different soils (Feldbach (F), Kirchlindach (K), Puch (P) and Neu-Eichenberg (N)). Panels show the first two dimensions of the NMDS of the Bray-Curtis dissimilarities performed on quantities of the microbial taxa over all four soils or for each of the three sick soils individually. Arrows indicate the fitted microbial quantities and the two phenotypic variables relative shoot dry weight ($SDW_{Rel.}$) and root rot index (RRI); the arrow length is scaled by the respective r^2 (goodness-of-fit with the ordination) of the variable. Ellipses correspond to the 95% confidence interval of the factor 'resistance level'. R^2 and significance levels of the factors tested in the PERMANOVA are provided: Analysis were performed with two models, either containing the factor 'genotype' or 'resistance level', and over all soils or for each of the three sick soils individually (• $p < .1$; * $p < .05$; ** $p < .01$; *** $p < .001$).

taxa,with correlations ranging from -0.36 to -0.65 in K and P soils. In K soil AMF were negatively correlated with *F. solani*, *R. solani*, *D. pinodella* and *F. oxysporum.* In P soil, negative correlations with *F. solani, F. oxysporum*, *F. avenaceum* and *A. euteiches* were evidenced. No relationship was found for AMF with other taxa in N soil. *C. rosea* showed a positive relationship with several pathogens in all three soils, with correlations ranging from 0.43 – 0.79. It was positively correlated with *F. solani* and *F. oxysporum* in all three soils and additionally with *A. euteiches* and *F. redolens* in P and N soils, respectively.

Spearman correlation analysis revealed that several pathogens were positively correlated with RRI and negatively correlated with $SDW_{Rel.}$ (Figure 4.8 B). Despite high amounts of *A. euteiches* detected in diseased roots from all three sick soils, this pathogen was only correlated negatively with $SDW_{Rel.}$ in K soil and with RRI in N soil. No correlation with the phenotypic variables was detected in K soil. *F. solani*, detected at high levels in K and P soils showed positive correlations in these soils, but not in N soil. *F. oxysporum* showed positive correlations with the phenotypic variables in K and P soil, but not in N soil where it was quantified at the highest level. In P soil, *F. avenaceum* showed a significantly negative correlation with $SDW_{Rel.}$. In K soil, *D. pinodella* showed significant correlations with both phenotypic variables. In all three soils, *R. solani* was significantly correlated with the two phenotypic variables. P. ultimum only showed a negative correlation with RRI in N soil. *F. redolens* did not correlate with the phenotypic variables in N soil, where it was only quantified. AMF generally showed positive correlations with $SDW_{Rel.}$ and negative correlations with RRI in all three sick soils. *C. rosea* showed a positive correlation with RRI in P and N soils, and a negative correlation with $SDW_{Rel.}$ in P soil.

Stepwise backward variable selection was used in order to account for the correlation among pathogens in the multiple regression. Overall, reducing variables by stepwise selection improved the multiple regression model fits: For $SDW_{Rel.}$, adjusted R^2 for the reduced models were 0.32, 0.29, 0.40 and 0.21 over the three sick and within K, P and N, respectively. In K soil, AMF and *F. solani* explained 20% (partial r^2) and 16% of the observed variance, respectively, (Figure 4.8 B). In P soil, AMF (37%) and *F. oxysporum* (7%) explained significant parts of the

variance. In N soil, AMF, *A. euteiches*, *F. redolens* and *R. solani* explained significant parts of the variance, with partial r^2 of 0.15, 0.05, 0.07 and 0.06, respectively. For RRI, adjusted R^2 were 0.46, 0.55, 0.57 and 0.45 over three sick and within K, P and N, respectively. Stepwise variable selection partly resulted in different microbial taxa retained in the final model (Figure 4.8 B). Residuals of the multiple regression of RRI were heteroscedastic, therefore these results have to be interpreted with caution.

4.4 DISCUSSION

Root-rot pathogens are well-known for their involvement in the pea yield depression syndrome, an important threat to pea cultivation in temperate zones (Rubiales *et al.*, 2015; Taheri *et al.*, 2017). Fuchs *et al.* (2014) developed a pot-based system, using sterilised soil as a disease-free control, that allows to simply evaluate the level of yield depression potential of agricultural soils. We applied this system and combined it with the quantification of ten microbial taxa to link disease pressure of the soil, plant resistance and microbial composition. We confirmed the pronounced pea root rot potential of three agricultural soils, all provoking stronger root rot symptoms than the presumably healthy soil F.

The K soil was previously described as a soil with strong legume yield depression syndrome (Wille *et al.*, 2020). In the present study, shoot biomass reduction on this soil was 32%. Growth reduction was even more pronounced in the P soil (40%) and to a lesser extent in N soil (20%). These values are in accordance with previously published data on growth reduction in controlled conditions inoculation assays (Pilet-Nayel *et al.*, 2005; Šišić *et al.*, 2018). Despite this strong disease pressure, we were able to confirm the high tolerance of pea lines selected from our recent larger resistance screen (Wille *et al.*, 2020). Gene bank accession S91, derived from a cross conducted by Unilever (UK) before 1960, grew best over all three sick soils, followed by the pea varieties G78 ('Roch') and C1 ('EFB.33'). Those three genotypes are full-leaf types with pigmented flowers.

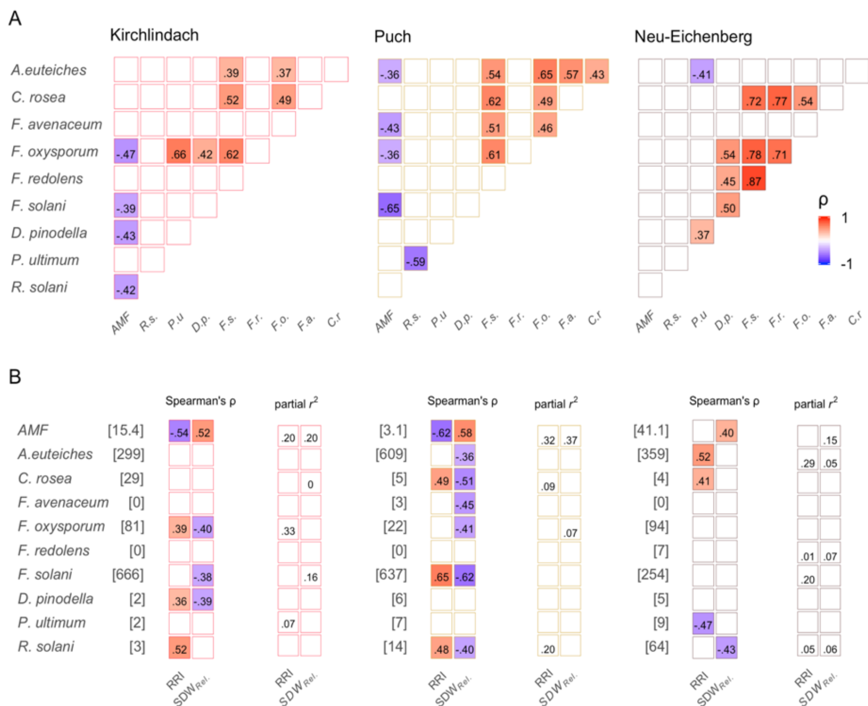

Figure 4.8. Relationship between ten microbial taxa in diseased pea roots assessed by quantitative real-time PCR and disease variables. A) Spearman correlations between abundances of ten microbial taxa within three sick soils (Kirchlindach, Puch, Neu-Eichenberg). The heatmaps show significant (p < .05) positive (pink) and negative (green) Spearman's *rho* for pairs of taxa. B) Relationship between root rot index (RRI) and relative shoot dry weight ($SDW_{Rel.}$) and the abundance of ten microbial taxa. Mean (eight pea lines, four replications) quantities for each microbial taxon within soils are indicated in square brackets (pg rct^{-1}; except for AMF where quantities are given in 10^3 copies rct^{-1}). The heatmaps show significant (p < .05) positive (pink) and negative (green) Spearman's *rho* (ρ). Furthermore, phenotypic variables were regressed on the microbial quantities; partial r^2 of retained variables after stepwise variable selection are presented.

Over the three sick soils, no significant soil × genotype, nor soil × resistance level interaction was found for $SDW_{Rel.}$. This suggest that resistance levels assessed on a given infested soil are transferable to other soils. This stands in contrast to other pea root rot resistance studies, where strong environment × genotype interactions have been reported (Weeden *et al.*, 2000; Hamon *et al.*, 2011; Desgroux *et al.*, 2016). Although the assessed soils were from different regions in Switzerland and Germany, they show certain similarities in edaphic characteristics and all managed according to certified organic farming. Moreover, controlled conditions of the growth chamber experiment in our study have certainly reduced parts of the interaction effects with the environment.

We estimated the importance of several major PRRC pathogens at the host level, employing molecular-based quantification. Previous studies have shown the health status of pea to be related to the fungal community present in diseased roots, but barely reflected by the fungal community in the soil (Xu *et al.*, 2012; Wille *et al.*, 2020). Therefore, we decided to quantify pathogens in the roots. In our study, multiple pathogens were simultaneously present at different levels in diseased roots, confirming similar observations from the field and corroborating the importance of the concept of the pea root rot complex (Xue, 2003a). The composition of the microbial taxa in diseased roots were significantly different between the four soils. The healthy status of the F soil was reflected by the lower total abundance of putative pathogens, particularly *A. euteiches* and *F. solani*, in the roots of plants grown in this soil and the clear-cut clustering in the NMDS. This significant soil effect is in line with repeated documentation on local environmental factors as strong drivers of plant-associated microbial composition (Chemidlin Prevost-Boure *et al.*, 2014; Xue *et al.*, 2018; Li *et al.*, 2019a).

In the three sick soils, *F. solani* and *A. euteiches* dominated the pathogen composition in diseased roots. Those pathogens are well-known members of the pea root rot complex, mutually facilitating plant infection and aggravating disease symptoms (Peters & Grau, 2002; Willsey *et al.*, 2018). *A. euteiches* is recognized as one of the most important pathogens of pea in Canada, France and Sweden (Gaulin *et al.*, 2007; Heyman *et al.*, 2007; Wu *et al.*, 2018). The abundance of both pathogens significantly correlated with each other and each negatively with $SDW_{Rel.}$ in K and P soils, corroborating their importance in the PRRC.

To the best of our knowledge, this is the first report on the presence of *A. euteiches* in pea roots grown in German and Swiss soils. This calls for a confirmation by isolation, using a targeted, oomycete-selective isolation protocol, because it has been shown that standard isolation procedures result in a strong underestimation of the frequency of *A. euteiches* in the field (Chatterton *et al.*, 2018). Furthermore, it would be advisable for future breeding and plant protection measurements to conduct a qPCR-based survey on *A. euteiches* pea root presence in central Europe.

F. oxysporum, *F. avenaceum* and *F. redolens* were detected at intermediate to low levels. Those three have repeatedly been confirmed as very prevalent pathogens associated with pea root rot in North America and Europe (Feng *et al.*, 2010; Pflughöft *et al.*, 2012; Chittem *et al.*, 2015).

F. oxysporum showed a negative correlation with $SDW_{Rel.}$ in K and P soils, but did not appear to have an effect on disease in N soil. *F. oxysporum* is a major pathogen of pea causing Fusarium wilt and root rots and is frequently isolated from root-rot infected fields (Kraft, 1994; Chatterton *et al.*, 2018). However, various strains including non-pathogenic forms of *F. oxysporum* are known, that can opportunistically co-infect a host and antagonise other pathogens (Oyarzun *et al.*, 1994). Experimental work suggests that *F. oxysporum* may not be a primary factor of root rots, but, as part of the PRRC, increase disease severity (Kerr, 1963; Chittem *et al.*, 2015). This opportunistic behaviour might be reflected by the strong positive correlations with diverse pathogens in all three sick soils evidenced in our study.

F. avenaceum was shown to be highly aggressive on pea in previous studies (Pflughöft *et al.*, 2012; Chittem *et al.*, 2015; Šišić *et al.*, 2018); its co-occurrence with relevant pathogens might explain the significantly higher disease pressure of the P soil compared to the N soil. In the P soil, correlations of *A. euteiches* and *F. avenaceum* with other taxa stand out; this is possibly due to the fact that these two taxa were quantified at higher levels in P soil compared to the other two soils. We cannot conclude on the role individual pathogens play in the P soil; however, it seems probable that the combination of *F. avenaceum*, *R. solani* and the high loads of *A. euteiches* and *F. solani* are the explanation for the high disease pressure of this soil.

F. redolens in turn, was only detected in diseased roots from N soil, which showed lower disease pressure compared with the P soil. *F. redolens* was detected at low levels, did not significantly correlate with RRI or $SDW_{Rel.}$ and explained only marginal portions of the variance in RRI or $SDW_{Rel.}$. Furthermore, *F. redolens* concentrations in diseased roots did not significantly differ between resistant and susceptible lines. We conclude that this pathogen is of minor importance in the PRRC. In the study by Chittem *et al.* (2015), *F. redolens* was a prevalent pathogen in pea fields together with other *Fusarium* species, however, showing low disease severity when inoculated under controlled conditions; this points at a subordinal role of this pathogen in the PRRC.

D. pinodella was detected at low levels in all three sick soils, but was significantly correlated with disease in the K soil only. It is a frequently isolated pea root rot pathogen in European cropping systems (Persson *et al.*, 1997; Pflughöft *et al.*, 2012). Based on frequent isolation from the N soil and confirmed aggressivity on a susceptible pea cultivar in sterilised sand, Baćanović-Šišić *et al.* (2018) concluded that *D. pinodella* is an important member of the PRRC in Germany. Our data does not allow to confirm nor reject these conclusions. Apparently, the level of infection with *D. pinodella* does not necessarily predict disease severity, as the pathogen was significantly correlated with the disease parameters despite its low level of detection in K soil. No relation to disease parameters was evident in the two other sick soils. This calls for follow-up experiments to elucidate the role *D. pinodella* in the PRRC; e.g. by testing different co-inoculation combinations and different inoculum levels.

P. ultimum were also detected at low levels, with a slight enrichment in the roots grown in the F soil. *Pythium* spp. are oomycetes and common root rot pathogens in European cropping systems, provoking severe pre- and post-emergence damping-off (Persson *et al.*, 1997; Pflughöft *et al.*, 2012; Alcala *et al.*, 2016). The enrichment of *P. ultimum* in roots grown in the healthy soil F is a possible indication for the poor emergence of pea line C2 in this soil, but otherwise no clear role of this oomycete was apparent with regard to PRRC. *Pythium* spp. have been shown to synergistically act with pathogenic *Fusarium* species, with a cultivar-dependent effect on plant growth (Kerr, 1963; Muehlbauer & Kraft, 1973). Low plant emergence and poor growth of pea line C2 was

confirmed in the field at site F (data not shown) and we aim at elucidating the responsible pathogens and their interaction in future experiments.

R. solani was detected throughout our study*,* with a clear presence in roots grown in the N soil. *R. solani* is frequent in pea fields in North Dakota, the Canadian prairies and Yunnan, China (Yang *et al.*, 2005; Mathew *et al.*, 2012; Melzer *et al.*, 2016). Studies on the relation of *R. solani* to pea diseases from Europe are scarce. This pathogen showed significant relations with the two disease parameters in all three sick soils. Because *R. solani* is known to be mainly related to seedling disease, we might hypothesise that this pathogen infects seedlings first and facilitates further infection by other pathogens (Gossen *et al.*, 2016; Chatterton *et al.*, 2018). Its above-average quantification in roots from the P soil and the associated high disease pressure of that soil support this hypothesis.

AMF and *C. rosea* were detected in root samples from all four soils. *C. rosea* is a mycoparasite able to protect pea from PRRC pathogens when applied as a biocontrol agent together with the seed (Xue, 2003a). Previously, we showed the fungus to be present in diseased pea roots using ITS-amplicon sequencing (Wille *et al.*, 2020). In the present study, *C. rosea* quantities in diseased roots were positively correlated with the most prominent pathogens: i.e. with *F. solani* and *F. oxysporum* in all three sick soils and additionally with *A. euteiches* and *F. redolens* in P and N soils, respectively. Further, it was positively related to disease variables in the P and N soils. There has been only one documented report on *C. rosea* as a pathogen on faba bean (Afshari & Hemmati, 2017). In biocontrol experiments, the strain AC941 is usually employed (Xue, 2003a; Gimeno *et al.*, 2019); and we do not know if the mycoparasitic lifestyle of this strain is extendable to the whole species or what factors determine the transition from commensalism to parasitism. *C. rosea* might thrive on other fungi present in and around the roots, thus co-occuring with them, or co-infect the roots with no direct effect on the pathogenic fungi or the plant.

Rank correlation analysis showed that AMF were overall negatively correlated with the abundance of those pathogen species which have a negative impact on $SDW_{Rel.}$ (Figure 4.8). AMF are well-known plant symbionts with antagonistic effects against plant pathogens (Azcón-Aguilar & Barea, 1996; Slezack *et al.*, 2000). Thygesen *et al.* (2004) co-inoculated pea seeds with *A. euteiches* oospores and two AMF species

and showed an AMF-dependent protection as early as 26 days after sowing. With our data, we can confirm that AMF can be readily detected inside roots at the seedling stage, 29 days after sowing. We also show that disease is negatively correlated with AMF quantities detected in the roots, and that the strongest explanatory effect on $SDW_{Rel.}$ was attributed to AMF in all three sick soils, confirming the field study by Xu *et al.* (2012) which showed that the health status of pea is largely reflected by the abundance of AMF in the roots. However, we cannot exclude that AMF are developing better in heathy plants or prefer healthy plants for colonisation and therefore might not be a causal reason for plant protection.

The distinct patterns of co-occurrence of ten microorganisms in the diseased roots among the soils are in line with the PERMANOVA results and corroborate the significant soil effect. These patterns, together with the observation that distinct taxa correlated negatively or positively with disease in the different soils, further emphasises the complexity of the root rot disease. We have to assume that the different microbes interact in various ways, ranging from synergism and facilitation among two or more species to commensalism and antagonism, in addition showing a temporal dynamic. For instance, *P. ultimum* and *R. solani* might infect the seedling first, weakening the plant and facilitating further infection. Except for the seminal work by Kerr (1963), which showed that *P. ultimum* eases the way for *F. oxysporum* infection, studies on the roles and the interactions between damping-off pathogens and pathogens that infect peas at later stages of the development are largely lacking (Lamichhane & Venturi, 2015). It has also been shown that the conjoint infection of root rot pathogens can break down resistance against individual pathogens (Shehata *et al.*, 1983). Willsey *et al.* (2018) inoculated pea seeds at planting with different combinations of *A. euteiches, F. solani, F. avenaceum* and *F. redolens* and used qPCR to monitor pathogen concentration in diseased roots. They showed that *A. euteiches* is the most severe pathogen alone or in combination with others and evidenced a synergistic interaction between *A. euteiches* and *F. solani* aggravating disease. *A. euteiches* was also able to facilitate root colonization by Fusaria. The high abundance of *A. euteiches* in roots from the highly infested P soil together with its co-occurence with *Fusarium* species, confirms their findings.

It has been shown that colonisation by non-pathogenic *F. equiseti* efficiently alleviate pea root rot caused by *F. avenaceum* or *D. pinodella* (Šišić *et al.*, 2017). Because *F. equiseti* readily colonised the root tissue it can be assumed that some sort of competition among the fungal species (e.g. niche exclusion) prevents the pathogen to infect the host and cause disease. Except for the clear negative correlations of AMF with several pathogens we do not have clear signs of antagonism in our study. Only in P soil, *R. solani* and *P. ultimum* are negatively correlated. Because both pathogens are related to seedling disease, competition for the colonisation of the young root might be at work between the two species.

Despite the presence of nearly all pathogens in diseased roots from the sick soils, their respective role and importance in disease development is likely to be different. Multiple regression showed that generally only small portions of the variance in the disease variable could be explained by pathogenic taxa. Furthermore, pathogens that significantly correlated with the disease expression were barely retained in the final model. We interpret this as an indication that the PRRC related disease phenotype cannot be reduced on to single microbial species. Pathogen isolation from diseased plant tissues and re-inoculation have been used to study multipartite pathogen systems in the past. Molecular based detection and quantification should be used to elucidate the role of individual taxa in the disease complex. For example, future experiments could quantify members of synthetically composed pathogen complexes at different time points of the plant development. In this regard, it would be particularly insightful to relate the starting inoculum with microbial quantities in the roots at later time points. By varying relative proportions of the microbes in the synthetic complex we could further improve the understanding of the pathogen complex and depict the roles of the different microorganisms.

Our study identified significant effects of the host genotype on the microbial composition in the roots, confirming precedent studies on this subject (Marschner *et al.*, 2001; Liu *et al.*, 2019). The factor genotype explained 14% of the variance in the microbial composition (over all four soils). This estimate of the importance of the host genotype in determining the microbial composition in the roots and the rhizosphere is in the range of previously published values for barley cultivars and wild relatives

(Bulgarelli *et al.*, 2015), sunflower cultivars (Leff *et al.*, 2017) or inbreed *Arabidopsis thaliana* accessions (Lundberg *et al.*, 2012).

Susceptible and resistant pea lines had different microbial compositions overall, with generally lower pathogen and higher AMF amounts in the roots of resistant lines. In this regard, especially the old variety S91 and the landrace S134 stand out; with particularly low total levels of pathogens in the diseased roots. Our results are in accordance with repeatedly documented impact of the host genotype on the soil and root-associated microbial community and its relation to disease resistance (Ding *et al.*, 2014; Yu *et al.*, 2016; Mendes *et al.*, 2018a; Mendes *et al.*, 2018b). It shows that information on the plant-associated microbiome in general, and monitoring microbial key players in the PRRC in particular, has the potential to be effectively included in resistance breeding. For instance, despite similar growth performance on the sick soils, the pathogen compositions between the resistant genotypes was apparently different (e.g. C1 grew as good as other resistant genotypes, but showed higher pathogen loads in the roots). Linking the pathogen composition with growth performance at latter stages of the development (and finally to yield), offers an additional selection criterion and might be an instrument to improve the prediction of the performance of the genotype in the field.

The link between certain key microbial taxa with plant resistance could provide plant breeding with microbial markers. Our study indicates that AMF play a role in resistance/tolerance of pea against root rot pathogens and it is particularly interesting that AMF showed significantly positive and negative correlations with growth and disease parameters, respectively, in all three sick soils. This suggest to follow-up on applications of AMF as biocontrol agents or to specifically breed crops for enhanced interactions with mycorrhiza (Hohmann & Messmer, 2017). In this regard, AMF could be a microbial marker for disease resistance and quantifying AMF in the roots already in the breeding nursery could be used to predict resistance of breeding lines and be used as a selection tool. Various examples of microbe-mediated disease resistance are known, therefore breeding for enhanced interaction with plant beneficial microbes has been proposed beyond plant-AMF interactions (Smith & Goodman, 1999; Bakker *et al.*, 2012; Wille *et al.*, 2019).

In conclusion, our results demonstrate that the composition of key microbial taxa of the root rot complex in diseased pea roots are determined by conjoint effects of the soil and plant genotype. In agriculture, both factors can be harnessed to enhance plant health, through specific management practises combined with resistant cultivars. Our data allowed to differentiate between microbial complexes of different pea genotypes with distinct levels of resistance. We show here, that molecular analysis of the root-associated microbiota can give further insights into these complexes and allow to identify microbial markers for plant resistance. More comprehensive research is needed to understand the factors that steer the recruitment of plant beneficial microbes and the dynamic interactions among root-associated microbes and the plant. It could prove particularly useful to follow the development of key microbes at several time points during the infection of different plant genotypes in distinct soil types. Moreover, the application of synthetic communities of multiple pathogenic and beneficial microbes could be a tool to better understand these complex interactions (Niu *et al.*, 2017). Addressing the complexity of plant resistance by entangling host plant and microbiome effects as suggested by Oyserman *et al.* (2018) will contribute to resistance breeding in the future.

5 | GENERAL DISCUSSION

Pea is the most important cool season grain legume offering locally produced plant-based proteins for food and feed in the temperate regions. The cultivation of pea and other grain legumes remains below expectations despite the dietary, agronomical and ecological benefits they present. Two major reasons are responsible for this situation in Europe: First, a combination of economic and political decisions hinders grain legumes to develop their potential (e.g. the inability to transfer beneficial external effects of legume cropping into economic benefits or indirect subsidies to competing crops; Zander *et al.*, 2016). Second, biotic (e.g. fungal diseases or parasitic weeds; Rubiales *et al.*, 2015) and abiotic stresses (e.g. cold or drought; Araújo *et al.*, 2015) cause important constraints on pea production. One of the main biotic stresses is the accumulation of phytopathogenic fungi in the soil causing severe root rots (Hagedorn, 1985), and resistance breeding was highlighted as one of the most promising approaches to face this challenge (Infantino *et al.*, 2006).

5.1 FIELD SOIL-BASED RESISTANCE SCREENING

The present thesis has assessed root rot resistance of pea in naturally infested field soil with the basic idea to assess resistance of various pea genotypes against the complex of pathogens present in the soil. As pointed out in *Chapter 1* and further developed in *Chapter 2*, root rot diseases are mainly the result of a conjoint action of several pathogens; hence the term *pea root rot complex* (PRRC) has established in the scientific community. Major achievements in resistance breeding against important diseases have been made in the last decades. Well-established controlled conditions screening protocols in combination with mapping or association studies have allowed to identify resistant germplasm and molecular markers linked with disease resistance against individual pathogens (Infantino *et al.*, 2006; Rubiales *et al.*, 2015). It can be speculated that the advent of sequencing technologies allowing the

generation of numerous genetic markers and the ambition to identify resistance loci have contributed to shifting research efforts away from the field towards reductionistic controlled conditions resistance screenings, namely with inoculations of single strains of selected pathogens (Nelson *et al.*, 2018). Despite the importance of these studies (see Coyne *et al.* (2019a), Li *et al.* (2016) or Barilli *et al.* (2016) for recent examples), the situation in the field is different with multiple pea root rot pathogens present in the same field (Chatterton *et al.*, 2018).

The initial task of this thesis was to implement a high-throughput resistance screening under controlled conditions and to test the reproducibility of a field soil-based screening assay. The approach of using field soil for controlled conditions experiments has also been proposed by Poorter *et al.* (2016) in order to close the gap between controlled environments and the field. The screening assay of this study can serve as (i) a basis for further studies on resistance and plant-microbe interactions and (ii) a model system for the development of a selection tool for breeding stations. Three hundred and twelve pea cultivars and breeding lines, provided by the breeding company *Getreidezüchtung Peter Kunz*, Switzerland and gene bank accessions from the *Germplasm Resources Information Network*, ARS-USDA, USA, including old cultivars, landraces and further collected material, have been screened under controlled conditions. Subsequently, a subset of genotypes with contrasting resistance levels were tested in the field (*Chapter 3*).

Preliminary testing confirmed the high root rot potential of the field soil and revealed the importance of a consistent watering regime that allows for the differentiation of resistance levels between pea lines. The experiment was divided into four replicates separated by time, due to practical feasibility. Replicates were further divided in five sowing/harvest blocks that allowed to harvest and assess one replicate per week. With this experimental design we solved the problem of limited space available in the growth chamber. Because the experimental blocks and sub-blocks were separated in time, more time was available for the sampling and measurements. For instance, in addition to 20 phenotypic variables assessed on each plant (roughly 400 individual plants per day) we also collected 132 root and rhizosphere soil samples per day. Therefore, growing experimental blocks sequentially allows for a

thorough sampling of phenotypic and microbiome data and is worth to consider for controlled conditions experiments.

The visual symptoms on the root and root-stem transition could not be scored according to previously established RRIs (Grünwald *et al.*, 2003; Moussart *et al.*, 2008; Pflughöft, 2008). Observed root rot was uniform overall and did not differentiate substantially between pea genotypes. The establishment of an appropriate root rot index (RRI) and its application was therefore a major challenge. The ideal resistance screen would allow to rapidly and easily assign an RRI to diseased plants and to clearly differentiate between resistance levels. It remains to be tested if this differentiation can be improved by adjusting the disease pressure of the substrate
(e.g. by adding sterile substrate, adapted watering or pot size). Furthermore, an RRI relevant for plant breeding must be related to plant growth and ultimately to yield. The RRI we defined and employed showed a significant negative correlation with relative shoot dry weight ($SDW_{Rel.}$), indicating its potential value for the assessment of disease resistance. For further experiments, we selected the pea genotypes mainly based on $SDW_{Rel.}$. This trait differentiated well between the tested genotypes, with several genotypes showing no growth reduction on the infested soil after 21 days.

A subset of pea genotypes was tested in the field from where the infested field soil was collected. The pea genotypes were selected based on the emergence, $SDW_{Rel.}$ and RRI in the resistance screen: Based on $SDW_{Rel.}$ and RRI either resistant or susceptible genotypes were selected and only pea genotypes with a high emergence rate on the diseased soil were included in the field experiment. At the beginning of the field season, two factors challenged the experiment. First, soon after emergence, plantlets were attacked by the pea leaf weevil (*Sitona lineatus*). An application of kaolin prevented further damage. Second, late spring/early summer was very dry in 2018, presenting favourable conditions for a healthy pea growth but not for the development of root rot. Despite this drawback, resistance ranking based on RRI in the field and under controlled conditions correlated significantly indicating the usefulness of the screening system.

The comparison of disease expression under controlled conditions and in the field showed that the established screening system could serve as a

valuable instrument to rapidly screen a high number of lines at an early stage of the development. In our experiment, the field root rot assessment was conducted 55 days after sowing (plant development stage BBCH 60 – 65; flowering) in comparison to the assessment in the juvenile stage (BBCH ~39; stem elongation) in the controlled conditions. This indicates that the controlled conditions resistance screen can indicate resistance that translates into resistance later on. However, we cannot make any conclusive statements on how the resistance traits ($SDW_{Rel.}$ and RRI) from our experiments would finally translate into yield. Conner *et al.* (2013) showed that plant biomass was a better predictor than RRI for final yield in a four years field trial investigating Aphanomyces root rot resistance. Clearly, we have to note that plants that emerge well and show vigorous growth in the first weeks of the development have a better potential to produce higher yields. Further experiments and field trials should therefore aim at validating the use of disease indices or biomass measurements (such as $SDW_{Rel.}$) in regard to yield. Because the development, and consequently the time of harvest, of modern varieties and genetic resources (e.g. old landraces) can vary substantially this would call for an extra labour input in the field when working with a diverse panel of pea genotypes. This, once more, shows how a selection tool would benefit from simple traits that can be assessed early in the development.

Experiments on root rot resistance flanked by investigations on the microbiome could provide such traits: If we manage to identify microbial key players in the root rot pathobiome by unambiguously relating those taxa to disease resistance or, preferably, stable yield then we would have a valuable instrument for plant breeding at hand. For instance, as evidenced in *Chapter 4*, arbuscular mycorrhizal fungi (AMF) colonisation in diseased roots are a good approximation for reduced RRI and high $SDW_{Rel.}$. If we can confirm that high AMF colonisation at early stages in the development of pea is linked to plant health, then quantifying AMF by qPCR in the roots of young plants would be a cost effective and swift solution for a robust evaluation of numerous breeding lines. It is noteworthy here, that AMF quantities were significantly related to biomass and disease over three geographically and microbiotically different sick soils.

Preliminary tests were run in order to implement the screening system at the breeding station of *Getreidezüchtung Peter Kunz* (GZPK). First efforts of the company staff to establish sick plots in 1 m^3 boxes by repeated culturing of peas or by mixing sick soil into the soil failed (Simon Dörr, personal communication). Currently, further experiments are running; but it clearly shows that the transfer of the system from the growth chamber to the breeding station can be challenging, with the control of the moisture level in the pots and watering as major challenges.

The established screening system provided valuable phenotypes that could be used in a genome-wide association study (GWAS). GWAS has recently been performed to identify pea root rot resistance loci and root architectural traits (Desgroux *et al.*, 2016; Desgroux *et al.*, 2018) or agronomic traits (Gali *et al.*, 2019). Whereas the first study used SSR markers and SNP markers from the GenoPea 13.2K array (Tayeh *et al.*, 2015a), the second study used genotyping-by-sequencing (GBS; double enzymatic digest using *Pst*I and *Msp*I) to generate the underlying SNP markers. The 197 USDA accessions evaluated in the present study have already been genotyped using the GenoPea array (Tayeh *et al.*, 2015a). However, we decided to apply GBS to all 312 studied genotypes. So far, we did not fully manage to genotype all accessions due to technical issues during library preparation and sequencing. Future work will finalise this activity. It will be revealing to compare detected loci significantly associated with disease resistance assessed on infested field soil with resistance loci from mapping or association studies on resistance against individual pathogens. Thereby, it is a hypothesis that a resistance locus detected on naturally infested field soil could be linked to a process between the plant and the associated microbiome (i.e. microbiota-mediated disease resistance) rather than being solely a process of plant innate defence mechanisms – this hypothesis is supported by recent findings in the field of plant-pathogen interactions (Berendsen *et al.*, 2018; Vannier *et al.*, 2019).

Future lines of research should aim at further elucidating the role the plant genotype plays in determining the root microbial composition and plant-microbe feedbacks. With a GWAS based on the microbiome sequencing data as the explanatory variables, plant loci responsible for the recruitment of beneficial taxa or the suppression of pathogenic key players could be identified. In addition, a 'holobiont-GWAS' where

disease resistance is simultaneously mapped on to the plant marker and the microbial taxa dataset could further contribute to disentangle the respective role of the plant host and the microbiome in conferring disease resistance. Clearly, only with thorough field experiments it would be possible to assess, if the integration of microbiome-information can advance the resistance breeding process.

5.2 ROOT ROT RESISTANCE ON DIFFERENT SOILS AND ASSESSMENT OF KEY MICROBIAL TAXA

The initial results on root rot resistance were validated in a second controlled conditions experiment that has served to (i) verify resistance levels on further soils and (ii) to assess the composition of key microbial taxa of the root rot pathobiome by quantitative real-time PCR (qPCR; *Chapter 4*). Four soils with different pathogen infestation levels were chosen: First, 'Kirchlindach' employed in the initial screen; second, 'Neu-Eichenberg' and third 'Puch' based on the personal communications with partner research groups, notably with Adnan Šišić (Department of Ecological Plant Protection, University of Kassel) and Andrea Winterling (The Bavarian State Research Center for Agriculture, Freising) – these soils also had confirmed root rot potential; and fourth, the healthy soil 'Feldbach' from the GZPK breeding station.

This second experiment revealed that resistance evaluated on one soil is transferable to other sick soils, highlighting the potential of the field soil-based resistance screening. As already stated in *Chapter 4*, however, it needs to be determined to what extent, in terms of edaphic and microbial factors, this transferability persists. The three sick soils were all (clay)-(silt)-loam soils from central Europe and under organic management regimes. It would be highly interesting to assess growth of the assessed genotypes on infested soils from the Canadian prairies or the U.S. Great Plains in order to compare resistance levels and the root-associated microbiota.

The second goal of this experiment was to compare resistance levels with the composition of key microbial taxa in the diseased roots. Ten taxa were selected based on the ITS-amplicon sequencing data from *Chapter 3* and availability of established qPCR protocols. The sequencing of the

fungal microbiome from diseased roots was performed on two pea genotypes grown under controlled conditions in a preliminary experiment. The sequencing data of the two genotypes were pooled for analysis and gave a first blueprint of the fungal pathobiome of our system. Several sequences could be attributed to known pea pathogens (e.g. *Fusarium* spp., *Didymellaceae*, *R. solani*), others to known plant beneficial taxa (e.g. *C. roseae* and AMF). This preliminary experiment was an important asset in the planning of the subsequent qPCR approach.

Sequencing of the microbial community, mostly either 16S (bacteria) or ITS (fungi), has been the state of the art and a common approach in microbiome analysis during the last ten years. Short read sequencing (e.g. Illumina MiSeq, as employed in *Chapter 3*), however, still lack the sensitivity that allows to attribute sequences to the species level. Long-read sequencing (e.g. with Oxford Nanopore or PacBio sequencing platforms) will set this restriction aside and allow for a reliable distinction also between closely related microbial strains (D'Andreano *et al.*, 2020). We choose an approach via qPCR to specifically target and quantify known pea root rot pathogens (fungi and oomycetes) and putative beneficials in addition to classic amplicon sequencing.

Established protocols for qPCR assays can be simply implemented, without much additional effort. Despite the availability of various qPCR assays for major pea root rot pathogens, these methods are only rarely adopted: For instance the 2014 published qPCR assay for *A. euteiches* has only twelve citations according to a *Web of Science* query[8]; none of these publications, however, use the assay for the detection of the pathogen in the field (Gangneux *et al.*, 2014). Recently, a large qPCR-based survey in Saskatchewan, Canada on *A. euteiches* confirmed its wide distribution (Karppinen *et al.*, 2020). Despite its acknowledged importance at least in France (Gaulin *et al.*, 2007), the Netherlands (Oyarzun & van Loon, 1989) Spain and Sweden (Levenfors, 2003) and its detection in various other European countries (CABI, 2020), there are no reports from Germany on *A. euteiches*, despite recent pathogen surveys (Pflughöft, 2008). In the present study we show its presence in two German pea fields in Bavaria and Hesse. This result calls for more specific surveys (i.e. including improved and specific culture-based isolation and molecular

[8] *https://apps.webofknowledge.com*, accessed on 26 March 2020

detection) if plant breeding and disease management want to incorporate this important pea root rot pathogen in their strategies.

Two major findings resulted from our Chapter 4 experiment. First, almost all eight pathogens were detected in diseased roots growing in all three sick soils, with a clear dominance of *F. solani* and *A. euteiches*. Second, resistant and susceptible pea genotypes had different compositions of the ten microbial taxa, with more *F. solani* and *A. euteiches* and less AMF in the roots of the susceptible genotypes. Because we did not quantify the microbials in the bulk soil prior to the experiment, we cannot make any statements on the inoculum potential of the soil and how this relates to the pathogen amounts in the roots. A simple conclusion would be to infer a high inoculum potential of a given pathogen where the pathogen is found at high levels in the roots (e.g. *A. euteiches* in 'Puch' soil). However, we hypothesize that the amount of a given pathogen in the roots is not only a function of its starting inoculum in the soil but rather determined by interactions with the plant and the whole microbial community in the rhizosphere. This hypothesis has been illustrated in a recent experiment by Wei *et al.* (2019). They showed that the health status of tomato plants grown in infested field soil depends on few non-pathogenic bacterial taxa present in the soil at the start of the experiment. Notably, few rare taxa determined if a plant becomes highly diseased or not. Similar phenomena are described in the context of 'suppressive soils' (Thuerig *et al.*, 2009). Having more such data at hand, where the disease can be linked to individual key microorganisms, plant breeders could specifically select plant genotypes that enrich beneficial key players in the rhizosphere to suppress disease.

ITS-amplicon sequencing is currently being done on the DNA samples from the experiment in *Chapter 4* in order to get a broader picture of the fungal community in the roots and to assess differences among soils and pea genotypes. Future experiments of this kind should include sampling and analysis of the microbial community in different compartments (bulk soil, rhizosphere soil and root) and at different time points (e.g. before planting, during germination and emergence and so forth). This would allow to describe the relationship among root rot pathogens in more detail and to what extend soil inoculum and the diversity within the pathobiome determine, together with the host genotype, disease severity. For instance, causally establishing the link between the inoculum concentration of a

microorganism *A* at time point t_0 (e.g. DNA amount in the soil before planting) and microorganism *B* demonstrably being responsible for disease at timepoint t_1 (e.g. *F. solani* DNA concentration in the roots 30 days after planting) would allow us to edge a tool for early plant selection and plant protection management; i.e. in analogy to the concept of 'indicator species'. However, as reasoned throughout this thesis, it probably will not be as simple as that, because microbes are embedded in the edaphic and microbial complexity of the soil and interact with the plant genotype. Consequently, we will have to identify indicator hubs, consisting of several microbial taxa or, even more complex, microbial taxa together with key abiotic factors and plant traits/genetic markers.

5.3 THE PLANT-ASSOCIATED MICROBIOME AND PLANT BREEDING

Chapter 2 elaborated on the importance the plant-associated microbiome has on plant health and experimental findings showing that the plant genotype has a significant influence on the microbial composition. Several examples have been cited that illustrate the impact plant domestication has on the plant-associated microbiome (Wissuwa *et al.*, 2009; Bulgarelli *et al.*, 2015; Leff *et al.*, 2017; Mendes *et al.*, 2018a). In that chapter, we developed the idea of considering the microbiome as an integral part of the environment and as an important element interacting with the host genotype; this concept has recently been formulated more explicitly and also demonstrated experimentally (Oyserman *et al.*, 2018; Oyserman *et al.*, 2019). Oyserman *et al.* (2019) use the term *microbiome-associated phenotypes* or *MAPs* to describe the phenotype emerging from the interaction between the plant genotype (G), the environment (E) and the microbiome (M). They showed in a simple three-factorial experiment that variance in root length of tomato is significantly explained by M and all interacting variables (GM, EM, GEM). Because the microbiome is a symbiotic, dynamic and evolving

part of the plant's environment, in contrast to the abiotic environment[9], this is an appropriate update of the basic G × E model.

The deepened insights into plant-microbe interactions have the potential to provide breeders and agronomists with instruments to make well-informed choices. Plant resistance breeding could use molecular methods to describe and quantify pathogen complexes in the soil (or in roots that have grown in there) in order to choose appropriate selection environments, as it is done for abiotic environmental factors (Schlaeppi & Bulgarelli, 2015). Likewise, initial breeding efforts and success could be verified in new environments: E.g. to test if a certain resistant plant genotype is still resistant when evaluated at a new site with different edaphic factors but comparable microbiome composition, or vice versa. Data from multiple sites could furthermore improve the prediction on the expected resistance level of a breeding line in a new environment.

Most plant-microbe interactions are too complex and too vaguely understood to serve as traits that the breeder uses for direct selection. Instead, the breeder will rely on surrogate traits. The traditional breeder selects plants by assessing traits he is ultimately interested in, such as grain yield, grain protein content or root rot resistance in the case of pea, not explicitly but rather indirectly considering the contribution of the microbiome. This individual trait selection has always been an integral part of plant breeding. In addition, breeders can consider 'ideotype breeding', where several traits are combined into one breeding objective, or a multidimensional breeding target (Donald, 1968; Duc *et al.*, 2015). A healthy root-associated microbiome might be one part of such a breeding target, and it is the breeder's task to define how this trait can be assessed (e.g. by identifying microbial key taxa or hubs) in order to serve as a selection criterion. In depth microbiome datasets recorded in distinct environments and linked to a certain plant phenotype (e.g. resistance) could allow to define key taxa in the microbiome that ultimately serve as markers for a given breeding target (Gilbert *et al.*, 2016; Chang *et al.*, 2017). Following the experimental results from *Chapter 4*, AMF and

[9] This is a simplification: The abiotic environment is evolving too, possibly even at short time intervals. Furthermore, plants influence and change their environment (e.g. by root exudation or by providing plant residues), however, this can only barely be compared with the living nature of the interaction between microbes and plants.

possibly *F. oxysporum*, *F. solani* and *R. solani* are potential marker candidates for resistance breeding in central Europe. In practise, a future pea breeding ideotype for central Europe could therefore be a combination of apparent agronomic traits (e.g. lodging resistance and yield) together with low DNA amounts of the microbial markers. The selection could be performed in parallel: The agronomic traits would be assessed in a healthy field over the whole growth period and the molecular assessment of the pathogens could be run in a sick plot or in the greenhouse. Because, once established, qPCR diagnostics is relatively simple, this strategy could also be adopted by small breeding companies in collaboration with research institutions.

5.4 FURTHER CONSIDERATIONS ON MANAGING ROOT ROT

Future disease management strategies of pea root rot should adopt a holistic view, because the integration of experimental evidence and technical know-how from different scientific fields will most likely strengthen plant protection and breeding, keeping it economically and environmentally sustainable in the long term. This holistic approach includes breeding for resistance against pathogen complexes as well as planning of suitable crop rotations. A careful selection of succeeding crops and a diversification of the crop rotation sequence is a strategy to manage a healthy rhizosphere microbial community. Niu *et al.* (2018) assessed the pea yield and the rhizosphere fungal community at the end of different four-years cropping systems, including pea, wheat, canola and lentil in Saskatchewan, Canada, one of the most important pea producing region in the world. They showed that the rotation with consecutive pulses (i.e. wheat – pea – lentil – pea) increased the proportion of pathogenic fungi in the rhizosphere soil. Pea grain yield at the end of this rotation was significantly lower than in rotations of wheat – canola – oat – pea or wheat – lentil – oat – pea (approx. 500 and 1000 kg ha^{-1}, respectively). Moussart *et al.* (2013) have shown that with the choice of the right legume species in successive planting the inoculum potential of a soil infested by the oomycete *A. euteiches* can be significantly modified: Whereas pea and lentil led to a build-up of the pathogen, faba bean and lupin reduced the inoculum potential. For faba

bean, however, this only held true when a resistant cultivar was planted. Aside of this PhD project, I planted lupin (cv. 'Feodora') on the infested field soil from Kirchlindach (*Chapter 3* & *4*) in the greenhouse and did not observe striking growth reduction or disease development in contrast to pea. This confirms findings by Moussart et al. (2013) that lupin is not susceptible to the same pathogen complex present in the soil and offers an alternative legume species for this field site.

Regarding an increased cultivation of grain legumes for food and feed, it is therefore advisable to include different legumes in well-planned crop rotation sequences. As proposed in precedent sections, microbiome surveys can assist the process of crop rotations planning and help in making appropriate decisions regarding crop species for a given field site. In addition to interactions within in the pathobiome, the importance of microbial species is linked to abiotic factors too. For instance, it was shown that soil moisture and total carbon and nitrogen content of the soil positively correlated with the qPCR-determined abundance of *A. euteiches* in the soil (Karppinen *et al.*, 2020). Besides underlining the importance of the earlier discussed E × M interaction, the latter study also illustrates how qPCR quantification of root rot pathogens could be used in combination with information on management practices and soil factors in order to select suitable field sites and plan crop rotations.

5.5 CONCLUSION

Pea will become an important food in the future, either consumed as a fresh vegetable, as dry pea or processed as a protein rich super-food. Pea also continues to play a role in locally produced feed contributing to a more sustainable livestock production. Resistance to soil-borne root rot pathogens is an important breeding goal to secure yield, and to eventually promote this crop in agricultural systems.

This thesis built on the observation that intensive pea cropping leads to a microbial dysbiosis in the rhizosphere, eventually leading to important crop losses, and it has shown how complex root rot diseases in pea are. It depicted the role of the rhizosphere microbiome for plant health and delineated the potential plant-microbe interactions have in pea breeding. At the core of the thesis was the implementation of an infested

field soil-based resistance screening. This tool allowed to reproducibly assess resistance of pea against a root rot pathogen complex. Resistance was verified in the field and on further infested soils. These validations, together with the ease of applicability, makes the controlled conditions screening a practical tool for future resistance breeding. The successful implementation of several qPCR assays targeting pea pathogens and beneficial microbes confirmed the utility of this molecular technique: I see an untapped potential of current molecular techniques for resistance breeding, the management of crop rotations and the use of the appropriate legume crop at a given site. However, further research is needed to understand the interaction between plants and pathogen complexes and to make this knowledge available and applicable for plant breeding and cultivation. In our experiment, the quantification of key microbial players in the root rot pathobiome confirmed the hypothesis that resistant pea genotypes have a different microbial composition in diseased roots than susceptible genotypes. Moreover, the study allowed to point out several key microbial taxa related to disease. These findings contribute to the field of plant pathology and emphasise the importance of integrated breeding approaches.

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APENDIX

A I SUPPLEMENTARY MATERIAL

Supplementary Table 1. Pea genotypes evaluate for root rot resistance. Leaf type: *'full'* = full leaf type; *'semi-l.'* = semi-leafless type.

ID	Acc./cv./Line ID	Origin of seed supply/Breeder	Leaf type
C1	EFB.33	Sativa Rheinau (CH)	full
C2	Respect	Agri Obtentions (FR)	semi-l.
G1	De 8 10106	GZPK (CH)	semi-l.
G2	De 8 10108	GZPK (CH)	semi-l.
G3	De 8 10111	GZPK (CH)	semi-l.
G4	De 8 10113	GZPK (CH)	semi-l.
G5	De 8 10206	GZPK (CH)	semi-l.
G6	De 8 10207	GZPK (CH)	semi-l.
G7	De 8 10215	GZPK (CH)	semi-l.
G8	De 8 10221	GZPK (CH)	semi-l.
G9	De 8 10222	GZPK (CH)	semi-l.
G10	Tarchalska	Danko Plant Breeding. (PL)	semi-l.
G11	De 9 15834	GZPK (CH)	semi-l.
G12	De 9 15841	GZPK (CH)	semi-l.
G13	De 9 15843	GZPK (CH)	semi-l.
G14	De 9 15850	GZPK (CH)	semi-l.
G15	De 9 15851	GZPK (CH)	semi-l.
G16	De 9 15852	GZPK (CH)	semi-l.
G17	De 9 15853	GZPK (CH)	semi-l.
G18	De 9 15859	GZPK (CH)	semi-l.
G19	De 9 15862	GZPK (CH)	semi-l.
G20	Gracliv.27	GZPK (CH)	semi-l.
G21	Lancom10_1_4	GZPK (CH)	semi-l.
G22	Maskle10_2_5	GZPK (CH)	semi-l.
G23	Conpro10_3_7	GZPK (CH)	full
G24	Aucnet 11_2_1	GZPK (CH)	semi-l.
G25	De 7 10401	GZPK (CH)	semi-l.
G26	De7 10407	GZPK (CH)	semi-l.
G27	De7 10414	GZPK (CH)	semi-l.

G28	De7 10421	GZPK (CH)	semi-l.
G29	De7 10431	GZPK (CH)	full
G30	De 7 10437	GZPK (CH)	semi-l.
G31	De 7 10450	GZPK (CH)	semi-l.
G32	De 7 10451	GZPK (CH)	semi-l.
G33	De7 10455	GZPK (CH)	semi-l.
G34	De7 10468	GZPK (CH)	semi-l.
G35	De7 10469	GZPK (CH)	semi-l.
G36	De7 10474	GZPK (CH)	semi-l.
G37	De7 10476	GZPK (CH)	semi-l.
G38	De7 10478	GZPK (CH)	semi-l.
G39	De7 10481	GZPK (CH)	semi-l.
G40	De7 10489	GZPK (CH)	semi-l.
G41	De7 10499	GZPK (CH)	semi-l.
G42	De7 10502	GZPK (CH)	semi-l.
G43	De7 1058	GZPK (CH)	semi-l.
G44	De7 10516	GZPK (CH)	semi-l.
G45	De7 10522	GZPK (CH)	semi-l.
G46	De7 10525	GZPK (CH)	semi-l.
G48	De7 10539	GZPK (CH)	semi-l.
G49	De7 10560	GZPK (CH)	semi-l.
G70	Grana	Nordsaat Saatzuchtgesellschaft M.B.H. (DE)	full
G71	Radley	National Centre for Plant Genetic Resources: Polish Genebank (PL)	semi-l.
G72	Terno	Selgen (CZ)	semi-l.
G73	Esso	Selgen (CZ)	semi-l.
G74	Kleopatra	Saatzucht Aschersleben (DE)	semi-l.
G75	Rocket	Donation Dr. Hans Rolf Späth	semi-l.
G77	Phönix	Donation Dr. Hans Rolf Späth	semi-l.
G78	Roch	Poznan Plant Breeding (PL)	full
G79	Hubal	Danko Plant Breeding. (PL)	full
G80	Pomorska	Poznan Plant Breeding (PL)	semi-l.
G81	Turnia	Poznan Plant Breeding (PL)	semi-l.

G82	Protecta	Selgen (CZ)	full
G83	Santana	KWS (DE)	semi-l.
G84	Proteal	Laboulet Semences (FR)	semi-l.
G85	Standal	Laboulet Semences (FR)	semi-l.
G86	Tip	Saatbau Linz (AT)	semi-l.
G87	Velvet	Selgen (CZ)	semi-l.
G88	Verbal	Laboulet Semences (FR)	semi-l.
G89	Volt	NPZ (DE)	semi-l.
G90	Mascara	KWS (DE)	semi-l.
G91	Akord	Poznan Plant Breeding(PL)	semi-l.
G92	Album	Laboulet Semences (FR)	semi-l.
G93	Alvesta	KWS (DE)	semi-l.
G94	Arvena	Danko Plant Breeding. (PL)	semi-l.
G95	Batuta	Danko Plant Breeding. (PL)	semi-l.
G96	Brylant	Danko Plant Breeding. (PL)	semi-l.
G98	Gambit	Selgen (CZ)	semi-l.
G99	Klif	Danko Plant Breeding. (PL)	full
G100	Natura	Selgen (CZ)	full
S2	PI103058	ARS GRIN (US)	full
S3	PI116056	ARS GRIN (US)	full
S4	PI116844	ARS GRIN (US)	full
S5	PI117264	ARS GRIN (US)	full
S6	PI117998	ARS GRIN (US)	full
S7	PI118501	ARS GRIN (US)	full
S8	PI121352	ARS GRIN (US)	full
S9	PI124478	ARS GRIN (US)	full
S10	PI125840	ARS GRIN (US)	full
S12	PI137119	ARS GRIN (US)	full
S13	PI140298	ARS GRIN (US)	full
S15	PI143485	ARS GRIN (US)	full
S16	PI156647	ARS GRIN (US)	full
S17	PI156720	ARS GRIN (US)	full
S18	PI162909	ARS GRIN (US)	full
S19	PI163126	ARS GRIN (US)	full
S20	PI163129	ARS GRIN (US)	full

S21	PI164548	ARS GRIN (US)	full
S22	PI164612	ARS GRIN (US)	full
S23	PI164971	ARS GRIN (US)	full
S25	PI169608	ARS GRIN (US)	full
S27	PI173840	ARS GRIN (US)	full
S28	PI179451	ARS GRIN (US)	full
S29	PI179459	ARS GRIN (US)	full
S30	PI179722	ARS GRIN (US)	full
S31	PI180329	ARS GRIN (US)	full
S33	PI180699	ARS GRIN (US)	full
S34	PI181799	ARS GRIN (US)	full
S35	PI181801	ARS GRIN (US)	full
S36	PI181958	ARS GRIN (US)	full
S37	PI184130	ARS GRIN (US)	full
S38	PI184784	ARS GRIN (US)	full
S39	PI193578	ARS GRIN (US)	full
S40	PI193584	ARS GRIN (US)	full
S41	PI193590	ARS GRIN (US)	full
S42	PI195020	ARS GRIN (US)	full
S43	PI195404	ARS GRIN (US)	full
S44	PI195631	ARS GRIN (US)	full
S45	PI197044	ARS GRIN (US)	full
S46	PI197990	ARS GRIN (US)	full
S47	PI198072	ARS GRIN (US)	full
S48	PI198074	ARS GRIN (US)	full
S49	PI198735	ARS GRIN (US)	full
S50	PI203067	ARS GRIN (US)	full
S51	PI203068	ARS GRIN (US)	full
S52	PI203069	ARS GRIN (US)	full
S53	PI204306	ARS GRIN (US)	full
S55	PI206838	ARS GRIN (US)	full
S56	PI206861	ARS GRIN (US)	full
S57	PI210558	ARS GRIN (US)	full
S58	PI210561	ARS GRIN (US)	full
S59	PI210583	ARS GRIN (US)	full
S60	PI212917	ARS GRIN (US)	full
S61	PI221697	ARS GRIN (US)	full
S62	PI227258	ARS GRIN (US)	full
S64	PI241593	ARS GRIN (US)	full
S65	PI242028	ARS GRIN (US)	full
S66	PI244093	ARS GRIN (US)	full
S67	PI244150	ARS GRIN (US)	full
S68	PI244175	ARS GRIN (US)	full
S70	PI248181	ARS GRIN (US)	full

S71	PI249645	ARS GRIN (US)	full
S72	PI250438	ARS GRIN (US)	full
S73	PI250439	ARS GRIN (US)	full
S74	PI250440	ARS GRIN (US)	full
S75	PI250441	ARS GRIN (US)	full
S77	PI250447	ARS GRIN (US)	full
S78	PI250448	ARS GRIN (US)	full
S79	PI257244	ARS GRIN (US)	full
S80	PI257592	ARS GRIN (US)	full
S82	PI261623	ARS GRIN (US)	full
S83	PI261624	ARS GRIN (US)	full
S84	PI261636	ARS GRIN (US)	full
S85	PI261671	ARS GRIN (US)	full
S87	PI263027	ARS GRIN (US)	full
S88	PI263030	ARS GRIN (US)	full
S89	PI263032	ARS GRIN (US)	full
S90	PI266070	ARS GRIN (US)	full
S91	PI269777	ARS GRIN (US)	full
S92	PI269778	ARS GRIN (US)	full
S93	PI269782	ARS GRIN (US)	full
S95	PI269798	ARS GRIN (US)	full
S96	PI269802	ARS GRIN (US)	full
S97	PI269804	ARS GRIN (US)	full
S98	PI269818	ARS GRIN (US)	full
S99	PI269822	ARS GRIN (US)	full
S100	PI269825	ARS GRIN (US)	full
S101	PI271035	ARS GRIN (US)	full
S102	PI271038	ARS GRIN (US)	full
S103	PI271116	ARS GRIN (US)	full
S104	PI271511	ARS GRIN (US)	full
S106	PI272175	ARS GRIN (US)	full
S107	PI272215	ARS GRIN (US)	full
S108	PI272218	ARS GRIN (US)	full
S109	PI273209	ARS GRIN (US)	full
S110	PI274584	ARS GRIN (US)	full
S111	PI275821	ARS GRIN (US)	full
S112	PI275822	ARS GRIN (US)	full
S113	PI275825	ARS GRIN (US)	full
S114	PI277852	ARS GRIN (US)	full
S116	PI279825	ARS GRIN (US)	full
S118	PI280609	ARS GRIN (US)	full
S119	PI280611	ARS GRIN (US)	full
S120	PI280613	ARS GRIN (US)	full
S122	PI280616	ARS GRIN (US)	full

S123	PI280617	ARS GRIN (US)	full
S124	PI280626	ARS GRIN (US)	full
S125	PI285710	ARS GRIN (US)	full
S126	PI285715	ARS GRIN (US)	full
S127	PI285718	ARS GRIN (US)	full
S129	PI285724	ARS GRIN (US)	full
S130	PI285727	ARS GRIN (US)	full
S131	PI285730	ARS GRIN (US)	full
S132	PI285740	ARS GRIN (US)	full
S133	PI285747	ARS GRIN (US)	full
S134	PI286430	ARS GRIN (US)	full
S135	PI286431	ARS GRIN (US)	full
S136	PI286607	ARS GRIN (US)	full
S137	PI288025	ARS GRIN (US)	full
S140	PI308796	ARS GRIN (US)	full
S142	PI314795	ARS GRIN (US)	full
S143	PI319374	ARS GRIN (US)	full
S145	PI324695	ARS GRIN (US)	full
S146	PI324700	ARS GRIN (US)	full
S147	PI324702	ARS GRIN (US)	full
S148	PI331413	ARS GRIN (US)	full
S149	PI331414	ARS GRIN (US)	full
S150	PI343292	ARS GRIN (US)	full
S152	PI343331	ARS GRIN (US)	full
S153	PI343338	ARS GRIN (US)	full
S154	PI343824	ARS GRIN (US)	full
S155	PI343958	ARS GRIN (US)	full
S156	PI343987	ARS GRIN (US)	full
S157	PI344003	ARS GRIN (US)	full
S161	PI347281	ARS GRIN (US)	full
S162	PI347295	ARS GRIN (US)	full
S163	PI347490	ARS GRIN (US)	full
S164	PI355906	ARS GRIN (US)	full
S165	PI356974	ARS GRIN (US)	full
S166	PI356984	ARS GRIN (US)	full
S167	PI356991	ARS GRIN (US)	full
S168	PI356992	ARS GRIN (US)	full
S169	PI357292	ARS GRIN (US)	full
S170	PI358300	ARS GRIN (US)	full
S172	PI358633	ARS GRIN (US)	full
S174	PI365419	ARS GRIN (US)	full
S175	PI371796	ARS GRIN (US)	full
S176	PI378157	ARS GRIN (US)	full
S177	PI381334	ARS GRIN (US)	full

S178	PI393489	ARS GRIN (US)	full
S180	PI411141	ARS GRIN (US)	full
S181	PI411142	ARS GRIN (US)	full
S182	PI413678	ARS GRIN (US)	full
S183	PI413683	ARS GRIN (US)	full
S184	PI413685	ARS GRIN (US)	full
S185	PI413688	ARS GRIN (US)	full
S186	PI413698	ARS GRIN (US)	full
S187	PI413703	ARS GRIN (US)	full
S188	PI429839	ARS GRIN (US)	full
S189	PI429843	ARS GRIN (US)	full
S190	PI429845	ARS GRIN (US)	full
S193	PI476413	ARS GRIN (US)	full
S194	PI477371	ARS GRIN (US)	full
S195	PI486131	ARS GRIN (US)	full
S196	PI494077	ARS GRIN (US)	full
S197	PI505080	ARS GRIN (US)	full
S198	PI505108	ARS GRIN (US)	full
S199	PI505122	ARS GRIN (US)	full
S200	PI505144	ARS GRIN (US)	full
U1	PI606703	ARS GRIN (US)	semi-l.
U2	PI606702	ARS GRIN (US)	semi-l.
U3	PI606701	ARS GRIN (US)	full
U4	PI606700	ARS GRIN (US)	full
U5	PI606699	ARS GRIN (US)	full
U6	PI619080	ARS GRIN (US)	full
U7	PI557502	ARS GRIN (US)	full
U8	PI557500	ARS GRIN (US)	semi-l.
U10	FP2091	Donation Dr. E. Gritton/M.-L. Pilet-Nayel	full
X1	Lisa	Sativa Rheinau AG (CH)	full
X2	Cooper	Innoseeds (NL)	semi-l.
X3	Frisson-WT	Donation Dr. H.Gamper	full
X4	Frisson-P2	Donation Dr. H.Gamper	full

ETH zürich

Mercator Research Program | Call 5

Improving disease resistance of pea through selection at the plant-soil interface

Background

Soil-borne diseases in legumes are one of the most severe problems in protein production. They cause severe damage and can lead up to total yield loss, especially in pea (*Pisum sativum* L.). The lack of adequate resistance in current pea varieties impedes pea cultivation worldwide. Sustainable solutions are needed, particularly for organic farmers who rely on this ecologically important nitrogen-fixing legume in crop rotations.

Objective

The overall goal is to improve the resistance of pea against soil-borne diseases to allow higher frequencies of grain legumes in organic farming systems. Investigating the potential role of root exudates in the inhibition or attraction of plant pathogen complexes as well as beneficial microbes will elucidate the physiological basis of disease resistance of pea genotypes.

Research Approach

- Development of a high-throughput screening system for resistance against soil-borne pathogen complexes and application of it to screen genetic resources of international origin as well as European pea breeding material
- Genome-wide association mapping, quantitative real-time PCR and HPTLC technology to unravel resistance mechanisms at the plant-soil interface
- On-farm experiments to verify resistance mechanisms in the field
- Survey among farmers to determine incentives needed to cultivate grain legumes

World Food System Center

Relevance and Expected Outcomes

A better understanding of the plant resistance mechanisms will aid in the development of screening tools for breeders to select genotypes resistant to soil-borne diseases taking account of the complex plant-soil interface. Breeding pea varieties with high resilience against soil-borne pathogens is a precondition for obtaining high and stable protein yields and is therefore crucial for the acceptance of new varieties by farmers. Resistant pea varieties will help to reduce the cultivation break of legumes in general as pea pathogens also affect other important legumes. Ultimately, outcomes of this project will be transferable to other legume cultivation systems.

Food System Challenges Addressed

Sustainable protein production, resistance mechanisms, resilience, organic plant breeding.

www.worldfoodsystem.ethz.ch/research/MRP →

Principal Investigator Prof. Bruno Studer, Molecular Plant Breeding

Co-Investigators Dr. Pierre Hohmann, Dr. Monika Messmer, FiBL

PhD Student tbc

Partners Prof. Gertrud Morlock (Giessen University), Agata Leska (GZPK)

Project Duration 2016-2019

Project Cost 271'670 CHF

Funding WFSC Mercator Research Program

C I PUBLICATIONS, CONFERENCE CONTRIBUTIONS & OUTREACH ACTIVITIES

1. Peer-reviewed publications in international scientific journals

Wille L., Messmer, M. M., Bodenhausen, N., Studer, B., Hohmann, P. (2020) Heritable variation in pea for resistance against a root rot complex and its characterisation by amplicon sequencing. *Frontiers in Plant Science*. 11:542153
Wille, L., Messmer, M. M., Studer, B., Hohmann, P. (2019) Insights to plant-microbe interactions provide opportunities to improve resistance breeding against root diseases in grain legumes. *Plant, Cell & Environment* 42: 20-40.
Besnard, G., Henry, P., Wille, L., Cooke, D., Chapuis, E. (2007) On the origin of the invasive olives (*Olea europaea* L., *Oleaceae*). Heredity 99: 649-657
Besnard, G., Wille, L., Henry, P., Chapuis E., Christin, P. A. (2007) Can microsatellite data allow identification of oleaster Plio-Pleistocene refuges zones in the Mediterranean Basin? *Journal of Biogeography* 34: 559-560
Croll D., Wille L., Gamper H., Nataranjan M., Lammers P. J., Corradi N., Sanders I. R., (2008) Genetic diversity and host plant preferences revealed by simple sequence repeat and mitochondrial markers in an arbuscular mycorrhizal fungal population of *Glomus intraradices*. *New Phytologist* 178: 672-687

2. Oral contributions to international conferences

Wille, L., Messmer, M. M., Studer, B., and Hohmann, P. (2019) Host genotype x soil interaction in the composition of pathogenic and beneficial fungi of pea lines screened for root rot resistance. Zurich-Basel Plant Science Center Symposium, Zürich (CH), 11.12.2019
Wille, L., Studer, B., Messmer, M. M. and Hohmann, P. (2019) Screening pea for resistance against a root rot complex on naturally infested field soil. 2nd EUCARPIA Workshop on Implementing Plant-Microbe Interactions in Plant Breeding, Tulln (AT), 6.12.2019

Wille, L., Studer, B., Messmer, M. M. and Hohmann, P. (2019) Resistant and susceptible pea lines harbour different root-rot pathogens and antagonistic fungi. Poster flash talk: MiCROPe, Vienna (AT), 2.-5.12.2019

Wille, L., Yates, S., Hohmann, P., Messmer, M. M. and Studer, B. (2019) Genome-wide association study for resistance of pea against a complex of root rot pathogens. 9th International Conference on Legume Genetics and Genomics, Dijon (FR), 5.12.2019

Wille, L., Hohmann, P., Messmer, M. M. and Studer, B. (2018) Erbse: Resistenzscreening gegenüber bodenbürtigen Pathogenen. 1. Biozüchtungstag, Research Institute of Organic Agriculture (FiBL), 19.7.2018

3. Poster presentations at international conferences (poster files are available on *www.orgprints.com*)

Wille, L., Messmer, M. M., Studer, B., and Hohmann, P. (2019) Host genotype x soil interaction in the composition of pathogenic and beneficial fungi of pea lines screened for root rot resistance. Zurich-Basel Plant Science Center Symposium, Zürich (CH), 11.12.2019; World Food System Center Symposium, Zürich (CH), 31.10.2019; MiCROPe, Vienna (AT), 2.-5.12.2019

Wille, L., Hohmann, P., Messmer, M. M. and Studer, B. (2018) Improving disease resistance of pea – clues from plant-microbe interactions. World Food System Center Symposium, Zürich (CH), 10.11.2018

Wille L., Messmer, M. M., Bodenhausen, N., Studer, B., Hohmann, P. (2018) Resistance screening of pea against a complex of root-rot pathogens. DIVERSIFOOD Congress, Rennes (FR), 8.11.2018

Wille, L., Hohmann, P., Messmer, M. M. and Studer, B. (2017) Improving disease resistance of pea – clues from plant-microbe interactions. World Food System Center Symposium, Zürich (CH), 25.10.2017

Wille, L., Hohmann, P., Messmer, M. M. and Studer, B. (2016) Improving disease resistance of pea through selection at the plant-soil interface. World Food System Center Symposium, Zürich (CH), 4.11.2016

4. Outreach activities (e.g. public engagement in science, technology and knowledge transfer activities, etc.)

Public engagement in science:

Wille, L., Hohmann, P., Messmer, Monika and Studer, Bruno (2019) Erbse: Resistenzscreening gegenüber bodenbürtigen Pathogenen. Short project presentation at the Körnerleguminosentag at GZPK 5.6.2019
Wille, L. (2018) ... das Problem an der Wurzel packen. Oral presentation: Gegenwartswoche Kanti Stadelhofen – PhD project presentation, 5.12.2018
Wille, L., Hohmann, P., Messmer, Monika and Studer, Bruno (2018) Erbse: Resistenzscreening gegenüber bodenbürtigen Pathogenen. Exhibition within the joint exhibition of the FiBL Plant Breeding Group during the "FiBL Tag der offen Tür", Frick, 19.8.2018
Wille, L., Hohmann, P., Messmer, Monika and Studer, Bruno (2018) Erbse: Resistenzscreening gegenüber bodenbürtigen Pathogenen. Short project presentation at the Körnerleguminosentag at GZPK 6.6.2018

Radio reports & social media:

Wille, L. and Messmer, Monika (2018) Interview Radio Interview for "Wissenschaftsmagazin", Swiss Radio and Television (DRS2): Neue Methoden zur Genveränderung: Umstrittene Möglichkeiten in der Pflanzenzucht, 12.5.2018 https://bit.ly/2Nfmc7o
Wille, L. (2019) Erbsen zählen mit ETH-Doktorand Lukas Wille. *higgs*, Scientist Takeover; 18.6.2019 www.higgs.ch/scientist-takeover-mit-lukas-wille/21948/

D I CURRICULUM VITAE

1. Personal information

First, second & family name: Lukas Felix Wille
Date & place of Birth: 30 March 1982, Bern
Nationality: Swiss
Contact Details: +41 (0)76 543 62 92;
lukas.wille@fibl.org

2. Education

2016 – 2020	Doctoral studies, Institute of Agricultural Sciences, ETH Zürich. Thesis titel «*The root rot complex of pea: Screening for resistance and quantification of microbial key players in the rhizosphere*». Supervision: Prof. B. Studer (IAS, ETHZ) & Dr. P. Hohmann (FiBL)
2012 – 2014	Teaching diploma for Gymnasium (grammar school), PHBern
2007	Master of Science in Biology, Evolution and Conservation. Master thesis: «*Genetic diversity and anastomosis in arbuscular mycorrhizal fungi*». Supervision: Prof. I. Sanders (DEE, UNIL)
2002 – 2007	Master studies, Faculty of Biology and Medicine, University of Lausanne Focus: Population Genetics, Conservation and Applied Ecology
2001	Matura, Literargymnasium Bern-Kirchenfeld, Bern

3. Employment history

Since 05/2020	Researcher in plant breeding and plant-microbe interactions, Department for Crop Sciences, Research Institute for organic agriculture (FIBL)
2016 – 2020	Research assistant, Institute of Agricultural Sciences, ETH Zürich
2007 – 2016	Biology & Chemistry teacher; part time, Gymnasium schools
2008 – 2014	Cycle messenger, dispatcher & member of the board of directors, Genossenschaft Velokurier Bern
2007	Research assistant, Department for Ecology and Evolution, UNIL, Lausanne

5. Supervision of graduate students

MSc students:	Mario Kurmann, IAS, ETH Zürich. Co-supervision with B. Studer & P. Hohmann

6. Awards

2018	Mercator Poster Award, World Food System Center Symposium, Zürich
2012	Swiss Cycle Messenger Champion, SUICMC 2012 Biel-Bienne