Quantification of selected pathogens of the root-rot complex of pea using qPCR

Master Thesis in Agricultural Sciences, ETH Zurich
Autumn Semester 2018

Mario Kurmann
Menzbergstrasse 33a
6130 Willisau
Switzerland

Supervision

Prof. Dr. Bruno Studer
Molecular Plant Breeding, ETHZ
LFW A 3
Universitätstrasse 2
8092 Zürich
Switzerland

Dr. Pierre Hohmann
Plant Breeding, FiBL
Ackerstrasse 113
CH-5070 Frick
Switzerland

Eidgenössische Technische Hochschule Zürich
Swiss Federal Institute of Technology Zurich

Eigenständigkeitserklärung

Die unterzeichnete Eigenständigkeitserklärung ist Bestandteil jeder während des Studiums verfassten Semester-, Bachelor- und Master-Arbeit oder anderen Abschlussarbeit (auch der jeweils elektronischen Version).

Die Dozentinnen und Dozenten können auch für andere bei ihnen verfasste schriftliche Arbeiten eine Eigenständigkeitserklärung verlangen.

Ich bestätige, die vorliegende Arbeit selbständig und in eigenen Worten verfasst zu haben. Davon ausgenommen sind sprachliche und inhaltliche Korrekturvorschläge durch die Betreuer und Betreuerinnen der Arbeit.

Titel der Arbeit (in Druckschrift):

Quantification of selected pathogens of the root-rot complex of pea using qPCR

Verfasst von (in Druckschrift):
Bei Gruppenarbeiten sind die Namen aller Verfasserinnen und Verfasser erforderlich.

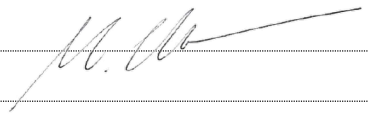
Name(n):	**Vorname(n):**
Kurmann	Mario

Ich bestätige mit meiner Unterschrift:

- Ich habe keine im Merkblatt „Zitier-Knigge“ beschriebene Form des Plagiats begangen.
- Ich habe alle Methoden, Daten und Arbeitsabläufe wahrheitsgetreu dokumentiert.
- Ich habe keine Daten manipuliert.
- Ich habe alle Personen erwähnt, welche die Arbeit wesentlich unterstützt haben.

Hier Text eingeben

Ich nehme zur Kenntnis, dass die Arbeit mit elektronischen Hilfsmitteln auf Plagiate überprüft werden kann.

Ort, Datum	**Unterschrift(en)**
Willisau, 31.03.2019	

Bei Gruppenarbeiten sind die Namen aller Verfasserinnen und Verfasser erforderlich. Durch die Unterschriften bürgen sie gemeinsam für den gesamten Inhalt dieser schriftlichen Arbeit.

Master Thesis project description: Quantification of selected pathogens of the root-rot complex of pea using qPCR - ETHZ, HS 2018

Master student:	Mario Kurmann, master studies in agricultural sciences (ETHZ)
Supervision:	Prof. Dr. Bruno Studer, head of the Molecular Plant Breeding group, ETHZ
	Dr. Pierre Hohmann, Senior Scientist Plant Breeding, FiBL
	Lukas Wille, doctoral student ETHZ-FiBL

Short description

Pea (*P. sativum*) is a valuable protein source for human food and animal feed and is the most important grain legume in agriculture of temperate climate zones. Pea cultivation is severely impeded due to a high susceptibility to soil-borne fungal pathogens. As part of the research project "resPEAact" (Doctoral Thesis of Lukas Wille), over 300 pea accessionss were screened under controlled conditions for resistance on a naturally infested field soil. Previous work showed an accumulation of pea specific fungal an oomycotan pathogens. The aim of future experiments of this project is to estimate if certain pea accessionss with different susceptibility levels show variation in the composition of pathogen and potential antagonists in the rhizosphere.

Research aims

Verification and quantification of selected fungal pathogens in the rhizosphere of diseased pea accessionss with the use of quantitative real-time PCR (qPCR).

Tasks

1. Set-up of quantitative qPCR: Implementation of previously published qPCR-assays in order to quantify selected pathogens in the roots of diseased pea plants.
2. Pot trial: Evaluation (disease score and biomass) of two tolerant and two susceptible pea accessionss on different sick soils. Verification and quantification of selected pathogens using implemented qPCR assays.
3. Verification of virulence: Inoculation of pea seedlings with selected fungal isolates previously isolated from infected pea roots and subsequent evaluation of disease characteristics.

Timetable

Start:	1. October 2018
Submission:	31. March 2019

Quantification of selected pathogens of the root-rot complex of pea using qPCR

Index

1. Introduction

1.1. Cultivation of pea

Pea (*Pisum sativum* L.) is a diploid (2n=14), self-pollinating crop belonging to the Fabaceae (or Leguminosae) family (Bodah et al., 2016). As a cool season annual crop, pea is planted from winter to early summer (Bodah et al., 2016; DAFF-SA, 2011). Pea plants are well known for their beneficial effects on soil fertility and are used for human food or animal feed (Bodah et al., 2016; Karkanis et al., 2016). Unfortunately, pea cultivation remains under the expectations concerning yields per acre and steadiness of yields due to several abiotic and biotic factors which severely affect pea cultivation (Foyer et al., 2016; Fuchs et al., 2014). Fortunately, researchers globally contribute to the process of finding sustainable solutions to secure pea cultivation in the future to meet the rising protein demand of a growing world population and face rising challenges in pea cultivation.

1.1.1. Nutritional characteristics

In pea, protein contents range between 13.7 to 30.7% of seed dry matter (Bodah et al., 2016; Dahl et al., 2012; Karkanis et al., 2016). Furthermore, essential amino acids as lysine and tryptophan are present in high concentrations in seeds (USDA, 2012). Phenolic compounds as tannins, phenolic acids and flavonoids are also present in peas and are reported to act as antioxidants (Dahl et al., 2012). Due to their nutritive composition, pea seeds traditionally have a solid position in human diet in different cultures and are widely used for animal feed (Joshi and Rao, 2017; Karkanis et al., 2016) (Castell and F.A., 1996). On a global scale, legumes are the second most relevant plant family of agricultural crop species after grasses (Watson et al., 2017). For low-income consumers in developing countries, whose major sources of protein are vegetable sources, use legumes as their main protein source (Joshi and Rao, 2017).

1.1.2. Ecosystem services

Besides the beneficial nutritional composition, pea cultivation provides crucial ecosystem services. As a member of the Fabacean family, pea plants perform, like a majority of this species (88% of the species examined to date), a symbiosis with rhizobium bacteria (Graham and Vance, 2003; Watson et al., 2017). Symbiotic rhizobium bacteria are gram negative soil bacteria which colonizes the roots of certain crops and are able to perform a fixation of atmospheric nitrogen and exchange with the pea plant for carbon (Hewins et al., 2015; Siczek et al., 2013). The expression of this mutualistic symbiosis depends on species and also on cultivar. Furthermore, environmental factors such as temperature, water availability, and soil characteristics (e.g. available soil mineral nitrogen) play a crucial role (Baddeley et al., 2013). Agricultural relevant (seed & forage) legumes fix an amount of 40 to 60 million metric tons (Mt) of nitrogen per year. Legumes in natural ecosystems fix another 3 to 5 million Mt per year (Graham and Vance, 2003). Moreover, the inclusion of legumes into cropping systems provides further advantages than nitrogen fixation. Soil fertility, soil organic matter, and biological activities in the soil can be improved (Carranca et al., 1999; Karkanis et al., 2016). Therefore, pea cultivation can contribute to an improvement of soil characteristics (Karkanis et al., 2016; Rubiales and Mikic, 2014; USDA, 2012; Watson et al., 2017).

1.1.3. Pea yield decaccessions

Pea is cultivated best on fertile, light-textured and well-drained soils (USDA, 2012). Generally, pea plants are adapted to many soil types but very sensitive to salinity and acidity (Karkanis et al., 2016; USDA, 2012). Pea is highly sensitive to numerous abiotic and biotic factors which can affect biological nitrogen fixation or physiological processes involved in plant growth (Bénézit et al., 2017; Fuchs et al., 2014). For example, nitrogen fixation driven by rhizobia, can be markedly reduced by soil compaction, drought or water logging or a lack of oxygen (Siczek et al., 2013). Nutritional deficiencies of phosphorous or potassium can also reduce nitrogen fixation (Siczek et al., 2013).

The most important factors affecting pea cultivation are fungal diseases infecting above- and belowground plant parts as infections can lead to high or complete yield losses (Biddle and Cattlin, 2007; Kraft and Pfleger, 2001). Belowground, pea is mainly affected by seedling damping-off, seed-, root- and foot rot caused by various soil- and seed-borne pathogens (Karkanis et al., 2016; Kraft and Pfleger, 2001).

Taken together these abiotic and biotic stresses have led to a constant decaccessions of pea cultivation worldwide over the last 50 years (Rubiales and Mikic, 2014). Despite the widely acknowledged ecosystem services and nutritional qualities of pea, and legumes in general, yields are relatively lower and more unstable when compared to cereal crops (Foyer et al., 2016).

1.2. Pea root rot complex

Field pea is susceptible to several soil-borne pathogens, with a high yield depression potential. These include various fungal pathogens such as several *Fusarium* spp., *Didymella pinodes* ((Berk. & A. Bloxam) Petr.), *Didymella pinodella* and *Rhizoctonia solani*, as well as the oomycetes *Pythium* spp. and *Aphanomyces euteiches*. (Baćanović-Šišić et al., 2017; Biddle, 2007; Chittem et al., 2015; Zitnick-Anderson et al., 2018). Soils that are used for frequent pea cultivation with relatively short cropping intervals, tend to favour the accumulation of these soil-borne pathogens leading to significant yield losses (Cesarano et al., 2017; Kraft and Pfleger, 2001; Nayyar et al., 2009).

The accumulation of soil-borne pathogens, which can influence the soil microbial community, in combination with crop yield depression is described with the term "soil fatigue" or "soil sickness" (Cesarano et al., 2017; Foyer et al., 2016). This term was first introduced in combination with unexplained yield depression after repetitive cultivation or perennial cultures (Fuchs et al., 2014). Pea yield losses of up to 60% have been reported in connection with fatigued soils, depending on pea variety, field management and environmental conditions (Willsey et al., 2018; Zitnick-Anderson et al., 2018). Symptoms such as seed decay, root- and foot rot, seedling blight and wilt are attributed to the infection by soil-borne pathogens. More than 20 different species of soil-borne fungal pathogens have been associated with the disease expression of pea root and foot rot (Baćanović-Šišić et al., 2017). This pathogen complex is frequently mentioned as pea root rot (PRR) complex (Chittem et al., 2015; Feng et al., 2010). The occurrence and development of PRR-complexes differ with year and location, depending on soil microbial community, climatic conditions, crop rotation and other agricultural management practices (Feng et al., 2010). Among the PRR-complex, *Fusarium* spp. are frequently isolated from diseased pea roots and reported to play a key role in the disease expression (Willsey et al., 2018).

1.3. *Fusarium* root rot

Fusarium root rot is characterised by brown to black lesions primarily in the epi- and hypocotyl regions. Red staining of the vascular tissue, extensive root decay, discoloured leaves and stem base are further symptoms (Biddle, 2007; Willsey et al., 2018). *Fusarium* spp. are able to survive saprophytically in the soil and on crop residues. Plants can be attacked at several development stages by different species (Willsey et al., 2018). Among the *Fusarium* species, *F. oxysporum*, *F. solani* and *F. avenaceum* are frequently detected and are reported to play a key role in the disease expression of the PRR-complex (Chittem et al., 2015).

1.3.1. *Fusarium oxysporum*

Fusarium oxysporum is the most wide-spread species within the *Fusarium* genus. *Fusarium oxysporum* occurs globally and can be found in most soils (Chittem et al., 2015; Leslie and Summerell, 2006). Pathogenic *F. oxysporum* strains are related to vascular wilts, damping-off, crown and root rot of many plant species worldwide and are in many cases host specific (Leslie and Summerell, 2006). In the case of chickpea, pea, lentil, common bean and alfalfa, *Fusarium* wilt is mainly caused by *F. oxysporum* (Rubiales et al., 2014). *F. oxysporum* is also a common soil saprophyte and can be dispersed on many different ways (wind, soil, seeds, insects or infected plant material) (Leslie and Summerell, 2006). Not only pathogenic strains of *F. oxysporum* exists, some isolates are proposed for the use in biological control programmes (Leslie and Summerell, 2006). Non-pathogenic *F. oxysporum* strains are found as an important component of a suppressive soil where non-pathogenic and pathogenic strains can co-occur and compete for nutrients or space (Leslie and Summerell, 2006; Xu et al., 2012). But the quantity of *F. oxysporum* species and isolates and the heterogeneity within *F. oxysporum* makes it difficult to interpret the literature in this area (Leslie and Summerell, 2006).

1.3.2. *Fusarium avenaceum*

Fusarium avenaceum is a predominantly soil-borne species and common in temperate regions worldwide. *F. avenaceum*, in association with other *Fusarium* species, can be a causal agent of foot and root rot diseases (Chittem et al., 2015; Leslie and Summerell, 2006). As a soil and seed-borne pathogen, *F. avenaceum* can severely affect pea cultivation (Chittem et al., 2015; Leslie and Summerell, 2006). Among the three *Fusarium* species, *F. avenaceum* is presumably the only species lacking taxonomic subgroups with a clear host specialisation (formae speciales), thus, able to infect multiple hosts (Leslie and Summerell, 2006; Šišić et al., 2016). Apart from pea, also faba bean, soybean, lentil, canola, potatoes and a range of several cereals have been reported to be susceptible to root diseases caused by *F. avenaceum* (Baćanović-Šišić et al., 2017). This complicates the control of *F. avenaceum* even more, as crop rotations need to exclude possible host crops. Furthermore, it is possible that the accumulation of *F. avenaceum* (in crop rotations favourable for the pathogen) occurs faster than the one of other pathogens with host specificity (Feng et al., 2010).

1.3.3. *Fusarium solani*

Fusarium solani is globally distributed and present in many soils (Leslie and Summerell, 2006) and has been specified as a single species in the genus *Fusarium*, section Martiella (Sisic et al., 2018). But a recent study revealed that *F. solani* is part of a species complex including 60 distinct phylogenetic species, forming the *Fusarium solani* species complex (Leslie and Summerell, 2006; Sisic et al., 2018). Due to the ecological plasticity, the *F. solani* complex as a whole has a broad host range (Sisic et al., 2018). However, individual *F. solani* species are often pathogenic to only one or a few host species (Sisic et al., 2018). Pathogenic species of pea are reported and *F. solani* is identified as a major pathogen of several legumes and can severely affect pea cultivation (Chittem et al., 2015; Leslie and Summerell, 2006). Moreover, *F. solani* species were reported to be specific pathogens of pea but are also able to infect chickpea and other non-legumes crops such as ginseng (*Panax ginseng* C. A. Mey.) and mulberry tree (*Morus alba* L.) (Sisic et al., 2018) affecting the fact that only *F. avenaceum* was reported of a lacking host specificity (Leslie and Summerell, 2006). This pathogenicity to several hosts would makes the control of such pathogens more difficult.

1.4. Controlling pea root rot and resistance breeding

1.4.1. Current management options of pea root rot

Control strategies for pea root rot are limited as the direct control of soil-borne pathogens is challenging. The application of fungicides (e.g. methyl bromide) through soil fumigation is highly regulated or banned by authorities in most nations. Technically demanding, such chemical control measures are virtually not feasible for most farmers. Moreover, they are not an option for organic certified farming systems. Beyond that, the awareness and understanding of the importance of a healthy soil microbiome rules out such drastic chemical interventions (Berendsen et al., 2012).

Seeds can serve as a reservoir of seed-borne pathogens and act as a first inoculum source for plant infection (Wilman et al., 2014). The use of quality seeds and the application of seed treatments are proposed management tools to tackle this issue and increase seedling emergence (Karkanis et al., 2016; Siddique et al., 2011; Wu et al., 2019). However, this approach only faces the problem of seed-borne pathogens but cannot supress infections by soil-borne pathogens.

To reduce the amount of inoculum in the soil, a well-chosen crop rotation is crucial and a common strategy for management of the PRR-complex (Feng et al., 2010; Karkanis et al., 2016; Siddique et al., 2011). To choose reasonable length of crop rotation breaks is challenging as certain pathogens can persist in the soil or on plant debris for several years, depending on the life cycle of the pathogens (Feng et al., 2010; Pflughöft et al., 2012). Therefore, crop rotations have to be long enough (in certain cases up to 10 years) to gain an effect of inoculum reduction (Karkanis et al., 2016). This makes it difficult to perform pea cultivation on a frequent basis.

Therefore, incorporation of resistant or more tolerant pea accessionss into the cropping system are an appropriate solution. Resistances against a few selected pathogens are available in certain cultivars, however, resistance in the field against pathogen complexes is scarce (Feng et al., 2010; Rubiales et al., 2014; Wille et al., 2018a). Breeding for resistance against pathogen complexes is crucial to maintain a profitable pea cultivation in the future.

1.4.2. Breeding for resistance against pea root rot complex

As there are several pathogens involved in the disease expression of the pea root rot complex (PRR), it is unlikely that merely one management tool can lead to a secured pea production. For managing the PRR-complex, a combination of several control strategies is needed. An essential management strategy is the use of resistant or tolerant pea accessionss. As mentioned before, resistances and tolerances are only available against single pathogens and not against pathogen complexes. QTLs have been identified in several pea accessionss explaining partial resistance to single pathogens of the PRR complex (Aphanomyces euteiches, M. pinodes,) (Fondevilla, 2011; Hamon et al., 2013). Also QTLs have been identified explaining partial resistance to *Fusarium* root rot (Coyne et al., 2015; Feng et al., 2011). Resistances which were found are mostly polygenetic, and therefore incorporation in breeding systems is challenging. Furthermore, resistant screening was mostly performed under controlled conditions, with selected pathogens and artificial inoculation. This does not represent the situation on the field or the situation with a complex growth medium. This makes it difficult to use these cultivars under field conditions where diverse pathogen complexes are involved. Apart from breeding against single pathogens, breeding for a beneficial microbiome is proposed as a possible solution, as there are several microorganisms that can act as antagonist against pathogens or enhance plant defence mechanisms (Fuchs et al., 2014; Hohmann et al., 2017; Mendes et al., 2018; Watson et al., 2017; Wille et al., 2018a). Therefore, inclusion of the microbiome is proposed as a new approach in resistance breeding (Hohmann et al., 2017). To drive the process of breeding for a beneficial microbiome and resistance against pathogen complexes forward, better insights into the microbiome composition and microorganism interactions are needed. Therefore, it is crucial to know which pathogens are involved in the disease expression of PRR and if certain pea accessionss interact differently with certain pathogens and other soil microorganism.

1.5. Methodologies to assess pea root rot

1.5.1. Assessing disease severity and resistance

Several techniques exist to assess pea root rot severity. Assessment techniques incorporate plant phenotypical data such as biomass ratios of different treatments (e.g. inoculated vs uninoculated or sick soil vs sterilised sick soil), visual assessment of disease symptoms and assessment of plant health status (Bacanovic, 2015; Infantino et al., 2006; Willsey et al., 2018). Plant health can be assessed by several measurement techniques i.e. leaf colour (chlorophyll content) or transpiration levels (Walter et al., 2015).

To assess resistance against pathogens, pea plants can be screened under field or controlled conditions. Screening under controlled conditions enables the focus on single pathogens or pathogen combinations (inoculation of single or multiple pathogens on sterile substrate) (Willsey et al., 2018; Zitnick-Anderson et al., 2018). For this, pathogens are isolated form diseased pea roots and then re-inoculated to healthy plants on sterile substrate. Effect of inoculation of pea growth performance can than be assessed. However, screening under axenic conditions has its limitations as resistance mechanisms can be overcome or differently expressed in the field due to the plant's complex interactions with a vast variety of soil microbes. Therefore, screening under field conditions enables the incorporation of a complex system into the screening assay. A combination of both approaches is possible with the use of a complex medium under controlled conditions. For this, soils with a natural microorganism composition can be used in pot trials. Information about resistance characteristic of pea accessionss, gained out of screening data from field or more realistic pot trials can give valuable information to breeders.

Sustainable management practices and effective breeding strategies call for a better understanding of the interplay of different pathogens involved in pea root rot complex. Traditionally, disease symptoms are linked to single pathogens (Biddle, 2007). But as there are several pathogens involved, linkage of disease symptoms to single pathogens is not clear and difficult.

1.5.2. Culture-based techniques

Classical techniques to identify plant pathogens include morphological identifications (Mirmajlessi et al., 2016; Vandemark, 2005). For this, pathogens are isolated form diseased plant material, cultivated and then identified according to their morphological characteristics. Such techniques require extensive knowledge of classical taxonomy and of morphological characteristics of single pathogens (Zitnick-Anderson et al., 2018). More importantly, culture-based identification of pathogens is biased to-

wards fungal species that are easily cultivable on a given medium and it may not always be possible to isolate the main agents of a disease (Lamichhane and Venturi, 2015). Furthermore, these techniques are laborious and time-consuming (Mirmajlessi et al., 2016). As *Fusarium* species are frequently confused (e.g. *F. solani* and *F. oxysporum*), a faster and more precise technique is needed (Leslie and Summerell, 2006; Mishra and Culham, 2003).

1.5.3. DNA-based techniques

An accurate and fast way to identify *Fusarium* spp. is the use of standard PCR. PCR is used for species identification, while quantitative real-time PCR (qPCR) can be used for quantification of target organisms (Zitnick-Anderson et al., 2018). qPCR enables a high throughput, high specificity and real-time monitoring. When DNA sequences of the target organism is known, it is possible to detect small amounts of target amplicons in complex samples. Melting curve analysis enables confirmation of specific amplifications (Mirmajlessi et al., 2016). Compared with culture-based techniques, qPCR reduces time needed for determining pathogen species and increases precision of the detection system (Mirmajlessi et al., 2016). No need for post processing (e.g. electrophoresis) and the possibility of quantitative measurements makes qPCR a practical alternative to traditional PCR (Mirmajlessi et al., 2016). However, DNA-based qPCR assays are prone to false positive results, as intact DNA of non-viable or dead cells can also be detected (Alemu, 2014). The setup of a qPCR assay requires high technical skills and the system is sensitive to external factors (e.g. DNA contamination). Nevertheless, qPCR is a state-of-the-art technique for pathogen identification in a complex medium in a reasonable time and under reasonable costs and is a central complement to culture based techniques.

1.6. Preliminary work

Within the framework of previous projects, pea root rot and pea soil fatigue was already in the focus of researchers at FiBL. In an attempt to investigate the cause of pea fatigue of 22 German soils, a two-level diagnostic test-system under controlled conditions was developed (Fuchs et al., 2014). Level one consisted of irradiation, nutrient addition and activated charcoal amendment to soils causing unexplained moderate to high pea yield losses. Results indicate the importance of soil organisms and their negative effect on pea growth and germination. Effect of nutrient deficiency and toxins were not found to be a major problem. Level two narrowed down the vast complexity of the soil microbes by using different fungicides to remove the effect of single or of pathogen groups. With this approach, it was possible to identify Oomycetes (such as Pythium ultimum or Aphanomyces euteiches) as a primary agent reducing germination rates and to some extend responsible for reduced growth of seedlings. On the other hand, rather groups of pathogens than single pathogens were identified to limit pea growth.

Within the framework of an ongoing PhD project (L. Wille), an analysis of the fungal root community of pea grown in an infested Swiss soil indicated the presence of several *Fusarium* spp. and Glomeromycota in susceptible and resistant plants, respectively (Wille et al., 2018b). *Fusarium* spp., as *F. solani* or *F. avenaceum* are well known for their potential to affect pea cultivation whereas Glomeromycota are known for their antagonistic, plant growth and health promoting characteristics (Hohmann and Messmer, 2017). Still, the extent of the contribution of individual pathogens to the disease expression of pea root rot is not yet clarified (Baćanović-Šišić et al., 2017). Also, the role and effect of antagonistic microorganism is not yet clear. To gain further insights in pea microbiome interactions and the performance of different pea accessionss on sick soils, a pea screening was recently conducted at FiBL/ETH. Three hundred and twelve pea accessionss were grown on one heavily infested Swiss soil. Plant biomass and disease ratings were assessed and it was possible to find different tolerance levels among the 312 pea accessionss (Wille et al., 2018b). If these tolerance levels are also found when these pea accessionss are grown on other sick soils needs to be clarified.

Fusarium spp. are frequently found in diseased roots and recent studies indicate their importance in the PRR complex (Chittem et al., 2015; Wille et al., 2018b; Willsey et al., 2018). Studies focussing on inoculation of pea plant with several *Fusarium* spp. are frequent but, to our state of knowledge, no studies are present where a complex medium was used for pea cultivation under controlled conditions. Therefore, screening pea accessionss on a complex medium under controlled conditions is a way to get deeper insights in the PRR complex.

2. Objectives

The objectives of this Master's thesis are to:

i) validate a pea screen for root rot tolerance using contrasting pea accessions on one heavily infested soil. Pea screen was previously conducted at FiBL (doctoral thesis L. Wille, FiBL & ETH Zurich)),
ii) detect and quantify the three *Fusarium* spp; *F. avenaceum*, *F. solani* and *F. oxysporum* in diseased roots of pea grown on four different soils and
iii) verify the pathogenicity of the three *Fusarium* spp. isolated from infected pea roots via re-inoculation of healthy pea plants in an axenic system.

3. Material & Methods

For quantification of the three pathogens *Fusarium solani*, *F. oxysporum* and *F. avenaceum* in diseased roots of pea, a pot trial was set up. Pea accessions, contrasting in their resistance against a naturally-occurring pathogen complex, were grown on four different soils for 29 days.

3.1. Pot Trial

3.1.1. Plant Material

Eight pea accessionss were used for the pot trial experiment. Three susceptible and three more tolerant pea accessionss to a pathogen complex of a heavily invested swiss soil were selected. Classification in susceptible and tolerant accessionss was based on a previous pea screening conducted at FiBL (L.Wille, data not shown). In addition to these six pea accessionss, two control accessionss were selected. For this, the commercial varieties EFB.33 (C1) and Respect (C2) were used as tolerant and susceptible check, respectively. Four out of the other six pea accessions were from the United States Department of Agriculture gene bank (USDA-accessionss) and labelled as S-accessionss in our pot trial. The other two accession are varieties form the organic plant breeder Peter Kunz (Getreidezüchtung Peter Kunz, GZPK) and labelled as G-accessionss in our pot trial.

Table 1: Additional information about the eight pea accession used for the pot trial

Name FiBl intern	Name	Status	Source	Plant_Id USDA	Origin USDA	Performance Pea Screen 2017
C1	EFB.33	Variety	Sativa Rheinau AG (CH)	-	-	tolerant
C2	Respect	Variety	Otto Hauenstein OHS	-	-	susceptible
G78	Roch	Variety	GZPK	-	-	Tolerant: Best biomass GZPK
G89	Volt	Variety	GZPK	-	-	Susceptible: second lowest biomass GZPK
S134	PI286430	GenBank -accession	USDA	G 12600	Nepal	Tolerant: best biomass in screen
S22	PI164612	GenBank -accession	USDA	Patani	India	Susceptible: lowest biomass in screen
S64	PI241593	GenBank -accession	USDA	G 6571	Taiwan	Tolerant: good biomass, high lesions
S91	PI269777	GenBank -accession	USDA	Aa87	UK	Tolerant: second best biomass in screen

3.1.2. Soils

The eight pea accessionss were grown on four field soils (Table 1), three of them showing moderate to strong signs of legume fatigue and one of them showing no fatigue. Pea plants were grown on the non-sterilised soils with a naturally occurring microorganism community and on a X-ray sterilised (approx. 70 kGy*) control of each soil (S). Takin into account all treatment levels and replicates, the pot trial consisted of 256 pots in total. Each pot was filled with approx. 600 ml of a soil-sand (2:1) mixture. Sterilised quartz sand was added to obtain a homogenous potting substrate and to obtain homogenous moisture levels in the pots.

Table 2: Additional information about the four soils used for the pot trial

"Häberli": collected in Kirchlindach (BE-CH), from field site Nüechtern
- Häberli is an organic managed field site with strong signs of legume fatigue. In 2014, a pea-barley mixed culture was sown with a total pea yield loss.
- Crop rotation (2012-2016): Lay, corn, pea-barley, wheat, potatoes

"Witzenhausen": obtained from Germany
- Witzenhausen is an experimental field site where different soil tillage systems and "Mulch" addition is tested.
- Crop rotation (2012-2015): Grass-clover, grass-clover, winter-wheat, cover crop-potatoes

"Lfl" Bayrische Landesanstalt für Landwirtschaft (Lfl): obtained from Germany
- Lfl is an experimental field site with repeated cultivation of different legumes over the last years. The soil used for our pot trial is actually from prior greenhouse trials, where it was tested which leguminous plants as pre crop have the worst effect on later pea cultivation
- Crop rotation (2012-2016): Wheat-clover, oats, clover-grass, intertillage: several legumes, spelt, pea, flied bean or blue lupine

GZPK – Getreidezüchtung Peter Kunz (= soil Feldbach)
- GZPK is a field site of an organic breeder
- Crop rotation (2014-2018): Wheat, grassland, vegetable, vegetables, lupines

The three soils Häberli, Witz. and Lfl are known for moderate to strong signs of legume fatigue. The soil Felbach shows no known pea cultivation issues and is therefore considered as 'healthy', control soil.

3.1.3. Experimental Design

The pot trial was arranged in a completely randomised block design with four replicates. Blocks were divided in non-sterilised soil (NS) and X-ray sterilised soil (S) and each replicate persisted of two tables (NS and S). Treatment combinations (Pea accessions x soil) were arranged in the same order on NS and S table. Tables in the climate chamber were moved and rotated on a daily basis to balance inequalities in the growth chamber.

3.1.4. Seeds – Sterilisation

Pea seeds were placed in 70 % EtOH for 30 seconds and then rinsed with demineralised water. Afterwards, seeds were placed in 2.5% Javel-water for 10 minutes. Pea seeds were then placed in water for 2x 20-30 min to remove Javel-water remains. The surface sterilised seeds were then used for sowing.

3.1.5. Growth conditions

The four replications were sowed on four on four consecutive days. One replicate with its sterile and non-sterile variant was sown per day. Seven seeds per pea accessions were planted individually per pot (0.66 l, TEKU, MXC 12, PP). Pots were previously prepared with a thin fleece covering the pot's base to prevent soil from leaking through pot holes. Pots were placed per replicate and treatment factor (sterile/non-sterile) on a flood and drain system. The tubs of the flood and drain system were previously covered with a thin fleece and foil. Plants were kept for 29 days in a growth chamber (15 m^2) under controlled conditions (58 W fluorescent lamps), temperature and humidity control system). Plants were grown at a 22 °C/19 °C day/night regime under 16 h/8 h light/dark conditions with relative air humidity ranging around 60 %. Plants were watered to saturation every 72-96 h with an automated watering system. If necessary, merging weeds were removed on a weekly basis. The number of plants was reduced to a maximum of five plants per pot after seven and 14 days after sowing. Reduction was performed by thinning out the least vigorous plants per pot to get an comparable number of plants.

3.1.6. Phenotyping

After 29 days of growing, plants were uprooted from pots and roots were thoroughly washed under tap water. An overall root disease score was assessed according to Wille et al. (in prep.). Pea roots were visually inspected for root disease symptoms. Individual plants were rated on a scale from 1 - 6, where 1 stands for healthy, symptom-free roots and 6 for a totally degraded/absent root system (Table 3). A root rot index (RRI) ranging from 0 to 6 was then calculated as the mean disease score of all plants in a pot. Shoot height was measured from the seed attachment to the terminal node. Shoots were then used for fresh and dry weight measurements (24 h at 105 °C). Shoot fresh and dry weight was assessed for single pots and mean values per plant were calculated per pot (biomass per pot divided by the number of plants).

Table 3: Root rot index for volume and appearance of pea roots according to Wille (in prep.)

Root biomass/-volume and appearance in general [Root Rot Index (RRI)]
1 – no symptoms, plant healthy 2 – Single isolated and small-area lesions 3 – Discoloration, root mass slightly reduced 4 – Discoloration, root mass heavily reduced 5 – (almost) only main root left 6 – complete root system destroyed or absent, plant dead

3.1.7. Sampling of diseased roots and DNA extraction

Roots were immediately frozen at -20 °C before being lyophilized in a freeze dryer (Harvest Right) for 24 h. Each sample was grinded at 30 Hz for 10 s to powder using the Retsch Mixer Mill MM200. Between 19 and 21 mg finely ground powder were weight into a 96well for DNA extraction. DNA extraction was performed with the plant and root Omega Mag Bind DNA DS Extraction kit (Omega Bio-Tek, Inc.). DNA extraction was performed according to the manufacturer's instructions. Cleaning steps were performed with a KingFisher™ Flex Purification System (KingFisher with 96 PCR head). The extracted DNA was quantified and quality checked with the NanoDrop 2000 System and stored at -20°C.

3.2. Quantitative real-time PCR (qPCR)

3.2.1. Implementation of previously published qPCR assays

The three primers pairs used in this thesis where previously published by Zitnick-Anderson, Simons et al. (2018) and Mishra and Culham (2003), Jiménez-Fernández, Montes-Borrego et al. (2010). Specificity of the primers was tested against *P. sativum*, *F. oxysporum*, *F. avenaceum* and *F. solani* DNA. *Fusarium* isolates of *F. oxysporum*, *F. avenaceum* and *F. solani* were obtained from A. Sisic (University of Kassel, Witzenhausen). For previous primer testing and adjustment of the reaction conditions, either the Rotor-Gene (Qiagen) quantitative real time PCR system or the CFX96 real time system (Bio-Rad) were used. During adjustments of the qPCR protocols, a reaction volume of 10 µl (5 µl SSO-SYBR (Bio Rad SSO AdvancedTM Universal SYBR Green Supermix), 2 µl Primer-Mix (forward & reverse Primer), 3 µl ultra-pure water and 1 µl sample DNA) was used. Gradient qPCRs (Bio-Rad, CFX96) were performed to determine the ideal annealing temperature for each primer and to eliminate unspecific amplifications.

3.2.2. qPCR Standards

Standards were set up with the fungal target DNA and axenic roots as background matrix. To obtain disease-free roots, plants were kept under axenic conditions without any contact to potential pathogen sources. For this, 10 seeds (pea accessions C1 (EFB.33)) were planted individually per pot according to the procedure under section 3.1.4. During set up, a special focus was placed on pea seed and material sterilisation. Therefore, higher concentrated Javel-water (5 % vs. 2.5 %) was used for seed and material sterilisation. Furthermore, sterilised quartz sand (105 °C/12h) was used instead of natural soil to reduce the risk of a potential contamination. Plants were kept for 14 days in a GroBank under controlled conditions (light (58 W fluorescent lamps), temperature and humidity control system). Plants were grown at 20 °C under 16 h/8 h light/dark conditions with relative air humidity ranging around

50 % and were watered to saturation every 48-72 h. After 14 days, plants were uprooted and roots were thoroughly washed under tap water, lyophilized in a freeze dryer (Martin Christ) for 8 h and then grinded at 30 Hz for 60 s to powder using the TissueLyser II (Qiagen) sample disruption system. DNA extraction and quantification was performed as described under section 3.1.7. Root material was then checked for amplification with the three *Fusarium* primers (Table 4) using the previously adjusted qPCR approach. Samples without a signal for the three *Fusarium* primers were used for qPCR standards. Axenic root DNA was then combined with fungal DNA. Fungal target DNA was extracted with the ZR Fungal/Bacterial DNA extraction kit (ZR Fungal/Bacterial DNA MicroPrepTM, ZYMO Research) according to the manufacturer's instructions. For the extraction, approx. 1 cm^2 of mycelia from fungal pure cultures grown at room temperature on potato dextrose agar (Potato Extract Glucose Agar, Carl Roth GmbH + Co. KG) was used. Mycelia was collected with a spatula and placed in a ZR BashingBead tube for further processing. The extracted DNA was quantified and quality checked on a 1% agarose gel and quantified with a NanoDrop 2000 (Thermo-Fischer Scientific). Finally, DNA extracts were standardised to 10 ng DNA μl^{-1}. Fungal DNA extracts were then added to axenic pea root extract to create fungal-DNA standards with pea roots as background matrix. 10-fold serial dilutions ranging from 10^1 to 10^{-4} ng target DNA μl^{-1} were set up for each *Fusarium* species.

3.2.3. Quantification of *Fusarium* species in diseased pea roots

For sample screening of the pot trial, reactions were conducted on a Rotor-Disc 100 using the Rotor-Gene (Qiagen) quantitative real time PCR system. qPCR setup was done with the automated QIAgility (QIAGEN) qPCR setup system. This system enables a rapid and high-precision setup of a PCR by pipetting all the relevant components. Each 13 µl reaction contained 6.6 µl SSO-SYBR (Bio Rad SSO AdvancedTM Universal SYBRR Green Supermix), 1.3 µl Primer-Mix (forward & reverse Primer), 4 µl ultra-pure water and 1 µl DNA. Each qPCR run was performed in two replicates. Each run consisted of 38-40 samples, water as negative control, axenic roots as negative check and five 10-fold serial dilutions ranging from 10^1 to 10^{-4} ng target DNA μl^{-1}. Standard curves were generated using fungal target DNA and sterile pea root reference DNA as background matrix. Runs were always conducted with two technical replicates. Cycling conditions were set as follows: Initial 98 °C for 3 min, followed by 50 cycles of 95 °C for 10 s, 30 s annealing at 62, 63 or 65 °C, depending on the primer (Table 4) and extension at 72 °C for 15 s. Resulting threshold cycle (Ct) values were analysed on the Rotor-Gene Q series software. Rotor-Gene Q series software automatically calculated run efficiency and DNA concentration for each sample. Results of each run were exported as and further data analysis was performed with the statistical software R (R-Core-Team 2017)

Table 4: Primers used for quantification of *Fusarium* spp. in diseased roots of pea.

F. solani (FSOL) (Zitnick-Anderson, Simons et al. 2018)		
SolF-SolR – 90 bp - EF-1α	annealing Temp.:	63° C
f: GCGCCTTACTATCCCACATC		
r: TTTTGTGACTCGGGAGAAGC		
F. avenaceum (FAVE) (Zitnick-Anderson, Simons et al. 2018)		
AveF-AveR – 100 bp - EF-1α	annealing Temp.:	62° C
f: GCTTATCTGCACTCGGAACC		
r: CGCGTAATCGAAGGGATATT		
F. oxysporum (FOX) (Mishra and Culham 2003, Jiménez-Fernández, Montes-Borrego et al. 2010)		
FOF1-FOR1 – 340bp – ITS	annealing Temp.:	65° C
f: 5'-ACATACCACTTGTTGCCTCG-3'		
r: 5'-CGCCAATCAATTTGAGGAACG-3'		
Primers commercially synthesized by Mycrosynth AG, Balgach, Switzerland		

3.3. Inoculation Trial

For verification of the pathogenicity of ten different fungal isolates to pea, an inoculation trial was set up. Pea variety Respect (C2) was used for the inoculation trial and was inoculated with the fungal isolates previously isolated from diseased pea roots. Fungal isolates were identified with the adjusted qPCR assay and pea performance after inoculation was assessed.

3.3.1. Fungal isolates

Three previously identified and seven unknown fungal isolates were selected for the inoculation of pea seedlings in sterile sand. The already identified isolates were classified as *F. oxysporum*, *F. avenaceum* and *F. solani* and were obtained from A. Sisic (University of Kassel, Witzenhausen). Unknown isolates were isolated from diseased pea roots grown in Häberli field soil at FiBL in 2016. All isolates were incubated on SNA (see attachment) for 22 days. Incubation under alternating cycles of 12 h UV-light and 12 h darkness at 19 ° C. After 22 days of incubation, the cultures were flooded with 10 ml demin. water and the fungal structures were dislodged with a cell spreader (Drigalskispatel). Spores were separated from mycelium by filtrating the mycelium suspension through milk filter paper. Spore concentration was determined by a haemocytometer and adjusted to $2.13*10^3$ spores g^{-1} substrate. Pots were then inoculated after sowing. Four seeds were planted individually per pot (0.66 l, TEKU, MXC 12, PP) filled with approx. 470 g previously heat (105 °C/12 h) sterilised sand. Pots were previously prepared with a thin fleece covering the pot's base to prevent soil from leaking through pot holes. Previously to sowing, pea seeds were surface sterilised as described before (section 3.1.4.). Also, all other materials as pots and trays had been sterilised previously to usage. Plants were kept for 28 days in a growth chamber under controlled conditions (light (58 W fluorescent lamps), temperature and humidity control system). Seedlings were grown at a 22 °C/19 °C day/night regime under 16 h/8 h light/dark conditions with relative air humidity ranging around 67 %. Plants were watered to saturation every 72-96 h with an automated watering system.

3.3.2. Phenotyping

After 28 days of growing, plants were uprooted from pots and roots were thoroughly washed under tap water. An overall root rot index was assessed according to Wille et al. (in prep.). Pea roots were visually inspected for root disease symptoms. Individual plants were rated on a scale from 1 - 6, where 1 stands for healthy, symptom-free roots and 6 for a totally degraded/absent root system (Table 3). A root rot index (RRI) ranging from 0 to 6 was then calculated as the mean disease score on all plants in a pot. Additionally, spread of lesions at shoot and root base were rated according to Pflughöft (2008).

3.4. Data analysis

Statistical analysis was conducted with the statistical software R (R-Core-Team 2017). A accessionsar model approach was used to analyse the effects of pea accession, soil and accession x soil interactions. *Fusarium* spp. concentrations were log-transformed prior to analysis. Data was checked for normal distribution using Shapiro-Wilk Normality Test. Normal distribution and homogeneity of variance of residuals were tested by generated diagnostic plots. Dunnett's Test (Dunn, O.J. 1964. Multiple comparisons using rank sums. Technometrics 6:241-252) was used for comparing different means within the dataset and calculated by "dunnTest()", package "FSA" (Ogle, Wheeler et al. 2018). Kruskal-Wallis test by rank was used for non-parametric tests, extended with the two-samples Wilcoxon test by „kruskal.test()" and „pairwise.wilcox.test()".

For comparison of shoot fresh weight of the sterile and non- sterile treatment, a shoot fresh weight ratio (sfw-ratio) was calculated by the sfw of the non-sterile divided by the sfw of the sterile treatment.

4. Results

4.1. Pot experiment

4.1.1. Reduced emergence & shoot fresh weight of pea plants grown on non-sterilised soil

For quantification of the three *Fusarium* spp. in diseased roots of pea and for validation of a screening system, a pot experiment was set up. Eight pea accessionss, contrasting in their resistance against a naturally-occurring pathogen complex, were grown on four different soils for 29 days.

Mean emergence rate over all four soils is at 92.11 % for sterile and at 82.71 % for non-sterile soils (assessed after seven days after sowing) **(Table 5)**. Mean emergence rates differ between soils and treatments within a range of 80 to 93.86 %. Emergence rates in non-sterilised soils were 86.14 % in Lfl, followed by 83.29 % in Feldbach, 80 % in Witzenhausen and 81.43 % in Häberli. Relative changes between emergence rate in sterile to non-sterile soil was with -11.26% significantly higher compared to the relative emergence rate changes of Lfl -3.98 % (Dunnet-test: p-value: 0.0177*).

For the four soils, shoot fresh weight reduction per plant from the sterilised to non-sterilised treatment is significantly and varied from -11.36 to -55.97 %. Mean reduction of sfw over all four soils was -37.02 %.

Shoot fresh weight of non-sterile Feldbach was significantly higher compared with the other soils (Dunnet-test: p-value: $<1e^{-07}$***) and rel. change was with -11.36 % significantly lower compared with -32.23 %, -44.74 % and -55.97 % for Witzenhausen, Häberli and Lfl, respectively.

Table 5: Mean emergence rate (%) after seven days after sowing (Mean ± SD) and mean shoot fresh weight per plant (Mean ± SD) of the eight pea accessionss grown for 29 days on the four different soils. Per pot, seven seeds were planted and emergence rate was assessed after seven and 14 days (Data not shown) of sowing. After assessment of emergence rate, pea plants per pot were reduced to five plants.

Treatment	Soil	Mean emergance % (±SD) 7 days		Mean sfw plant (±SD) 29 days	
S	Feldbach	93.86	(±9.9)	2.64	(±0.88)
NS	Feldbach	83.29	(±16.7)	2.34	(±0.68)
rel.		-11.26%		-11.36 %	
S	Häberli	91.57	(±10.9)	2.28	(±0.78)
NS	Häberli	81.43	(±20.3)	1.26	(±0.50)
rel. change		-11.07%		-44.74 %	
S	Lfl	89.71	(±11.0)	3.18	(±0.96)
NS	Lfl	86.14	(±20.0)	1.40	(±0.59)
rel. change		-3.98 %		-55.97 %	
S	Witz.	93.29	(±8.1)	2.11	(±0.79)
NS	Witz.	80.00	(±23.9)	1.43	(±0.57)
rel. change		-14.25 %		-32.23 %	
overall Mean					
S		92.11	(±1.62)	2.55	(±0.41)
NS		82.71	(±2.30)	1.61	(±0.43)
		-10.21 %		-37.02 %	

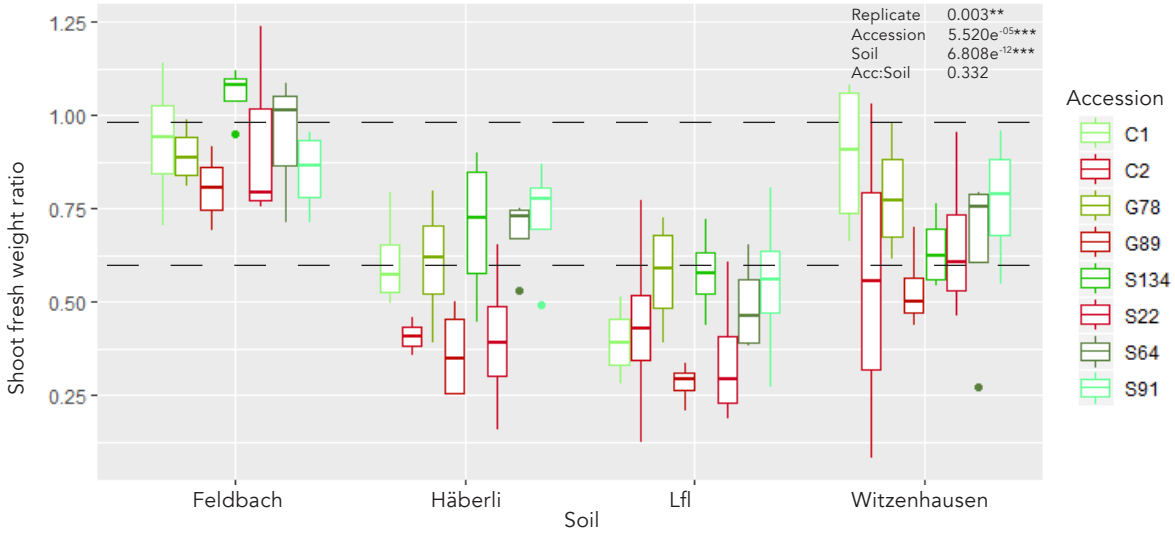


Figure 1: Shoot fresh weight ratio (sfw-ratio) between sterile and non-sterile treatment assessed for eight different pea accessions grown for 29 days on four different soils. Classification in susceptible (red) and tolerant (green) accessions is based on pea screening data 2017 (L. Wille). Four soils: Feldbach = healthy control soil, Häberli =sick soil form CH, Lfl and Witzenhausen = sick soils form DE. Mean sfw-ratio for Feldbach soil is 0.97 (±0.33 SD, upper accessions) and mean sfw-ratio for the three sick soils is 0.57 (± 0.22 SD, lower accessions).

4.1.2. Shoot fresh weight ratio

To gain a direct comparison between the performance of a pea accession grown on sterilised vs. non-sterilised soil, the shoot fresh weight ratio was calculated (sfw-ratio) as described under material and methods.

Significant differences for sfw-ratio were found between accessions (p-value: $7.776e^{-06}$***) and soils (p-value: $2.414e^{-13}$ ***) over the complete dataset **(Fig. 1)**. Significant differences were also observed between replicates (p-value: 0.0156*). An accession x soil interaction was not significant (0.332). Overall, sfw-ratios for accessions grown on Feldbach soil (overall mean sfw-ratio: 0.97 (upper accessions)) were significantly higher than (Dunnett-test: p-value < $1e^{-05}$***) the accessions grown on the three sick soils (overall mean sfw-ratio: 0.57 (lower accessions)). Within the soils, differences between accession are significant for Häberli (p-value: 0.007**). More susceptible pea accessions (red) show lower sfw-ratios than the tolerant pea accessions (green) **(Fig. 1 & Table 6)**. This difference is apparent and significant in the Häberli (p-value: $1.124e^{-05}$***) and Lfl (p-value: 0.01122*) soil, where mean values for susceptible soils are -44.59 % and -31.33 % lower, respectively. Tendencies are visible in the other two soils as well, where susceptible pea accessionss show a weaker performance but differences are not significant.

Table 6: mean shoot fresh weight ratios of the four different soils grouped according to theire tolerance to the pea root rot -complex

Soil	tolerant (t)/ susceptible (s)	Mean sfw-ratio		±SD
Feldbach	tolerant	0.895		0.225
Feldbach	susceptible	1.15		0.483
		Diff. t/s	+28.49 %	
Häberli	tolerant	0.666		0.151
Häberli	susceptible	0.369		0.147
		Diff. t/s	-44.59 %	
Lfl	tolerant	0.517		0.15
Lfl	susceptible	0.355		0.185
		Diff. t/s	-31.33 %	
Witz.	tolerant	0.746		0.192
Witz.	susceptible	0.589		0.271
		Diff. t/s	-21.05 %	

Correlation Screen Data 2017 and Pot Trial Data 2018

The setup of the pot trial 18 was based on the findings of the pea screening of 2017. Classification in susceptible and tolerant pea accessions was done due to the performance of the pea accessions in the screen. A subset of eight pea accession (out of 312) was used in the pot trial 2018 and also grown on Häberli soil, plus two other soils (Lfl and Witz.) A significant correlation of the sfw-ratio means of the eight pea genotypes between screen data 2017 and pot trial data 2018 (grown on Häberli soil) was found according to Spearman's rank correlation coefficient (r_s = 0.94***) **(Fig. 2)**. Also a significant correlation was found between the screen data 2017 and Lfl soil (r_s = 0.81*). But no significant correlation for screen data 2017 and Witzenhausen **(Table 7)**.

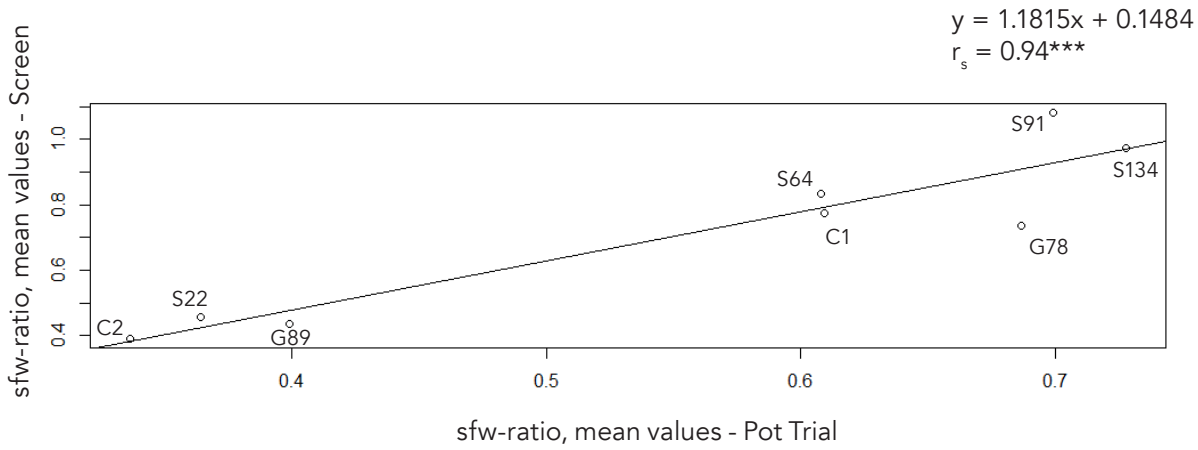


Figure 2: Correlation between shoot fresh weight-ratio of the eight selected pea accessions grown on Häberli soil from screen data 2017 (Lukas Wille) and pot trial 18. Correlation is calculated using rank correlation by Spearman.

Table 7: Correlation between shoot fresh weight-ratio of the eight selected pea accessions grown on Häberli soil from screen data 2017 (Lukas Wille) and pot trial 18 (Häberli, Lfl and Witzenhausen soil). Correlation is calculated using rank correlation by Spearman (rs).

Soil	r_s	p-value
Häberli	0.94	$6.379e^{-04}$***
Lfl	0.81	0.015*
Witz.	0.53	0.176

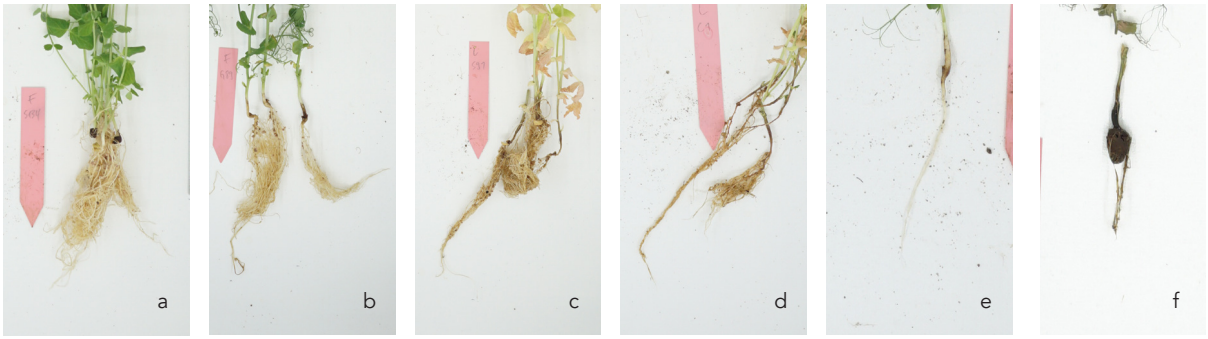


Figure 3 (a-f): Field pea root rot rating scale for disease symptoms induced in *Pisum sativum* accessions contrasting in their tolerance against pea root rot-complex. Pictures of pea plants were taken after 29 days of growing on four different soils. A to f show the six different disease stages (scale from 1 - 6, where 1 stands for healthy, symptom-free roots and 6 for a totally degraded/absent root system, a = 1 and f = 6). Detailed information to root rot ratings can be found under M&M, Table 3.

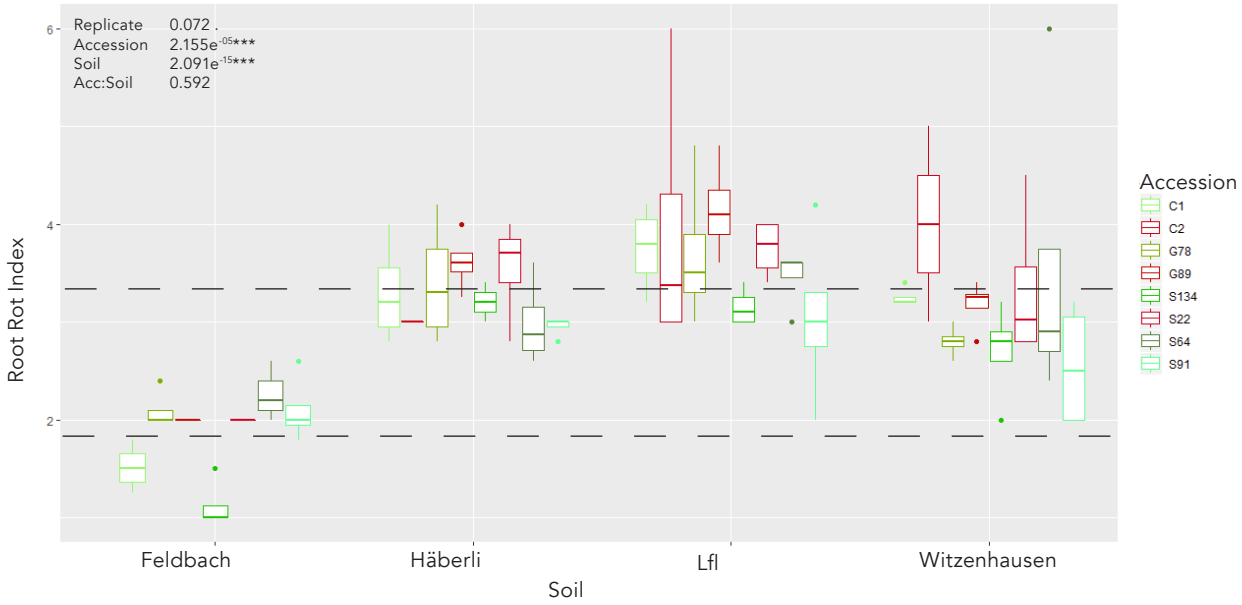


Figure 4: Root rot rating assessed for eight different pea accessions grown for 29 days on four different soils. Individual plants were rated on a scale from 1-6 (Figure 5), where 1 stands for healthy, symptom-free roots and 6 for a totally degraded/absent root system. A root rot index (RRI) ranging from 1 to 6 was then calculated as the mean disease score of all plants in a pot. Classification in susceptible (red) and tolerant (green) accessionss is based on pea screening data (L. Wille). Four soils: Feldbach = health control soil, Häberli =sick soil form CH, Lfl and Witzenhausen = sick soils form DE. Mean RRI for Feldbach soil is 1.83 (± 0.45 SD, lower accessions) and mean RRI for the three sick soils is 3.34 (± 0.71 SD, upper accessions).

4.1.3. Root Rot Index

An overall root rot rating was assessed according to Wille et al. (in prep.) after 29 days **(Fig. 4)**. Globally, root rot ratings differ significantly between accession (p-value: $5.183e^{-05}$***) and soils (p-value: $1.217e^{-15}$***) taking the complete data set into account. Differences are also significant if Feldbach is excluded. No significant replicate effect (p-value: 0.090) and no significant accession-soil interaction (p-value: 0.343) was found.

Ratings for accessions grown on Feldbach soil are around a score of 2 with lower values for C1 and S134 (significant difference of S134 to the accession with a RRI ≥ 2). Significantly lower mean root rot values are found for accessions grown on Feldbach soil compared to the other three sick soils (Dunnet-test: p-value $<1e^{-10}$***). Mean root rot index (RRI) for Feldbach is 1.83 (± 0.45 SD, lower accessions) and 3.34 (± 0.71 SD, upper accessions) for the three sick soils (Häberli, Lfl, Witzenhausen). RRI mean values of susceptible pea accessionss (red) are significantly higher than the one of tolerant (green) pea accessionss in Häberli (0.03*) and Lfl (0.04*) soil with differences between 10.96 to 13.42 % **(Table 8)**.

Table 8: Mean (±SD) root rot vales for susceptible and tolerant pea accession grown for 29 days on the four different soils.

Soil	toleant (t) / susceptible (s)	Mean RRI		±SD
Feldbach	tolerant	1.81		±0.489
Feldbach	susceptible	1.88		±0.354
		rel. Diff. t/s	3.72 %	
Häberli	tolerant	3.17		±0.428
Häberli	susceptible	3.56		±0.51
		rel. Diff. t/s	10.96 %	
Lfl	tolerant	3.42		±0.598
Lfl	susceptible	3.95		±0.819
		rel. Diff. t/s	13.42 %	
Witz.	tolerant	2.97		±0.829
Witz.	susceptible	3.42		±0.748
		rel. Diff. t/s	13.16 %	

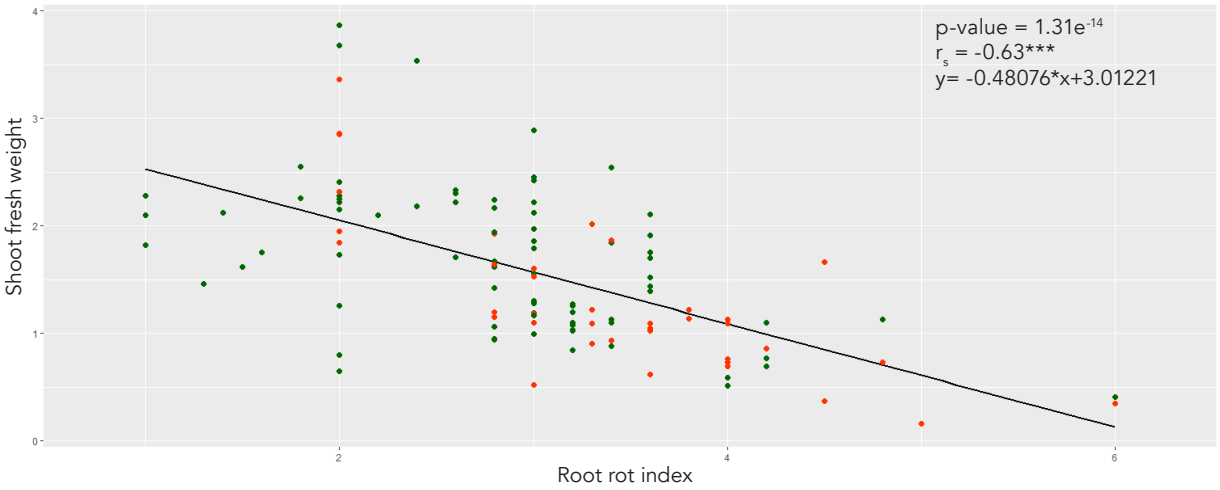


Figure 5: Correlation between shoot fresh weight per plant and root rot index of all four soils calculated using rank correlation by Spearman. Plants grown for 29 days on non sterilised soils. Red points indicate more susceptible pea accessions and green points indicate more tolerant pea accessions.

Root Rot Index correlates with shoot fresh weight per plant

Correlation between shoot fresh weigth per plant and disease score index is calculated using rank correlation by Spearman resulting in a correlation coefficient of -0.63. Root rot index (RRI) correlates significantly (p-value: $1.31e^{-14}$***) with sfw per plant. Higher disease score ratings results in lower shoot biomass (sfw per plant = -0.48076*(RRI) +3.01221)

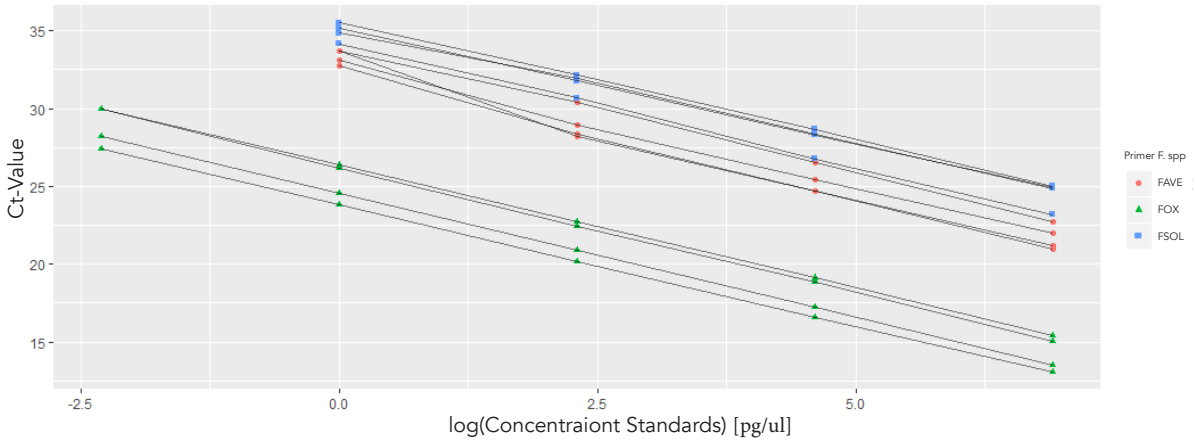
4.2. Quantification of *Fusarium* spp

4.2.1. DNA extraction

As DNA extraction is a critical part of qPCR, extracted DNA was checked for quantity an purity **(Table 9)**. DNA amount of samples used for qPCR ranged from 34.1 to 675.6 ng/ul. Samples with an DNA amount of 0 were excluded previous to data analysis. Remaining samples have a mean DNA amount of 308.6 ng/ul. The 260/280 ratio was between 1.71 and 2.07. A 260/280 ratio around 1.8 is considered as „pure" for DNA. The 260/230 ratio was between 1.23 and 2.48 where a 260/230-ratio between 1.8 and 2.2 is accounted for„pure" for DNA. Ratios under 1.8 may indicate the presence of copurified contaminants.

Table 9: DNA measurements performed with the NanoDrop2000. Samples with an DNA amount of 0 were excluded from analysis.

DNA	Mean (±SD)	Min	Max
DNA amount	308.6 ng/ul (±129)	34.1 ng/ul	675.6 ng/ul
260/280-ratio	1.85 (±0.72)	1.71	2.07
260/230-ratio	2.104 (±0.41)	1.23	2.48



FAVE	R^2 = 0.994	E = 0.87 Ct(y) = -3.675*log(x) + 32.89
FOX	R^2 = 0.999	E = 0.90 Ct(y) = -3.589*log(x) + 23.81
FSOL	R^2 = 0.998	E = 0.93 Ct(y) = -3.498*log(x) + 35.59

Figure 6: Standard curves (Fungal DNA plus plant matrix) with a 10-fold serial dilution ranging from 10^3 to 10^{-1} pg target DNA ul^{-1} were used for *Fusarium oxysporum* (green triangle). For *F. avenaceum* (red point) and *F. solani* (blue square), 10-fold serial dilution ranging from 10^3 to 10 pg target DNA ul^{-1} were used. R^2 = coefficient of determination; E = Efficiency of qPCR Run

4.2.2. Standard curves

Standard curve regression accessionss calculated for all qPCR runs show reproducible amplifications. A efficiency between 75-99 % was calculated for all qPCR Runs. One single qPCR run has a lower efficiency than 80 %. All the other Runs have an efficiency over 80 %. High coefficient of determination (R^2 = 0.98-0.99) indicate a good fit of the data to the regression accessions. Reproducibility of high R^2 was possible. qPCR runs were conducted per Primer per day with all four replicates. Standard curves are accessionsarly within a run, but standard curves differ between the replicates.

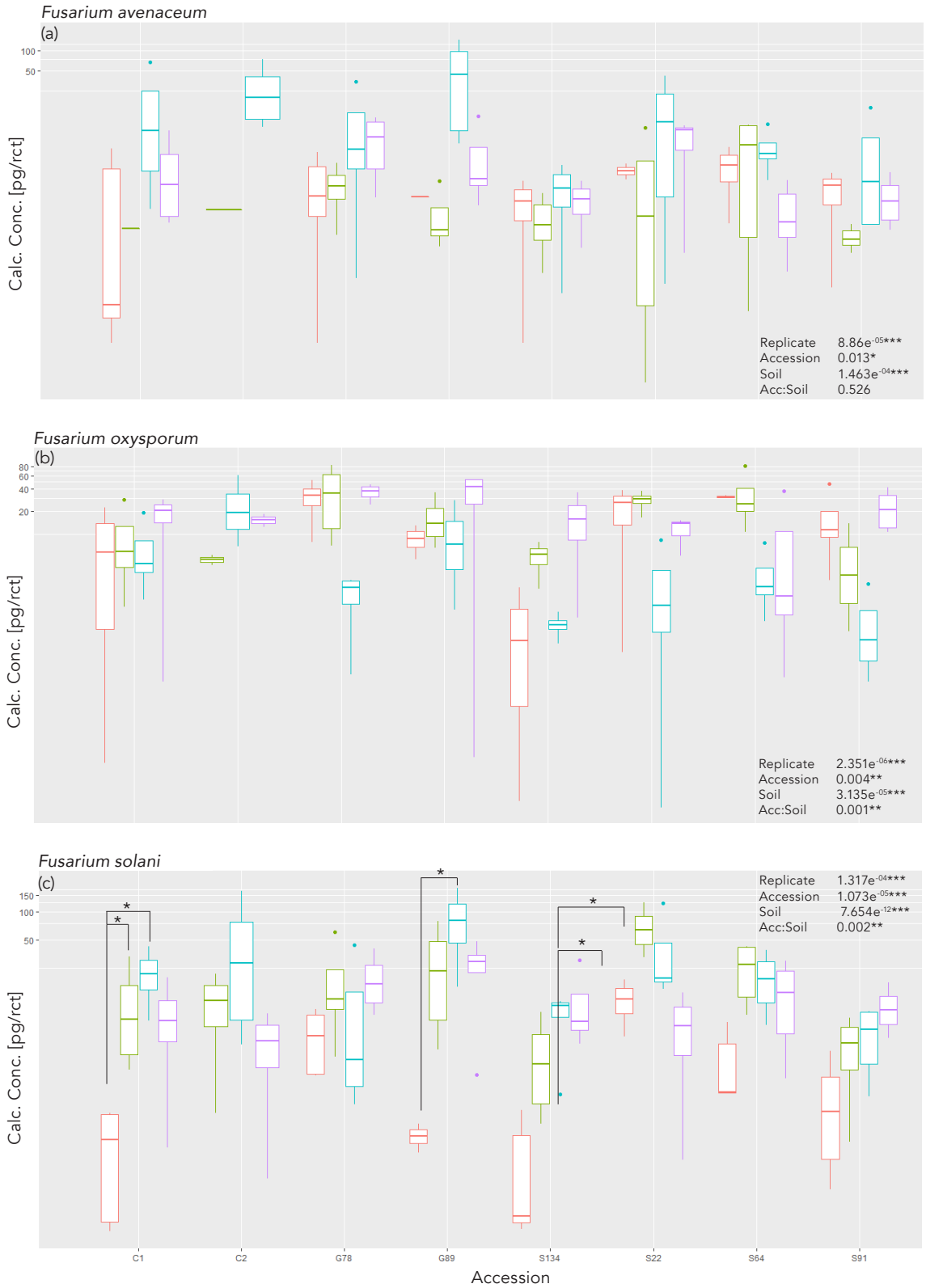


Figure 7: Quantification of the three *Fusarium* species ((a) *F. avenaceum*, (b) *F. oxysporum* and (c) *F. solani*) in diseased roots of pea. Pea accessions contrasting in their tolerance level against pea root rot complex were grown for 29 days on four different soils (Feldbach = health control soil, Häberli = sick soil form CH, Lfl and Witzenhausen = sick soils form DE). Classification in susceptible (red) and tolerant (green) accessionss is based on pea screening data (L. Wille).

Table 10: Mean *Fusarium* DNA concentration (Mean ±SD) of the three *Fusarium* spp. per soil and mean concentration per species.

Fusarium spp	Soil	Mean Calc. Conc. (pg/rct)	±SD	
F. avenaceum	Feldbach	3.55	±10.6	
	Häberli	1.27	±2.38	
	Lfl	18.5	±32.7	Mean per species (±SD)
	Witz.	2.40	±3.27	7.44 pg/rct (±20.3)
				Min: 0.001 pg/rct
				Max: 146.3 pg/rct
F. oxysporum	Feldbach	15.3	±16.6	
	Häberli	18.6	±21.7	
	Lfl	6.68	±12.3	Mean per species (±SD)
	Witz.	21.6	±16.7	15.5 pg/rct (±17.9)
				Min: 0.002 pg/rct
				Max: 83.93 pg/rct
F. solani	Feldbach	2.35	±4.11	
	Häberli	22.8	±29.8	
	Lfl	32.4	±46.6	Mean per species (±SD)
	Witz.	13.4	±12.6	18.9 pg/rct (± 31.2)
				Min: 0.036 pg/rct
				Max: 180.3 pg/rct

4.2.3. Calculated concentration of *Fusarium* species in diseased pea roots

Concentration of the *Fusarium* species can be calculated with use of the equations in **figure 6**. In our case, the concentrations were automatically calculated by the Rotor-Gene Q series software and then used for analysis. For analysis, the values for Feldbach C2 were excluded. Calculated concentrations of the three *Fusarium* spp. are shown in **figure 7** and mean concentrations of each *Fusarium* spp. are shown in **table 10**.

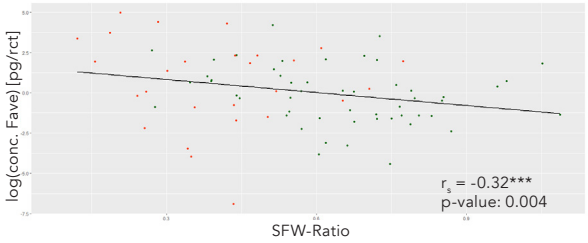
Overall, detected Fusairum DNA ranges from 0.001 pg/rct to 180.3 pg/rct. Detected maximal and minimal values are different for the three *Fusarium* spp. **(Table 10)** with highest mean values for *F. solani* and *F. oxysporum* concentrations (mean conc.: 18.9 (±31.2) and 15.5 (±17.9), respectively) and with 2-2.5 fold lower mean values for *F. avenaceum* (7.44 (±20.3)) concentration.

Differences between replicates of all three *Fusarium* spp. are significant (*F. ave*: $8.86e^{-05}$ ***, *F. ox*: $2.351e^{-06}$***, *F. sol*: $1.317e^{-04}$***). Also differences between soils of all three *Fusarium* spp. are significant (*F. ave*: $1.463e^{-04}$*** , *F. ox*: $3.135e^{-05}$***, *F. sol*: $7.654e^{-12}$***). For *F. avenaceum*, DNA concentration of Lfl differs significantly to the concentration of the other three soils. For *F. oxysporum*, the concentration differs significantly between Lfl and Häberli and Witzenhausen. For *F. solani*, difference is significant between Feldbach and Lfl. Differences among the accessions in all *Fusarium* spp. are significant too (*F. ave*: 0.013* , *F. ox*: 0.004**, *F. sol*: $1.073e^{-05}$***). A significant accession-soil interaction can be found for *F. oxysporum* and *F. solani* (0.001** and 0.002**, respectively). Within accessions, significant differences were only found for *F. solani* DNA between soils for C1, G89 and S134. There, *F. solani* DNA concentration differs significantly between Feldbach and the labelled soils **(Fig. 7 (c))**.

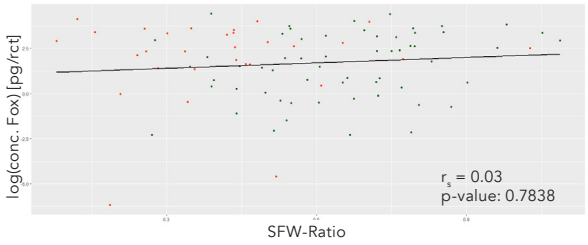
In general, the results are quite variable and differences between accession, soils and replicates were found for most *Fusarium* species. To gain an idea of interactions between DNA concentration and pea performance, correlation between *Fusarium* spp. and shoot fresh weight-ration & root rot ratings were calculated **(Fig. 8)**.

Fusarium spp. concentration ~ Shoot fresh weight ratio

Fusarium avenaceum



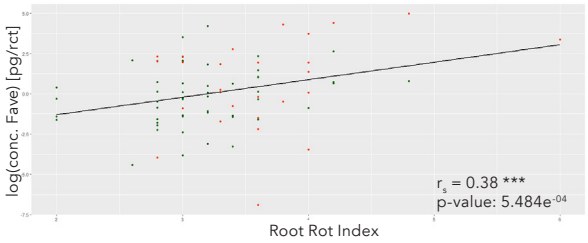
Fusarium oxysporum



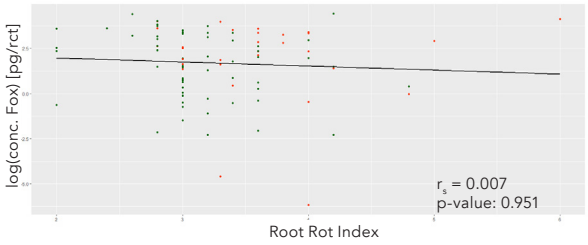
Fusarium solani

Fusarium spp. concentration ~ Root rot rating

Fusarium avenaceum



Fusarium oxysporum



Fusarium solani

Figure 8: Correlation between *Fusarium* DNA in roots of diseased peas grown for 29 day on the four non-sterilised soils and sfw-ratio & root rot rating. Correlation is calculated using rank correlation by Spearman. Red dots indicate pea accession which are characterised as susceptible and green dots indicate pea accession which are more tolerant.

Table 11: Calculated correlations using rank correlation by Spearman. Overall correlations for the three sick soils and correlations for the single soils were assessed.

Fave-sfw-ratio		
Soil	Correlation	p-value
Sick Soils	-0.32	0.004*
Feldbach	-0.15	0.569
Häberli	-0.21	0.326
Lfl	-0.32	0.075
Witzenhausen	-0.21	0.331

Fox-sfw-ratio		
Soil	Correlation	p-value
Sick Soils	0.03	0.784
Feldbach	-0.13	0.532
Häberli	-0.15	0.431
Lfl	-0.43	0.013
Witzenhausen	0.02	0.917

Fsol-sfw-ratio		
Soil	Correlation	p-value
Sick Soils	-0.45	1.048e^{-05}***
Feldbach	-0.33	0.139
Häberli	-0.51	0.004*
Lfl	-0.54	0.002*
Witzenhausen	-0.04	0.854

FAVE-RRI		
Soil	Correlation	p-value
Sick Soils	0.38	5.484e^{-04}***
Feldbach	0.23	0.395
Häberli	0.07	0.748
Lfl	0.35	0.057
Witzenhausen	0.01	0.972

FOX-RRI		
Soil	Correlation	p-value
Sick Soils	0.007	0.951
Feldbach	0.52	0.001*
Häberli	0.10	0.616
Lfl	0.56	8.746e^{-4}***
Witzenhausen	-0.08	0.709

Fsol-RRI		
Soil	Correlation	p-value
Sick Soils	0.56	1.559e^{-10}***
Feldbach	0.25	0.255
Häberli	0.53	0.003*
Lfl	0.68	2.147e^{-05}***
Witzenhausen	-0.05	0.807

4.2.4. Correlation between *Fusarium* DNA and disease score rating & shoot fresh weight

At a first step, correlations were assessed on a global level, including all three sick soils. Results can be seen in **figure 8** and spearman correlation coefficients can be found in **table 11**. Correlation plots (a) with the correlation between *Fusarium* DNA and shoot fresh weight (sfw)-ration and (b) with the correlation of *Fusarium* DNA and root rot index (RRI). Red dots indicate accessions with a higher susceptibility to pea root rot than the green dots (more tolerant pea accessions). Significant correlations for shoot fresh weight-ratio and *Fusarium* DNA concentration were found for *F. avenaceum* and *F. solani*. Sfw-ratio is in both cases negatively correlated with *Fusarium* concentration. Hence, a higher *Fusarium* concentration in the roots results in a lower sfw-ratio. Spearman rank correlation is -0.45 for *F. solani* and -0.32 for *F. avenaceum*, the correlation is for both significant (p-values: $1.048e^{-05}$*** & 0.004*, respectively). No significant correlation was observed for *F. oxysporum* and sfw-ratio (p-value: 0.8001).

Also no significant correlation was found for *F. oxysporum* DNA and root rot ratings for the three soils. Whereas a significant correlation was found for *F. avenaceum* (r_s = 0.38***) and *F. solani* (r_s = 0.56***). Here, *Fusarium* concentration is positively correlated with RRI. Means that a higher concentration of *Fusarium* DNA in the roots of pea results in a higher RRI.

With a more detailed look at the different soils, results become less consistent. For *F. avenaceum* DNA ~ sfwratio and *F. avenaceum* ~ RRI, no significant correlation for single soils was observed. Whereas for *F. oxysporum* DNA ~ RRI the two soils Feldbach and Lfl show a significant correlation (p-value: 0.001* and $8.746e^{-04}$*** ,respectively) where beforehand no overall correlation (over all three sick soils) was found. *F. solani* DNA ~ sfwratio shows a significant correlation for Häberli and Lfl soil (p-values: 0.004* and 0.002*, respectively) and also the correlations for *F. solani* DNA ~ RRI in the Häberli and Lfl (p-values: 0.003 * and $2.147e^{-05}$***,respectively) soil are significant. Overall, no significant correlation was found for Witzenhausen and sfw or RRI.

4.3. Inoculation Trial

4.3.1. Identification of fungal isolates

Ten different fungal isolates, previously isolated from diseased roots grown on Häberli soil, were used for the inoculation of pea plants grown on a sterile substrate. Pea plants were inoculated with one single pathogen isolate per pot. Three out of ten isolates were already identified as *F. solani*, *F. avenaceum* and *F. oxysporum* (identified by Adnan Šišić; University of Witzenhausen). The other seven isolates were not yet identified.

It was possible to show, that the three previously identified *Fusarium* spp. were correctly identified due to their morphological characteristics. Furthermore, five out of the seven isolates were identified as *F. oxysporum* and the other two remained unknown. qPCR assay included *F. solani*, *F. avenaceum* and *F. oxysporum* primer.

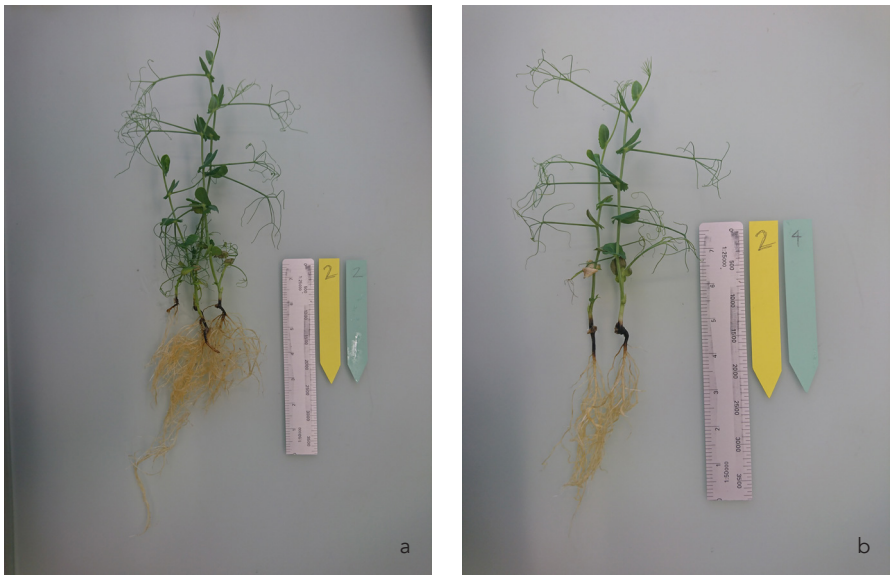


Figure 9: (a and b) Disease symptoms induced in *Pisum sativum* variety C2. Pictures of pea plants were taken after 28 days of growing on a sterile substrate but inoculated at day of sowing with fungal isolats.

4.3.2. Inoculation of pea variety Respect with fungal isolates reduces plant biomass

Shoot fresh weight (sfw) per plant was highest in the non-inoculated control and is significalntly higher compared to the inoculated variant (p-value: 0.020*) **(Fig. 10)**. Inoculation with the ten different isolates resulted in lower sfw-ratios per plant and lower emergence rates **(Table 12 & Fig. 10).**

Disease score was assessed according to Pflughöft (2008) after 28 days of growing on a sterile substrate. First, the attempt was made to use the same disease rating system used for the pot trial (according to L.W.). But after investigation of the first roots, it was evident that another assessment system is needed. Roots grown in a complex medium showed symptoms all over the root system **(Fig. 3, a-f, page 15)**, whereas roots grown on sterile medium and only inoculated with one pathogen isolate showed only detectable symptoms around root and shoot base **(Fig. 9)**. Symptoms from brown to black lesion from covering only small parts of the root or stem to completely surrounding root and stem were observed. Relative lower disease scores can be seen for the inoculation with *F. avenaceum* (mean RRI of 1.5) but differences are not significant **(Fig. 11 and Table 12)**. Inoculation for *F. avenaceum* differed compared to the other isolates, as not enough spores were produced for inoculation, mycelium was directly added to the pots. Inoculation with *F. oxysporum* and *F. solani* resulted in similar disease scores (mean RRI between 4.8-6). The diseases symptoms caused by the unknown isolate shows similar disease score ratings as *F. oxysporum*.

For correlation test between sfw per pot and RRI, not emerged plants were rated with a disease score of 9. Disease score ratings correlate with sfw per pot **(Fig. 12)**. A Spearman rank correlation of -0.76 (p-value: $3.773e^{-05***}$) was calculated. Spearman rank correlation is not significant without rating of not emerged seed with a disease score of 9 (r_s = -0.30, p-value: 0.115).

Differces between emergance rates among the isolates are not significant according to Kruskal (Kruskal-Wallis multiple comparison p-values adjusted with the Benjamini-Hochberg method). Howerver, sample size is to small to draw a clear conclusion.

Table 12: Mean emergance rates and mean root rot index after 14 days after sowing. Four seeds were planted per pot.

Inoculated with:	Mean emergance (%) after 14 days	Mean RRI (±SD) after 14 days		Mean sfw (±SD) after 14 days	
(none) Control	100	1.0	(±0)	0.76	(±0.12)
F. ave	33.33	1.5	(±0.71)	0.39	(±0.13)
F. sol	75.00	5.7	(±0.58)	0.44	(±0.11)
F. oxy	50.00	5.3	(±0)	0.21	Na
F. oxy 1	66.67	4.8	(±0.38)	0.56	(±0.32)
F. oxy 2	66.67	4.8	(±0.24)	0.53	(±0.11)
F. oxy 3	75.00	4.4	(±1.50)	0.42	(±0.31)
F. oxy 4	58.33	4.1	(±1.64)	0.52	(±0.09)
F. oxy 5	50.00	6.0	(±1.41)	0.22	(±0.18)
Unknown 1	50.00	4.5	(±1.50)	0.52	(±0.17)

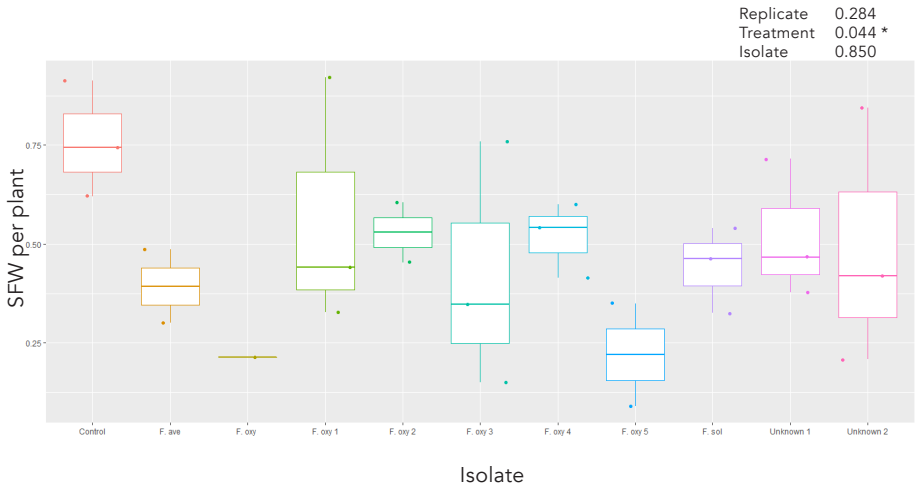


Figure 10: Shoot fresh weigth per plant assessed after 28 days of growing on a sterile substrate and inoculated with fungal isolate at the day of sowing.
Treatment = inoculated/non-inoculated

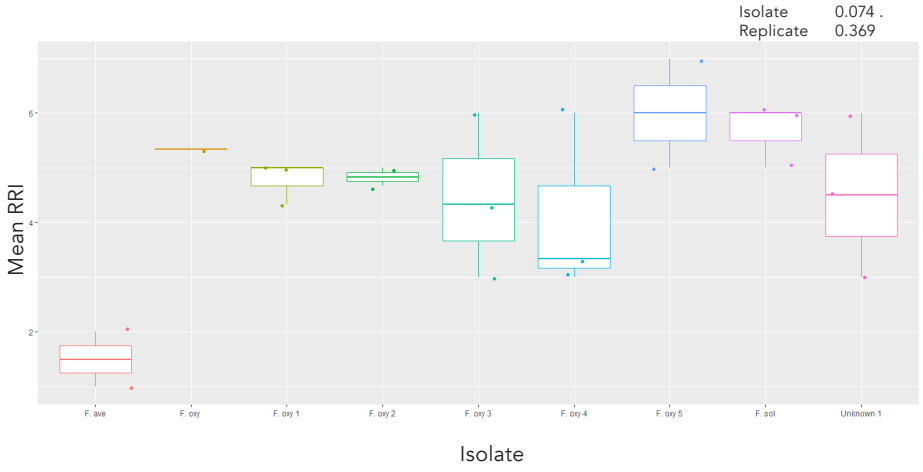


Figure 11: Mean disease score per pot assessed after 28 days of growing on a sterile substrate and inoculated at day of sowing.

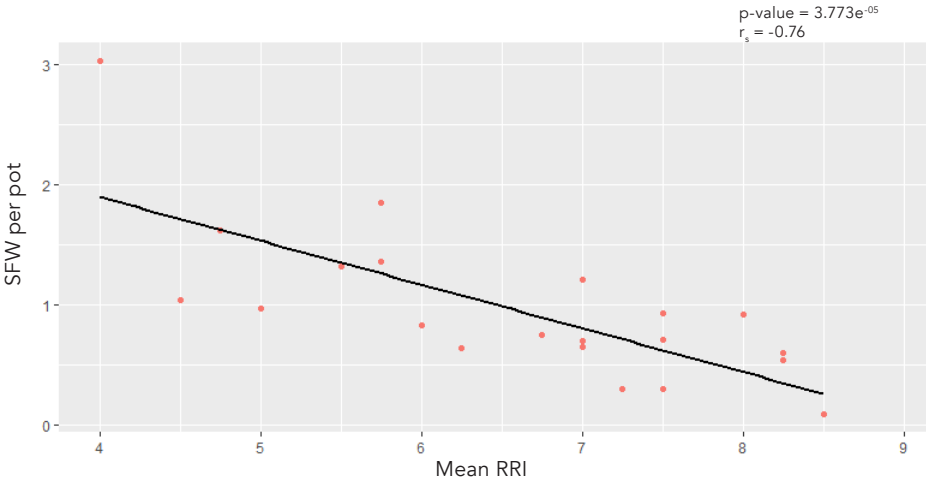


Figure 12: Correlation of shoot fresh weight (sfw) per pot with mean root rot rating (RRI) per pot calculated using rank correlation by Spearman.

5. Discussion

Resistance against root-rot pathogens is often assessed under controlled conditions, using single spore isolates to inoculate pea plants (Willsey et al., 2018; Zitnick-Anderson et al., 2018). Resistance ranking of evaluated accessionss can be done upon plant biomass measurements, disease index or plant health assessments (Infantino et al., 2006; Walter et al., 2015). Alternatively, resistance screens of up to hundreds of accessionss are performed in the field with a known predominance of a single major pathogen species or strain. These approaches have led to the identification of resistance breeding material and resistance loci in the pea genome (Coyne et al., 2015; Desgroux et al., 2016; Pilet-Nayel et al., 2017). However, screening against single pathogens has its limitations as resistance mechanisms can be overcome or differently expressed in the field due to the plant's complex interactions with a vast variety of soil microbes. Therefore, the pea screening 2017 was conducted with a complex growth media under controlled conditions to combine the advantages of a controlled environment and the inclusion of a complex microorganism community.

The present study successfully verified a soil-based screening assay of 312 pea accessions with a subset of eight accessions selected based on their contrasting resistance capacities. Furthermore, the eight pea accession were also evaluated on two sick soils from Germany and on one healthy Swiss soil, indicating that the resistance ranking assessed on one sick swiss soil is, to some extent, also applicable to the other two sick soils used in this study. Furthermore, the study showed that it is possible to detect and quantify the three different *Fusarium* spp. in the roots of diseased pea plants with the implemented qPCR assay and that certain *Fusarium* spp. quantities correlate with the assessed biomass measurements and disease ratings, indicating the importance of *F. avenaceum* and *F. solani* in the disease expression of pea root rot (PRR) in young seedlings. Furthermore, the results from our inoculation trial displayed that besides *F. avenaceum*, *F. oxysporum* and *F. solani* other pathogens must be involved in the disease expression in our soils. Root rot symptoms were different between pea plants inoculated with only one *Fusarium* spp. compared to the root rot symptoms induces by a complex of different pathogens in our soils.

Sterilisation of soils increased seed emergence, suggesting that a biological factor is involved in disease expression and reduction of seed emergence, confirming that the three soils are sick. Another study revealed similar effects on emergence rates, which were close to 100 % after sterilisation of soils (Fuchs et al., 2014). Pathogens involved in the pea root rot complex are reported to affect pea emergence and growth at early plant stages (Fuchs et al., 2014; Rubiales et al., 2014). Reduced seedling growth and emergence rate is described as damping-off and in pea most frequently caused by *Phytium ultimum* and *Rhizoctonia solani* (Rubiales et al., 2014). Also a recent study at FiBL identified Oomycetes as key agents for reducing seed emergence rates and growth of seedlings (Fuchs et al., 2014). In addition to Oomycetes, several *Fusarium* spp., *Macrophomina phaseolina* and *Phytophthora* spp are known for their potential to contribute to damping off. Within *Fusarium*, *Fusarium solani* is reported to be the main agent of damping-off (Rubiales et al., 2014). As Fusarium spp. were found in the roots of diseased pea plants, and as also *F. solani* was detected, a contribution to a reduction in seed emergence and seedling growth could be possible. To narrow down the effect of reduced seed emergence to single pathogens, an inoculation trial was set up to determine pathogenicity of single *Fusarium* isolates. Results give an indication that *Fusarium* spp. might have an impact on seed emergence, as inoculation with different *Fusarium* isolates reduced emergence rates of pea seeds. However, as it was not possible to point out significant differences between the isolates, a novel inoculation trial with a higher sample size would be needed to draw clear conclusions.

Although not reported as the main agent among *Fusarium* spp. to cause damping-off, *F. avenaceum* showed the highest impact on emergence rate in our inoculation trial, with only 33 % of the seeds emerged. Contrastingly, *F. solani* is reported to be the main agent among *Fusarium* spp. to reduce seed emergence (Rubiales et al., 2014), in this study, *F. solani* inoculation reduced emergence rate only by 25 % . Inoculation with *Fusarium* isolates not only affected seed emergence but also shoot fresh weight of pea seedlings grown on sterile substrate which is discussed later on under section inoculation trial.

A negative effect on shoot fresh weight (sfw) per plant was also observed in the soil-based pot trial, were biomass was reduced between 11-56 % when grown on non-sterilised compared to sterilised soil. A sfw-ratio was assessed to gain a direct comparison between the performance of the pea accessions on sterilised vs. non-sterilised soil. Shoot fresh weight ratios for accessions grown on Feldbach soil were higher than the ones grown on the three sick soils. These findings are consistent with our expectations, as Feldbach soil is known to have no pea cultivation issues. A significant reduction of sfw-ratios for more susceptible pea accession was found in the Häberli and Lfl soil. Performance of the pea accessions on the other two soils than Häberli and Lfl revealed similar trends. Furthermore, sfw-ratios of our pot trial and screen data 2017 show significant correlations, indicating that the reproducibility of the screen 2017 is possible. Highest correlation was found for Häberli soil, followed by Lfl soil and no significant correlation for Witzenhausen. The here presented experiment did not reveal statistically significant soil x pea accession effects, thus indicating that resistance ranking performed on a sick soil might be applicable to other soils. This would breeder allow to select on one heavily infested soil for resistance against a pathogen complex with a possible expression of the resistance also on other soils.

Along shoot fresh weight ratios, also root rot ratings were assessed after 29 days of growing. Root appearance among the eight tested pea accession in the four soils ranged from healthy voluminous roots to completely decayed root systems. Observed disease symptoms (Fig 3, a-f) appeared to be very similar to the symptoms reported recently by Willsey et al. (2018) (Fig. 1). Willsey et al. (2018) performed inoculation of pea plants with several single pathogens or a combination of them (*Aphanomyces euteiches*, *Fusarium redolens*, *Fusarium solani*, *Fusarium avenaceum*) and found an increase in severity when multiple pathogens were inoculated together. In our pot trial, healthy looking roots were only found in Feldbach soil compared to roots of pea plants grown in the other three soils with higher root rot ratings. Overall, root rot ratings correlate significantly with sfw-ratios, confirming resistance rankings of the tested accession.

Root rot ratings for Feldbach soil were lower compared to the ones of the three sick soils. Higher mean root rot ratings were found in pea accession rated as susceptible to the pea root rot complex compared to the tolerant accessions. Differentiation between the eight pea accessions was less obvious for root rot ratings than for shoot fresh weight ratio. Nevertheless, mean root rot values for susceptible accessions were in every soil higher than the ones from tolerant and significantly different in Häberli and Lfl soil. Lower root rot ratings indicate a lower disease pressure or effect of soil microbial community on pea performance. This is supported by our qPCR results where we found a positive significant correlation between *Fusarium* DNA quantity and root rot for certain soils indicating the importance of these *Fusarium* spp. in the disease expression. Similar findings were also observed by Xue (2002) and Zitnick-Anderson et al. (2018), where the quantity of fungal soil community correlated with disease score ratings.

In our soils *Fusarium avenaceum*, *F. oxysporum* and *F. solani* quantity was successfully determined with the established qPCR assay. Different amounts of *Fusarium* spp. DNA were detected in the roots of diseased pea plants with generally higher amounts for *F. oxysporum* and *F. solani* compared to *F. avenaceum*. If differences between the *Fusarium* DNA quantity is due to a biological or technical reason is unclear but all three *Fusarium* spp. are reported to be frequently isolated from diseased pea roots and play a key role in the expression of pea root rot (Chittem et al., 2015; Feng et al., 2010; Xu et al., 2012). Besides root rot ratings, correlations were also assessed for *Fusarium* quantity and shoot fresh weight ratios. Take together these results (corr. for sfw-ratio & RRI) indicate that *F. solani* is important for disease expression in Häberli soil. For Lfl, *F. solani* seems also to play an important role but also *F. avenaceum* may contribute to the disease expression. Furthermore, none of the three quantified pathogens correlates with RRI and sfw in the Witzenhausen soil. Thus, indicating that other pathogens responsible for root disease could be important in this soil (e.g. *Didymella* spp.) (Pflughöft et al., 2012). Therefore, we might have different compositions of virulent pathogens in our four soils. Resistance mechanism of our more tolerant accessions might therefore only be valid on soils with a similar pathogen complex and similar virulent pathogens. Correlation between screen data 2017 and pot trial 18 indicate, that our resistance ranking is applicable when a similar microorganism composition is present. This would partially hamper our thesis, that it might possible to breed on one single soil for resistance to different pathogen complexes.

To get a deeper insight in soil pathogen community, and to get more information about resistance mechanism of our pea accessions, the inclusion of further pathogens accredited to the pea root complex would be a next step. Furthermore, inclusion of certain antagonist or health and growth promoting microorganism as arbuscular mycorrhiza fungi could be a further step. To forward the analysis with the qPCR assay, the inclusion of a multiplex assay would reduce time needed for sample screening.

Nonetheless, importance of *F. solani* and *F. avenaceum* are highlighted by our findings where a significant correlation for *Fusarium* quantity with RRI and sfw was found. Importance and effect of the two *Fusarium* species on pea cultivation is reported (Bodah et al., 2016) and similar findings were observed by Zitnick-Anderson et al. (2018), where also a positive correlation for *Fusarium* DNA quantity and RRI was found. Also Vandemark and Barker (2003) found a significant correlation between quantified pathogen DNA (for Aphanomyces euteiches) in the roots of pea and disease severity ratings.

But a high amount of fungal DNA in diseased roots does not necessarily support the pathogenicity and effect of a pathogen to the plant. As all three *Fusarium* spp. were detected and quantified in the roots of diseased pea but with different contributions to the expression of pea root rot, we can assume to have different virulences among the three *Fusarium* spp. to pea. Another explanation could be the occurrence of interspecific competition between or among *Fusarium* spp (Velluti et al., 2000). Different colonization rates of combined inoculated *Fusarium* spp. were previously detected by Zitnick-Anderson et al. (2018), suggesting competition between *Fusarium* strains. Furthermore, *F. oxysporum* is reported to be ubiquitous and present in many soils worldwide and commonly colonizes already necrotic roots and can therefore be mistaken as primary agent of necrosis (Chittem et al., 2015; Leslie and Summerell, 2006). Therefore, *F. oxysporum* isolates should be tested for their pathogenicity to pea before conclusion can be drawn about their contribution to the disease symptoms (Leslie and Summerell, 2006). This also accounts for the other *Fusarium* spp. detected in the roots of our pea plants and therefore we set up an inoculation trial including several *Fusarium* isolates.

Identification of the so far unknown isolates was possible with the established qPCR assay. Prior identification of three isolates as *F. solani*, *F. avenaceum* and *F. oxysporum* was confirmed and another five out of seven isolates were identified as *F. oxysporum*. Identification of two other isolates was not possible as only the three *Fusarium* primers (FOX, FSOL and FAVE) were used.

Results from our inoculation trial show a reduction of sfw due to an inoculation with the different *Fusarium* isolates. *Fusarium oxysporum* may induce weak to strong disease symptoms similar to *F. solani*. Shoot fresh weight reduction due to an inoculation with *F. avenaceum* is in the range of the other *F. oxysporum* isolates but disease score ratings assessed for pea plants inoculated with *F. avenacuem* are considerably lower than the ratings for *F. oxysporum* and *F. solani*.

F. oxysporum isolates may induce disease symptoms in pea roots grown on a sterile substrate, but our findings of the pot trial indicate that *F. oxysporum* is not a primary pathogen in inducing disease symptoms in pea roots of plants grown on a complex medium. Infection might be possible as a second invader of already necrotic roots but interspecific competition between or among *Fusarium* spp. may reduce the effect of *F. oxysporum* in a complex system (Leslie and Summerell, 2006; Velluti et al., 2000; Willsey et al., 2018). Disease symptoms induced by the complex microorganism community in our soils look similar to the ones reported by Willsey et al. (2018) for inoculation with several pathogens. Disease symptoms in roots grown on our complex medium support the presence of other pathogenic microorganism than *Fusarium* spp. as single inoculation with *Fusarium avenaceum*, *F. oxysporum* and *F. solani* revealed different symptoms in the roots of pea. Combination of different *Fusarium* spp. and other soil-borne pathogens may lead to increased disease symptoms in the roots of pea as reported by Willsey et al. (2018). Once more, this indicates that other pathogens could be important in the soils used for our soil-based pot trial. (Pflughöft et al., 2012)

Our findings support the fact that the use of culture based identification techniques is an adequate method to isolate pathogens from diseased roots of pea plants, but it may not always be possible to isolate the main agents of a disease and isolated pathogens do not necessarily contribute to a disease expression under natural conditions with competition between microorganism for root space and resources (Lamichhane and Venturi, 2015).

In conclusion, it was only partially possible to perform the verification of the pathogenicity of the three *Fusarium* spp. However, the results from our inoculation trial displayed that besides *F. avenaceum*, *F. oxysporum* and *F. solani* other pathogens must be involved in the disease expression. Nevertheless, it was possible to verify the soil-based screening assay with a subset of eight accessions selected based on their contrasting resistance capacities and to evaluated the eight pea accession on two sick soils and on one healthy soil, indicating that the resistance levels assessed on one sick soil is partially applicable to the other two sick soils used in this study. Moreover, the study displayed that it is possible to detect and quantify the three different *Fusarium* spp. in the roots of diseased pea plants with the implemented qPCR assay.

Acknowledgements

The author is thankful to Lukas Wille, doctoral student in the Molecular Plant Breeding Group ETHZ/ Plant Breeding FiBL for technical assistance in the lab and support during the complete master thesis, to Thomas Oberhänsli for support in the lab and to Agata Leska, Daniel Ortler and Anja Wille for support during sampling of the pot trial.

Special thanks to Prof. Dr. Bruno Studer, Molecular Plant Breeding Group ETHZ, and Dr. Pierre Hohmann, Plant Breeding FiBL for supervision.

Besides the official thesis supervisors, I would also like to acknowledge Monika Messmer, head of the and Variety Testing group at FiBL.

6. References

Alemu, K. (2014). Real-Time PCR and Its Application in Plant Disease Diagnostics. Advances in Life Science and Technology.

Baćanović-Šišić, J., Šišić, A., Schmidt, J. H., and Finckh, M. R. (2017). Identification and characterization of pathogens associated with root rot of winter peas grown under organic management in Germany. European Journal of Plant Pathology 151, 745-755.

Baćanović, J. (2015). Pathogens occurring in the winter pea – maize – winter wheat rotation, their host specificity and the potential of compost in suppressing foot and root disease of peas. .

Baddeley, J. A., Jones, S., Topp, C. F. E., Watson, C. A., Helming, J., and Stoddard, F. L. (2013). Biological nitrogen fixation (BNF) by legume crops in Europe.

Bénézit, M., Biarnès, V., and Jeuffroy, M.-H. (2017). Impact of climate and diseases on pea yields: what perspectives with climate change? Ocl 24.

Berendsen, R. L., Pieterse, C. M., and Bakker, P. A. (2012). The rhizosphere microbiome and plant health. Trends Plant Sci 17, 478-86.

Biddle, A. J., and Cattlin, N. D. (2007). „Pests, Diseases and Disorders of Pea and Beans."

Biddle, A. J. C., Nigel D. (2007). „Pests, Diseases and Disorders of Peas and Beans - A Colour Handbook."

Bodah, E. T., Porter, L. D., Chaves, B., and Dhingra, A. (2016). Evaluation of pea accessions and commercial cultivars for fusarium root rot resistance. Euphytica 208, 63-72.

Carranca, C., de Varennes, A., and Rolston, D. (1999). Biological nitrogen fixation by fababean, pea and chickpea, under field conditions, estimated by the 15N isotope dilution technique. European Journal of Agronomy 10, 49–56.

Castell, A. G. G., W., and F.A., I. (1996). Nutritive value of peas for nonruminant diets. Animal Feed Science Technology 60.

Cesarano, G., Zotti, M., Antignani, V., Marra, R., Scala, F., and Bonanomi, G. (2017). Soil sickness and negative plant-soil feedback: A reappreaisal of hypotheses

Chittem, K., Mathew, F. M., Gregoire, M., Lamppa, R. S., Chang, Y. W., Markell, S. G., Bradley, C. A., Barasubiye, T., and Goswami, R. S. (2015). Identification and characterization of Fusarium spp. associated with root rots of field pea in North Dakota. European Journal of Plant Pathology 143, 641-649.

Coyne, C. J., Pilet-Nayel, M.-L., McGee, R. J., Porter, L. D., Smýkal, P., Grünwald, N. J., and Link, W. (2015a). Identification of QTL controlling high levels of partial resistance toFusarium solanif. sp.pisiin pea. Plant Breeding 134, 446-453.

Coyne, C. J., Pilet-Nayel, M.-L. L., McGee, R. J., Porter, L. D., Smýkal, P., and Grünwald, N. J. (2015b). Identification of QTL controlling high levels of partial resistance to Fusarium solani f. sp. pisi in pea. Plant Breeding 134, 446-453.

DAFF-SA (2011). Production Guideaccessionss - Garden Peas

Department of Agriculture, Forestry and Fisheries, South Africa.

Dahl, W. J., Foster, L. M., and Tyler, R. T. (2012). Review of the health benefits of peas (Pisum sativum L.). Br J Nutr 108 Suppl 1, S3-10.

Desgroux, A., Anthoëne, V. L., Roux-Duparque, M., Rivière, J.-p., Aubert, G., Tayeh, N., Moussart, A., Mangin, P., Vetel, P., Piriou, C., Mcgee, R. J., Coyne, C. J., Burstin, J., Baranger, A., Manzanares-dauleux, M., and Bourion, V. (2016). Genome-wide association mapping of partial resistance to Aphanomyces euteiches in pea. BMC Genomics, 1-21.

Feng, J., Hwang, R., Chang, K. F., Conner, R. L., Hwang, S. F., Strelkov, S. E., Gossen, B. D., McLaren, D. L., and Xue, A. G. (2011). Identification of microsatellite markers linked to quantitative trait loci controlling resistance to Fusarium root rot in field pea. Canadian Journal of Plant Science 91, 199-204.

Feng, J., Hwang, R., Chang, K. F., Hwang, S. F., Strelkov, S. E., Gossen, B. D., and Conner, R. L. (2010). Genetic variation in Fusarium avenaceum causing root rot on field pea.

Fondevilla, S., Küster, H., Krajinski, F., Cubero, J. I., and Rubiales, D. (2011). Identification of genes differentially expressed in a resistant reaction to Mycosphaerella pinodes in pea using microarray technology.

Foyer, C. H., Lam, H. M., Nguyen, H. T., Siddique, K. H., Varshney, R. K., Colmer, T. D., Cowling, W., Bramley, H., Mori, T. A., Hodgson, J. M., Cooper, J. W., Miller, A. J., Kunert, K., Vorster, J., Cullis, C., Ozga, J. A., Wahlqvist, M. L., Liang, Y., Shou, H., Shi, K., Yu, J., Fodor, N., Kaiser, B. N., Wong, F. L., Valliyodan, B., and Considine, M. J. (2016). Neglecting legumes has compromised human health and sustainable food production. Nat Plants 2, 16112.

Fuchs, J. G., Thuerig, B., Brandhuber, R., Bruns, C., Finckh, M. R., Fließbach, A., Mäder, P., Schmidt, H., Vogt-Kaute, W., Wilbois, K.-P., and Lucius, T. (2014). Evaluation of the causes of legume yield depression syndrome using an improved diagnostic tool. Applied Soil Ecology 79, 26-36.

Graham, P. H., and Vance, C. P. (2003). Legumes: importance and constraints to greater use. Plant Physiol 131, 872-7.

Hamon, C., Coyne, C. J., McGee, R. J., Lesne, A., Esnault, R., Mangin, P., Herve, M., Le Goff, I., Deniot, G., Roux-Duparque, M., Morin, G., McPhee, K. E., Delourme, R., Baranger, A., and Pilet-Nayel, M. L. (2013). QTL meta-analysis provides a comprehensive view of loci controlling partial resistance to Aphanomyces euteiches in four sources of resistance in pea. BMC Plant Biol 13, 45.

Hewins, C. R., Carrino-Kyker, S. R., and Burke, D. J. (2015). Seasonal variation in mycorrhizal fungi colonizing roots of Allium tricoccum (wild leek) in a mature mixed hardwood forest. Mycorrhiza 25, 469-83.

Hohmann, P., and Messmer, M. M. (2017). Breeding for mycorrhizal symbiosis: focus on disease resistance. Euphytica 213.

Hohmann, P., Wille, L., Studer, B., Bodenhausen, N., and Messmer, M. (2017). Breeding for microbiome-mediated disease resistance. In „International Symposium on Microbe-assisted crop production – opportunities, challenges & needs", pp. S. 48.

Infantino, A., Kharrat, M., Riccioni, L., Coyne, C. J., McPhee, K. E., and Grünwald, N. J. (2006). Screening techniques and sources of resistance to root diseases in cool season food legumes. Euphytica 147, 201-221.

Jiménez-Fernández, D., Montes-Borrego, M., Navas-Cortés, J. A., Jiménez-Díaz, R. M., and Landa, B. B. (2010). Identification and quantification of Fusarium oxysporum in planta and soil by means of an improved specific and quantitative PCR assay. Applied Soil Ecology 46, 372-382.

Joshi, P. K., and Rao, P. P. (2017). Global pulses scenario: status and outlook. Ann N Y Acad Sci 1392, 6-17.

Karkanis, A., Ntatsi, G., Kontopoulou, C.-K., Pristeri, A., Bilalis, D., and Savvas, D. (2016). Field Pea in European Cropping Systems: Adaptability, Biological Nitrogen Fixation and Cultivation Practices. Notulae Botanicae Horti Agrobotanici Cluj-Napoca 44.

Kraft, J. M., and Pfleger, F. L. (2001). „Compendium of pea diseases and pests. ."

Lamichhane, J. R., and Venturi, V. (2015). Synergisms between microbial pathogens in plant disease complexes: a growing trend. Front Plant Sci 6, 385.

Leslie, J. F., and Summerell, B. A. (2006). „The Fusarium Laboratory Manual „.

Mendes, L. W., Raaijmakers, J. M., de Hollander, M., Mendes, R., and Tsai, S. M. (2018). Influence of resistance breeding in common bean on rhizosphere microbiome composition and function. ISME J 12, 212-224.

Mirmajlessi, S. M., Loit, E., Mänd, M., and Mansouripour, S. M. (2016). Real-time PCR applied to study on plant pathogens: potential applications in diagnosis – a review. Plant Protection Science 51, 177-190.

Mishra, P. K. F., Roland T.V., and Culham, A. (2003). Development of a PCR-based assay for rapid and reliable identi¢cation of pathogenic Fusaria. FEMS Microbiology Letters 218, 329^332.

Nayyar, A., Hamel, C., Lafond, G., Gossen, B. D., Hanson, K., and Germida, J. (2009). Soil microbial quality associated with yield reduction in continuous-pea. Applied Soil Ecology 43, 115-121.

Ogle, D. H., Wheeler, P., and Dinno, A. (2018). FSA: Fisheries Stock Analysis.

Omega-BioTek (2019). mag-bind-plant-dna-ds-96-kit_protocoll.

Pflughöft, O. (2008). Pilzkrankheiten in Körnerfuttererbsen (Pisum sativum L.) – Diagnose, Epidemiologie, Ertragsrelevanz und Bekämpfung.

Pflughöft, O., Merker, C., von Tiedemann, A., and Schäfer, B. C. (2012). Zur Verbreitung und Bedeutung von Pilzkrankheiten in Körnerfuttererbsen (Pisum sativum L.) in Deutschland. Gesunde Pflanzen 64, 39-48.

Pilet-Nayel, M.-L., Moury, B., Caffier, V., Montarry, J., Kerlan, M.-C., Fournet, S., Durel, C.-E., and Delourme, R. (2017). Quantitative Resistance to Plant Pathogens in Pyramiding Strategies for Durable Crop Protection. Frontiers in Plant Science 8, 1-9.

R-Core-Team (2017). R: A language and environment for statistical computing. . R Foundation for Statistical Computing, Vienna, Austria.
Rubiales, D., Fondevilla, S., Chen, W., Gentzbittel, L., Higgins, T. J. V., Castillejo, M. A., Singh, K. B., and Rispail, N. (2014). Achievements and Challenges in Legume Breeding for Pest and Disease Resistance. Critical Reviews in Plant Sciences 34, 195-236.
Rubiales, D., and Mikic, A. (2014). Introduction: Legumes in Sustainable Agriculture. Critical Reviews in Plant Sciences 34, 2-3.
Siczek, A., Lipiec, J., Wielbo, J., Szarlip, P., and Kidaj, D. (2013). Pea growth and symbiotic activity response to Nod factors (lipo-chitooligosaccharides) and soil compaction. Applied Soil Ecology 72, 181-186.
Siddique, K. H. M., Johansen, C., Turner, N. C., Jeuffroy, M.-H., Hashem, A., Sakar, D., Gan, Y., and Alghamdi, S. S. (2011). Innovations in agronomy for food legumes. A review. Agronomy for Sustainable Development 32, 45-64.
Šišić, A., Baćanović-Šišić, J., Al-Hatmi, A. M. S., Karlovsky, P., Ahmed, S. A., Maier, W., de Hoog, G. S., and Finckh, M. R. (2018). The ‚forma specialis' issue in Fusarium: A case study in Fusarium solani f. sp. pisi. Sci Rep 8, 1252.
Šišić, A., Baćanović, J., and Finckh, M. R. (2016). Endophytic Fusarium equiseti stimulates plant growth and reduces root rot disease of pea (Pisum sativum L.) caused by Fusarium avenaceum and Peyronellaea pinodella. European Journal of Plant Pathology 148, 271-282.
USDA (2012). Plant Guide - PEA Pisum sativum L.
Vandemark, G. J., and Barker, B. M. (2003). Quantifying the relationship between disease severity and the amount ofAphanomyces euteichesdetected in roots of alfalfa and pea with a real-time PCR assay. Archives Of Phytopathology And Plant Protection 36, 81-93.
Vandemark, G. J. G., Niklaus J. (2005). Use of real-time PCR to examine the relationship between disease severity in pea and Aphanomyces euteiches DNA content in roots. European Journal of Plant Pathology 111, 309-316.
Velluti, A., Martın, S., Bettucci, L., Ramos, A. J., and Sanchis, V. (2000). The effect of fungal competition on colonization of maize grain by Fusarium moniliforme, F. proliferatum and F. graminearum and on fumonisin B and zearalenone formation. International Journal of Food Microbiology 59, 59-66.
Walter, A., Liebisch, F., and Hund, A. (2015). Plant phenotyping: from bean weighing to image analysis. Plant Methods 11, 14.
Watson, C. A., Reckling, M., Preissel, S., Bachinger, J., Bergkvist, G., Kuhlman, T., Lindström, K., Nemecek, T., Topp, C. F. E., Vanhatalo, A., Zander, P., Murphy-Bokern, D., and Stoddard, F. L. (2017). Grain Legume Production and Use in European Agricultural Systems. pp. 235-303.
Wille, L., Messmer, M. M., Studer, B., and Hohmann, P. (2018a). Insights to plant-microbe interactions provide opportunities to improve resistance breeding against root diseases in grain legumes. Plant Cell Environ.
Wille, L., Studer, B., Messmer, M., Bodenhausen, N., and Hohmann, P. (2018b). Resistance screening of pea against a complex of root-rot pathogens. Poster at: DIVERSIFOOD Final Congress: Cultivating diversity and food quality, Rennes, Frances, 10. - 12.12.2018.
Willsey, T. L., Chattertona, S., Heynena, M., and Ericksona, A. (2018). Detection of interactions between the pea root rot pathogens Aphanomyces euteiches and Fusarium spp. using a multiplex qPCR assay. Plant Pathology 67, 1912–1923.
Wilman, K., Stepien, L., Fabianska, I., and Kachlicki, P. (2014). Plant-pathogenic fungi in seeds of different pea cultivars in Poland. Arh Hig Rada Toksikol 65, 329-38.
Wu, L., Chang, K.-F., Hwang, S.-F., Conner, R., Fredua-Agyeman, R., Feindel, D., and Strelkov, S. E. (2019). Evaluation of host resistance and fungicide application as tools for the management of root rot of field pea caused by Aphanomyces euteiches. The Crop Journal 7, 38-48.
Xu, L., Ravnskov, S., Larsen, J., and Nicolaisen, M. (2012). Linking fungal communities in roots, rhizosphere, and soil to the health status of Pisum sativum. FEMS Microbiol Ecol 82, 736-45.
Xue, A. G. (2002). Biological Control of Pathogens Causing Root Rot Complex in Field Pea Using Clonostachys rosea Strain ACM941.
Zitnick-Anderson, K., Simons, K., and Pasche, J. S. (2018). Detection and qPCR quantification of seven Fusarium species associated with the root rot complex in field pea. Canadian Journal of Plant Pathology 40, 261-271.
Zymo_Research (2019). ZR-Fungal_Bacterial DNA MiniPrep Kit.

7. Annex

7.1. Annex - Design & Planning Pot-Trial „Verification Screen"

Pea accessions:
- EFB, Respect
- 9 susceptible
- 9 tolerant

4 soils (2x4 factor levels):
- Häberli Sterile (S)/Non-Sterile (NS)
- Witzenhausen, S/NS
- Lfl, S/NS
- GZPK, Feldbach, S/NS

Design:
20 Accessions x 3 Soils x 2 steril x 4 Reps =
20 x 3 x 2 x 4

Completely Randomised Block Design,
Blocks = S/NS
352 Pots (Verification Screen und Comparison Soils - unbalanced):

20 Accessions x 2 S/NS x 1 Häberli soil x 4 Reps (20x2x1x4=160) +
8 Accessions x 2 S/NS x 3 (Witzenhausen/Lfl/GZPK/) x 4 Reps (8x2x3x4=192)

the EIGHT are also in the 20 and also in the Field trial

Total: 352 pots

Selection pea accessions:

All 20 Accessions:
„C1", „C2", „G78", „G85", „G89", „S64", „S134", „S14", „S22", „S91", "S177", „S111", „S80", „S150", „G24", „S118", „S5", „G27", „G1", „S51"

Subset, which is tested on three soils:
„C1", „C2", „G78", „G89", „S64", „S134", „S22", „S91"

For 20 accessions:
7 Seeds/Pot x 2 S/NS x 4 Reps x 1 Soil = 56 seeds total / accession

For 8 accessions:
8 Seeds/Pot x 2 S/NS x 4 Reps x 3 Soils = 192 seeds aditional to the 64 / accession = 256

- von 12 Linien 64/4 Reps = 16 + 14 Reserve = 30 Samen / Linie / Replikat
- von 8 Linien 256/4 Reps = 64 + 16 Reserve = 80 Samen / Linie / Replikat

Calculation Substrate:

Substrate Häberliboden:
160x600ml = 96L = 48L (NS) + 48L (S)
-> each 32L Soil + 16L Sand per treatment (without reserve)
160x1000ml = 160L = 80L (NS) + 80L (S)
-> je 53.3L soil + 26.6L sand per treatment

Substrate other soils:
64x600ml = 38.4L = 19.2L (NS) + 19.2L (S) for each soil -> 12.8L soil + 6.4L Sand pro treatment
64x1000ml = 64L = 32L (NS) + 32L (S) je für jeden Boden -> 21.3L Erde + 10.6L Sand per treatment

Implementation:

4x1 Reps -> 4x1d Sowing -> 5W growth
-> 4d harvest (72 pots/d)

Practical planning

-> 2/3 soil + 1/3 Sand, 12 cm pots (600 ml),
7 seeds /Topf

Max. grow chamber: 10 tables (à max. (or max-max. 28) 12er pots = 240 (280))

7.2. Annex - Culture media

PDA potato dextrose agar
39 g of medium plus 1 liter of destilled water

(Potato Extract Glucose Agar,
Carl Roth GmbH + Co. KG)

Kartoffel-Infus	4 g/l
Glucose	20 g/l
Agar	15 g/l
pH-Wert	5,6 ±0,2

SNA for 1 l

KH2PO4	1 g
KNO3	1 g
MgSO4*7 H2O	0.5 g
KCL	0.5 g
Glucose	0.2 g
Sucrose	0.2 g
Agar	20 g
H2O	1 l

7.3. Annex - Molecular laboratory equipment

- KingFisher™ Flex Purification System (KingFisher with 96 PCR head).
- Rotor-Gene (Qiagen) quantitative real time PCR system
- Bio-Rad, CFX96 real time system (Bio-Rad, CFX96 Real Time System, C100 Touch Thermal Cycler, CFX96 Optics Module)
- QIAgility (QIAGEN) qPCR setup system
- Omega Mag Bind DNS DS Extraction Omega Bio-Tek, Inc.
- TissueLyser II (Qiagen) sample disruption system
- Retsch Mixer Mill MM200
- NanoDrop™ 2000/2000c Spectrophotometers, Thermo Fischer Scienific
- Low Bind Greiner Bio-One GmbH Reaction Tubes, 1.5 ml, PP, graduated Attached cap, natural
- Standard Nolato Treff AG Tube, CaopLock, PP, CLEAR, 1.5 ml
- 200 ul tubes for dilution series
- Potato Extract Glucose Agar Ph.Eur., for Microbiologie ROTH, Carl Roth GmbH + Co. KG
- Bio Rad SSO AdvancedTM Universal SYBRR Green Supermix
- TEKU, MXC 12 (0475), PP, Durchm. 12 cm, Höhe 9 cm , Vol.: 0.66 l

7.4. Annex - Impressions pot trial

Pea accessions G89 and S64 grown on sterile and non-sterilised soil (Witzenhausen and Lfl) after seven, 14 and 21 days after sowing.

Pea accession G89 after seven days of growing on Witzenhausen soil. Sterile treatment

Pea accession G89 after seven days of growing on Witzenhausen soil. Non-Sterile treatment

Pea accession S64 after seven days of growing on Lfl soil. Sterile treatment

Pea accession S64 after seven days of growing on Lfl soil. Non-Sterile treatment

Pea accession G89 after 14 days of growing on Witzenhausen soil. Sterile treatment

Pea accession G89 after 14 days of growing on Witzenhausen soil. Non-Sterile treatment

Pea accession S64 after 14 days of growing on Lfl soil. Sterile treatment

Pea accession S64 after 14 days of growing on Lfl soil. Non-Sterile treatment

Pea accession G89 after 21 days of growing on Witzenhausen soil. Sterile treatment

Pea accession G89 after 21 days of growing on Witzenhausen soil. Non-Sterile treatment

Pea accession S64 after 21 days of growing on Lfl soil. Sterile treatment

Pea accession S64 after 21 days of growing on Lfl soil. Non-Sterile treatment