Evaluation of Pathological Risks Associated with Use of Leguminous Cover crop and Living mulch species

DISSERTATION

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VON

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In the essence of our existence, our actions and our survival, few factors liberate the soul of the man while still keeping him, the man, in constant contact with that fruit of 'His'.

I begin my research on the topic "Evaluation of Pathological Risks Associated with Use of Leguminous Cover crop and Living mulch species" with the bejt of Bosnian Sheikh Husejn Lamekamija, who said:

"When a droplet reaches a sea, it vanishes

A droplet is indeed water divided from the water"

This work is part of the endless sea of thoughts and struggles for authentic research, and my individual path towards an all encompassing awareness of 'Him' through my contribution to the natural sciences.

Author

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CHAPTER 1: General Introduction

1.1 Introduction

Modern cropping systems rely on growing a narrow range of crop species and genotypes that have usually been selected under high fertility soil conditions and pesticide inputs with a primary focus on yield (Benckiser and Schnell 2007). This has promoted large scale farming systems over small and medium sized farms, with highly specialized production units of essentially identical plant species *e.g*. monocultures. However, the ecological impacts of monoculture farming have led to loss of natural biodiversity and ecosystem simplification over vast areas of land (Altieri and Nicholis, 2005). Consequently, artificial agroecosystems have been created which are highly dependent on non-renewable external inputs and require constant human interventions. Rather than to more efficiently utilize diverse on farm resources and aim to restore ecosystem functions, farmers struggle to ensure stable yields and impose loss of biodiversity through high fertilizer inputs, frequent pesticide use and base their production on cheap fossil energy. Although this approach has enabled the farmers to achieve high crop yields in the short term (Østergård et al., 2009), it is likely to fail in the long term due to limited energy resources and decline in soil fertility.

The International Assessment of Agricultural Knowledge, Science and Technology for Development states that the worlds agriculture is at a crossroad and that far-reaching changes are required to cope with the global increase of human population and constant increase in demand for food, land, and energy (IAASTD 2009). Fossil fuels are becoming ever scarcer and more expensive leading to increasing costs of transport, chemicals and fertilizers. Thus, current developments within agriculture which lead to loss of biodiversity, soil erosion, polluted and restricted water supplies combined with natural resource and fossil fuel limitation, threaten the food security of the increasing human population. Losses in soil physical, chemical and biological structure including the loss of nutrient value for crops are forcing farmers to become concerned about the problems of soil degradation (Doran and Zeiss, 2000; Brussaard et al., 2007). Therefore, a more holistic approach is needed that will minimize negative environmental impacts and integrate ecosystem management strategies which have the potential to maintain and enhance current agricultural production in the long term in a way that is economically viable and socially acceptable in the short term.

1.2 An agro-ecosystem approach – harnessing and improving biodiversity

The conservation of biodiversity is vital for ecosystem functioning and agricultural sustainability (Cardinale et al., 2012). Biodiversity plays a central role for several ecosystem services needed for food production, such as soil formation and conservation, water use efficiency, biological pest and disease control, nutrient cycling, and crop pollination (Sandifer et al., 2015). Numerous studies have provided valuable insights into how biodiversity contributes to the resilience and stability of the farming systems worldwide (Bommarco et al., 2013; Berry, 2009; Altieri, 1999; Hanson et al., 2007). In general, more diverse agroecosystems are shown to be more resilient, with greater ability to suppress pest and pathogen outbreaks and alleviate adverse environmental fluctuations which are predicted to increase under future climate scenarios.

Soil is the fundamental medium for crop growth in all production systems. Thus, improvement in agricultural sustainability requires effective management of soil heath and soil quality, the two key components on which resilient and sustainable agricultural systems should be built. Both, soil health and soil quality, rely on biological processes that integrate soil ecosystem health and its productive capacity. Biologically diverse soils have greater carbon transformation ability, higher soil organic matter content (SOM) and better structure that contributes to increased crop nutrient supply, decreased pest, disease or weed pressure, and decontamination and bioremediation of pollutants (Brussaard et al., 2007; Bardgett and van der Putten, 2014).

Despite this central role of soil biological diversity, the majority of studies addressing biodiversity have had an aboveground focus (Bardgett and van der Putten, 2014). While these studies have increased our knowledge on the functional consequences of aboveground biodiversity loss, we are only beginning to elucidate the importance and the influence of belowground biodiversity on ecosystem functions (Jing et al., 2015). Several studies indicate that belowground diversity can have profound influence on plant community composition both, in positive or negative ways. Drought adapted soil microbial communities for example, have been shown to increase plant fitness under drought stress (Lau and Lennon, 2012). Similarly, in greenhouse studies conducted in macrocosm bioassays established over two growing seasons which simulated European calcareous grassland and North American old field ecosystems, van der Heijden et al. (1998) reported that alteration in the composition and

number of arbuscular mycorrhizal fungal (AMF) taxa led to large fluctuations in the structure and composition of the plant communities. Furthermore, the authors found that a considerable number of plant species were almost completely dependent on the presence of AMF in the system. A more recent study of Wagg et al. (2014) illustrated that reduction in soil biodiversity and simplification of the soil communities, such as nematodes, bacteria, mycorrhizal and non-mycorrhizal fungi led to a strong decline of plant species diversity. In addition, a strong decline in total biomass of legumes and forbs was observed as soil biodiversity was depleted, whereas grass biomass production increased significantly, contributing up to 92% to the net primary productivity in the most simplified soil communities. Numerous studies have also shown an increase in diseases caused by soil borne plant pathogens due to biological soil simplification as agroecosystems are being deprived of basic functional components (Ratnadass et al., 2012). Therefore, the capacity of soil to perform functional processes and sustain plant growth depends crucially upon management strategies that promote and enhance positive soil biology.

1.3 Crop rotations, legumes and agricultural sustainability

Crop rotations are fundamental components of the systems that focus on biological soil management. Spatial (mixed cropping) and temporal (crop rotations) crop diversification stimulates aboveground and belowground biodiversity and creates higher availability of resources for soil communities contributing to overall system resilience (Kremen and Miles, 2012). Appropriate rotations in the long term can lead to higher crop yields and overall farm profitability by influencing soil structure and soil organic matter content, nutrient availability, root distribution, and plant diseases and weeds (Honermeier, 2007). Better soil structure improves water infiltration, reduces risks of water logging and increases supply of water to the crops during the drought periods. Higher content of soil organic matter improves water holding capacity and nutrient retention that in turn reduces pesticide and synthetic fertilizer requirements. Rotations with non-host crops are still one of the most widely recommended practices for the control of pests, diseases and parasitic weeds. Diversified crop rotations are also an important component of climate change mitigation strategies (Paterson, 2009). Therefore, crop rotations provide many important agronomic, economic and environmental benefits to agroecosystems.

Legumes can play a key role in more diversified and sustainable cropping systems. When grown as cover crops, intercropped, or under-sown (living mulch) with other main crops, legumes can enhance yields and overall net primary productivity of agroecosystems (Fletcher et al., 2016; Crews et al., 2016). Positive yield responses of crops grown in rotations with legumes are a well known fact which has been utilized for centuries by farmers (Emerich and Krishnan, 2009). Depending on the amount of nitrogen (N) returned to the soil and the duration that N_2-fixing plants are grown, yield increases of crops planted after legumes have been estimated to reach equivalent values of those expected from application of as much as 180 kg fertilizer N ha^{-1} (generally N fertilizer equivalences are in the range between 15 to 148 kg of inorganic N ha^{-1}) (Kumar and Goh, 1999). Yield of wheat, for example, is reported to be on average 18 to 24% higher in North America and the EU and up to 50% in south Asia and Australia when wheat is grown in rotations with legumes compared to non-fertilized monocultures (Kirkegaard et al., 2008). Similar results have also been reported for other cereals such as maize and rice (Rahman et al., 2014; Karpenstein-Machan and Stuelpnagel, 2000).

The yield improvements in crops grown after legumes are mainly attributed to the ability of legumes to improve soil N fertility through their unique ability to form symbiotic relationships with the N-fixing soil bacteria of the genus *Rhizobium*. The actual amount of N fixed by legumes is difficult to predict as it depends on many factors including legume species, farm management, weather conditions, and the age of the crop (Peoples et al., 2012, 1995). However, if the legumes are healthy and productive values estimated commonly fall in the range of 200–300 kg of N ha^{-1} $year^{-1}$ (Peoples et al., 1995). In contrast to inorganic N which is prone to leaching and increases overall H^+ concentration in the soils directly contributing to soil acidification, one of the most serious soil degradation processes, the N_2 fixation process by legumes itself does not acidify soils, and the N is usually deposited in the form of semi stable organic materials with low to medium C to N ratio which stimulates overall soil biology (Usman et al., 2013; Peoples et al., 2009; Cobo et al., 2002).

In addition to positive effects on yields, legumes in agricultural systems perform a range of ecosystem services beyond biological N fixation. When planned properly in terms of adequate diversity and appropriate density, legume based species mixtures and crop rotations can enhance physical, chemical and biological characteristics of soils, reduce weed populations,

and break the cycles of pests and pathogens (Fletcher et al., 2016; Emerich and Krishnan, 2009). Perennial legumes such as alfalfa, and many clover species form deep and extensive root systems which can contribute to break soil compaction, improve water infiltration and build-up of soil fertility by assimilating and transporting nutrients from deeper soil layers (Fageria, 2013; McCallum et al., 2004). The root system of alfalfa has been shown to reach depths greater than 3.5 meters in some soils (Brun and Worcester, 1975). Specialized proteoid roots of white lupine (*Lupinus albus*) exude citric and malic acid which enhance the phosphorous (P) nutrition of the plant and contribute to improved availability of P to subsequent crops (Dissanayaka et al., 2016). Legumes such as soybean (*Glycine max* L.) can form symbiotic relationships with root-nodule rhizobia that lack or have low activity of a hydrogenase uptake enzyme (commonly referred to as HUP^-), and substantial amounts of Hydrogen (H_2) gas, an obligate by-product of the symbiotic N_2 fixing process, can be exuded into the soil (up to 5000 L of H_2 ha^{-1} day^{-1}). It has been shown that diffused H_2 from the nodules induces rapid multiplication of soil organisms capable of utilizing H_2 as energy source which in turn by mechanisms not yet understood can induce disease suppression, enhance plant disease resistance and promote plant growth (Kirkegaard et al., 2008; Peoples et al., 2008).

Despite of all these positive effects of legumes for agro-ecosystems, increasing their frequency in crop rotations has to be considered carefully as many of the species of interest share important soil-borne pathogens among each other and with some important cash crops. This is one of the major threats to the long-term sustainability of legume intensive growing systems. Thus, the success of legume production and their ability to provide important ecosystem services depends crucially on legume root health, and the potential agronomic and environmental benefits can only become effective if the associated pathological risks are thoroughly assessed and solutions for potential problems identified.

While there is a need to broaden the spectrum of species that can be used in crop rotations, it is also important to carefully select appropriate legumes suitable for such agro-ecosystem intensification. Cover crops, for example, should be fast growing and highly productive to fit in the off-season between main crops, whereas living mulch should not be too competitive with main crops. Of additional concern is however, that many of the legumes that have the potential to be used in agriculture are not commercially attractive for farmers without some degree of animal production present. Thus, increase of legumes in rotations, particularly in

cooler areas, requires an integrated approach that should also include higher frequencies of legume cash crops. In Germany, pea and faba bean are of particular interest that in addition to important ecological services, provide valuable sources of proteins for human and animal nutrition. However, despite this importance, their production in Germany and many other European countries is in long-term decline (EUROSTAT, 2017; DAFA, 2012; Guddat et al., 2010; Sass, 2009). Since pea and faba bean share important soil-borne diseases (van Emden et al., 1988), farmers are often forced to grow these species in wide rotations that usually only fit into five or more year rotation plans.

1.4 Soil borne legume pathogens

Root rot caused by soil-borne plant pathogens poses significant challenges to growers and compromises sustainability of legume production worldwide (Feng et al., 2010; Summerell et al., 2011; Holtz et al., 2011; Arias et al., 2013b). Several species of *Fusarium, Aphanomyces, Phoma, Didymella, Pythium, Rhizoctonia, Sclerotinia* (Finckh et al., 2015b), commonly referred to as the root rot complex pathogens, are among the most frequent causal agents of the disease. In addition to root rot and inhibition of root development, symptoms include poor seed germination, pre-emergence death of seedlings, post-emergence damping off, stunted growth, foot rot, vascular wilt, seedling blight, and post-harvest seed decay (Finckh et al., 2015b).

Species of the genus *Fusarium* are of particular interest as they can efficiently spread along crop rotations, and often infect a wide range of plants under diverse environmental conditions (Summerell et al., 2010). More than 20 *Fusarium* species have been associated with roots (leaves and pods) of leguminous crops (Arias et al., 2013b; Leslie and Summerell, 2006; Clarkson, 1978). The dominance and importance of the single species vary greatly and depend mainly on the legume host plant and the climatic conditions. For example, in southern hemisphere countries, Bosch et al. (1989) and Lauren et al. (1988, 1992) reported prevalence of *F. avenaceum, F. sambucinum* and *F. crookwellense* in New Zealand pasture species, whereas *F. acuminatum, F. avenaceum F. equiseti* and *F. oxysporum* dominated South African annual medic pastures (Lamprecht et al., 1988). In Australia, *F. acuminatum F. oxysporum,* and *F. equiseti* were prevalent in annual medic pastures (Bretag, 1985). In surveys of Swedish and Finish red clover fields the most dominating *Fusarium* species was *F. avenaceum* (Yli-Mattila et al., 2010; Lager and Gerhardson, 2002; Rufelt, 1986). Persson et al. (1997) regularly isolated

F. solani, F. oxysporum, F. redolens, F. avenaceum and *F. culmorum* from infected pea roots in Sweden and Denmark, and reported that among these *F. avenaceum* was highly aggressive in a greenhouse assay. Recent surveys conducted by Pflughöft et al. (2012) indicate that *F. redolens* together with *F. oxysporum* and *F. avenaceum* are dominant *Fusarium* species of the pea root rot complex in Germany. Similar results have also been reported from field surveys conducted in central Alberta, Canada (Feng et. al. 2010 and Holtz et al. 2011), and North Dakota, USA (Chittem et al., 2015) where *F. avenaceum* and *F. oxysporum* were prevalent *Fusarium* species in pea and lupine roots. In an extensive survey of soybean sudden death syndrome pathogens, Aoki et al. (2003) found that the disease is caused by two phylogenetically and morphologically distinct species of the *F. solani* species complex, *F. virguliforme* and *F. tucumaniae* in North and South America, respectively. Recent studies of Arias et al. (2013) and Chitrampalam and Nelson (2014) suggest potential emerging importance of *F. gramineraum* and *F. tricinctum* in soybean root rot in some areas of the USA.

In addition to the complex ecology and difficulties to precisely determine combinations of soil and environmental conditions that influence individual densities of particular species, the *Fusarium* genus has a controversial taxonomic history and many of the species that have been formerly considered as a single morpho-species are today recognized as a species complex (Watanabe et al., 2011; Summerell and Leslie, 2011; Laurence et al., 2016). Each species complex comprises numerous saprophytic, endophytic, and pathogenic strains revealing a highly complex picture and the ability of particular strains to occupy diverse ecological niches. Thus, detection of *Fusarium* species in the plant tissue is not proof of ongoing disease infection, and often additional screening tests are needed to confirm pathogenicity of recovered isolates. Therefore, to fully understand the ecological significance of the *Fusarium* species associated with a given host often a multi-focal approach is needed.

1.5 Questions and Objectives of the thesis:

Diversity and aggressiveness of *Fusarium* species associated with roots of leguminous cover crops and living mulches in different agro-climatic regions of Europe – Chapter 2

While a number of different *Fusarium* species have been associated with roots of leguminous crops, their occurrence with respect to clover and vetch roots has not been well documented in Europe. Which species are of importance? Is there potentially host or geographic exclusivity of *Fusarium* species associated with these hosts? What are potential risks for crop rotations in legume intensive cropping systems?

Objective 1: To conduct a comprehensive survey of *Fusarium* species that infect the roots of clover and vetch species grown across Europe

Objective 2: To determine possible (underlying) patterns of host preference and geographical distribution

Objective 3: To characterize the aggressiveness of predominant *Fusarium* species on pea, and determine the potential role of the studied legumes as alternate hosts of pathogens of importance to a main legume cash crop

The *Fusarium solani* species complex - Chapter 3

A collection of *Fusarium solani* isolates recovered from Chapter 2 and the follow-up surveys were subjected to molecular genotyping and the characterization of aggressiveness in greenhouse bioassays. Previously recognized as a single morpho-species, *F. solani* includes multiple phylogenetic species comprising a diverse species complex. Are the *F. solani* isolates from the studied hosts a single phylogenetic species? Are they new species in the complex? Do they correspond to one or more already known phylogenetic species?

Objective 4: To determine the diversity and potential geographical or host preference of *Fusarium solani* species complex isolates

Objective 5: To compare the aggressiveness and determine if isolates of the *Fusarium solani* species complex from non-pea hosts are capable of cau sing disease on pea under greenhouse conditions

Objective 6: To clarify the host range of *F. solani* f. sp. *pisi* within several leguminous species

Ecological and pathological interactions between *Fusarium equiseti, F. avenaceum* and *Peyronellaea pinodella* in pea – Chapter 4

Fusarium equiseti is a naturally occurring endophyte in diverse ecosystems that lacks strict host adaptation. However, there are contradicting reports of the role of *F. equiseti* in agro-ecosystems as the fungus has been reported as pathogen, endophyte and saprophyte. Several *F. equiseti* isolates that were apparently endophytic in their hosts of origin and pea were identified in Chapter 2. Their potential role in pea root rot health was studied in Chapter 4 including their interactions with well documented pathogens of pea root rot disease.

Objective 7: To determine the effect of root colonization by *F. equiseti* on pea growth, and on disease development in the presence or absence of *F. avenaceum* and *Peyronellaea pinodella*

Objective 8: To determine the influence of endophytic colonization of *F. equiseti* on colonization and proliferation of the pathogens in the rhizodermis and the root cortex of pea plants

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CHAPTER 2: Diversity and aggressiveness of *Fusarium* species associated with roots of leguminous cover crops

Manuscript in preparation

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Abstract

Leguminous cover crop and living mulch species show not only great potential for providing multiple beneficial services to agro-ecosystems, but may also present pathological risks for crop rotations. These risks should be thoroughly assessed in order to promote effective use. The present study was conducted in 2013 and 2014 and included evaluation of frequency, distribution and aggressiveness of *Fusarium* spp. recovered from the roots of subterranean clover, white clover, winter and summer vetch grown as cover crop and living mulch species across five European sites. Although the samples originated from Mediterranean to Nemoral environmental zones of Europe, no strong separation in *Fusarium* community structure was observed between the sites. The most frequently isolated species in both years from all hosts were *F. oxysporum* and *F. avenaceum* accounting for 68.5% of total isolation percentage. They were common at most of the sites, particularly at Switzerland. In Sweden, *F. oxysporum* dominated, while *F. avenaceum* occurred rarely. In aggressiveness tests, disease severity and impact on pea biomass varied among and within tested isolates of six *Fusarium* spp. *Fusarium avenaceum* caused the highest biomass reductions and most severe root rot symptoms suggesting that it could emerge as potential risk in intensive legume cropping systems.

2.1 Introduction

In the past 10 to 15 years, there has been a tendency in Europe to switch from cereal based cropping systems, with winter and summer fallow, to cropping systems which combine greater crop diversity with maximum soil cover (Kertész and Madarász, 2014; Basch et al., 2011). Currently, an increasing number of different legumes are being grown in rotations with cereals (Fletcher et al., 2016; Hayes et al., 2016; Kremen and Miles, 2012). In addition to being an important protein source for animal and human nutrition worldwide, legumes provide multiple ecological services to agricultural systems. The main benefit of their inclusion in cropping systems is their ability to fix atmospheric nitrogen (Trydeman-Knudsen et al., 2004; Hauggaard-Nielsen et al., 2008). Growing legumes as cover crops, intercropped or under-sown (living mulch) with cereals may also contribute to mobilization and remineralization of nutrients (Mueller and Thorup-Kristensen, 2001), improved soil structure (Roldan et al., 2003), increased water infiltration and prevention of soil erosion (Langdale et al., 1991; Meisinger et al., 1991), and to weed suppression (Teasdale et al., 2007).

However, changes towards legume rich crop rotations raise concerns as many of the species of interest share important soil-borne pathogens with some important main crops, thereby increasing their prevalence in rotations, which may likely limit production due to increased disease pressure. Thus, the potential agronomic and environmental benefits of leguminous cover crops and living mulches can only become effective if the pathological risks are thoroughly assessed and solutions for potential problems identified.

Species of the genus *Fusarium* are of particular interest as they can efficiently spread along crop rotations, and often infect a wide range of plants under diverse environmental conditions (Summerell et al., 2010). Different *Fusarium* species, mainly *F. oxysporum*, *F. solani, F. avenaceum, F. redolens*, *F. culmorum*, are associated with roots of leguminous crops (Finckh et al., 2015a; Pflughöft et al., 2012; Satyaprasad et al., 2000; Kraft, 1994), and usually occur as a complex rather than alone. Prevalence, dominance and importance of the single pathogens vary greatly depending on location, climate, and agricultural practice (West et al., 2012) and some shifts in importance have occurred over time. For example, *F. solani* has been described as one of the major and most aggressive pathogens of pea (*Pisum sativum* L.) during the 1980s and 1990s (Persson et al., 1997; Kraft, 1984). However, recent surveys conducted in Germany indicate that *F. redolens* together with *F. oxysporum* and *F. avenaceum* dominated among

Fusarim spp. (Pflughöft et al., 2012). Similar results have also been reported from several other recent studies. In disease surveys conducted in central Alberta, Feng (2010) and Holtz et al., (2011) reported that *Fusarium* species were most frequently associated with root rot of pea and lupines (*Lupinus angustifolius*), and *F. avenaceum* dominated the complex. In both studies, members of the *F. oxysporum* complex were isolated two to three times more often than *F. solani* from diseased pea roots.

Although there is a certain host specialization, many of the species associated with roots of legumes are also part of the pathogen complex causing economically important diseases on cereals, like Fusarium Head Blight (FHB) or Fusarium Crown Rot (FCR) (Bottalico and Perrone, 2002). In addition, *Fusarium* spp. are a highly successful group of fungi that produce mycotoxins harmful for animal and human health (Ferrigo et al., 2016) .

In the current study, root samples of subterranean clover, white clover, winter vetch, and summer vetch grown in rotation or association with wheat were collected in 2013 and 2014 from field sites across Europe. The 2013 survey focused on assessing the composition of the fungal community at genus level with the aim to determine the most prevalent root infecting fungi and establish their importance. As most fungi recovered from the roots belonged to the genus *Fusarium,* the subsequent work focused on the evaluation of diversity and aggressiveness of species belonging to this genus. Thus, the main objectives of the study were: (i) to assess possible underlying patterns of host preference and geographical distribution of *Fusarium* species; and (ii) to characterize the aggressiveness of predominant *Fusarium* species to pea, in order to determine the potential role of clover and vetch species as alternate hosts of pathogens of importance to a main legume crop. In this article, the term aggressiveness refers to relative ability of pathogen/isolate to colonize and cause damage to plants (D'Arcy et al., 2001).

2.2 Material and methods

2.2.1 Sampling sites and plant material

Roots of subterranean clover (*Trifolium subterraneum* L.), white clover (*T. repens* L.), winter vetch (*Vicia villosa* Roth) and summer vetch (*V. sativa* L.) intercropped with wheat as living mulch or grown as cover crops after wheat were collected during 2013 and spring 2014. The samples were collected from field experiments set up jointly in five European sites representing different agro-climatic zones and soil conditions: University of Tuscia (UNITUS, Italy), Agroscope (AGS, Switzerland), Technical University Munich (TUM, Germany), University of Kassel (KU, Germany), and Swedish University of Agricultural Sciences (SLU, Sweden) (Table 2.1). Plant samples were randomly collected at each site, sent to University of Kassel laboratory, and stored at -20°C until further use. Summer vetch was grown exclusively in 2013 at KU. Roots of subterranean clover, white clover and winter vetch from south Germany (TUM) were only available in 2013 (Table 2.1).

Table 2.1. Site-specific pedo-climatic characteristics, plant species, sampling dates and number of root samples processed per site and year.

Site[1], climate zone[2]	Temp.[3] (°C)	Ppt.[4] (mm)	Soil Type	pH	% OM	Plant species[5], use[6] (2013/14)	2013		2014	
							n[7]	Sampling time	n	Sampling time
Italy 42°25'N, 12°05'E (MDN)	11.6	845	Typic Xero-fluvent	6.7	1.2	Subclover LM/CC	120	26. Apr	120	23. Apr
						W. vetch CC	-	-	120	23. Apr
Switzerland 47°30'N, 8°55'E (CON)	9.5	1111	Hapludalf	7.1	2.0	Subclover LM/CC	120	01. July	120	14. Apr
						W. vetch CC	-	-	120	14. Apr
South Germany 11° 41'E, 48° 23' N (CON)	8.3	805	Cambisol	6.9	1.6	Subclover LM	80	17. Dec	-	-
						W. clover LM	80	17. Dec	-	-
						W. vetch CC	80	17. Dec	-	-
Central Germany 51°22'N, 9°54'E (ATN)	9.4	644	Typic Haplu-dalf	6.2	2.0	Subclover LM/LM	80	30. Oct	80	16. June
						W. clover LM/LM	80	30. Oct	80	16. June
						S. vetch CC	80	30. Oct	-	-
Sweden 59°49'N 17°42'E (NEM)	8.2	598	Incepti-sol	5.7	5.3	W. clover LM/CC	40	23. Oct	40	14. Apr
						W. vetch CC	-	-	40	14. Apr

[1]Experimental fields of: Italy = University of Tuscia, Switzerland = Agroscope, South Germany = Technical University Munich, Central Germany = University of Kassel, Sweden = Swedish University of Agricultural Sciences; [2]MDN = Mediterranean North, CON = Continental, ATN = Atlantic North, NEM = Nemoral according to Jongman et al., 2006; [3]Average annual temperature; [4]Average annual precipitation; [5]Subclover = subterranean clover, W. vetch = winter vetch, W. clover = white clover, S. vetch = summer vetch; [6]LM = living mulch species, CC = cover crop species; [7]Number of roots analyzed.

2.2.2 Identification of fungi

Root samples were surface sterilized with 3% sodium hypochlorite for 10 s, rinsed in distilled water and placed on filter paper under a laminar flow hood for 1 h to dry. Three approximately 1 cm pieces were selected randomly, placed in Petri dishes containing Coon´s medium (4 g/l Maltose, 2 g/l KNO_3, 1.20 g/l $MgSO_4$ x 7 H_2O, 2.68 g/l KH_2PO_4, and 20 g/l agar) and incubated under alternating cycles of 12 h blacklight blue (BLB) fluorescent light (F40; range 315-400 nm with the peak at 365 nm) and 12 h darkness. Fungal colonies developing from the root pieces were sub-cultured separately in Petri dishes containing half-strength potato dextrose agar (19 g/l Difco PDA and 10 g/l agar).

Fusarium like colonies were then transferred on PDA and Synthetic Nutrient-Poor Agar (SNA, 1 g/l KH_2PO_4, 1 g/l KNO_3, 0.5 g/l $MgSO_4 \times 7\ H_2O$, 0.5 g/l KCl, 0.2 g/l sucrose, 0.2 g/l glucose, and 20 g/l agar (Nirenberg, 1976) and identified based on cultural appearance (colony color and pigmentation) and microscopic examination of conidiogenous cells according to Leslie and Summerell (2006). Other fungi were identified to genus level according to colony and conidia characteristics following the methods described by Watanabe (2002) and Boerema et al., (2004). Data concerning overall occurrence of root associated fungi on the genus level are presented for 2013, while data from both 2013 and 2014 are presented for the occurrence of *Fusarium* species.

Isolates representing each species of *Fusarium* based on morphological characteristics (with the exception of *F. redolens*) that appeared in the survey more than five times were chosen at random from the collection for molecular confirmation. Single spore cultures were used for molecular analysis. Dilution plating was done from cultures growing on SNA. A small piece of culture material was added to 10 ml distilled water. Dilution series of this suspension up to 10^{-6} were plated on 2% water agar. After 24 h of incubation at room temperature, single conidia were transferred to half-strength PDA using a reverse microscope.

The identity of selected isolates of *F. culmorum, F. graminearum, F. avenaceum, F. tricinctum, F. acuminatum, F. equiseti* and *F. oxysporum* was confirmed by real-time PCR. DNA was extracted from freeze-dried mycelia using a CTAB method (Brandfass and Karlovsky, 2006). Real-time PCR was carried out under conditions optimized for each species (Dastjerdi, 2014). Primers used for *F. culmorum, F. graminearum, F. avenaceum, F. poae, F. tricinctum, F. equiseti*

and *F. oxysporum* were OPT18 F and OPT18 R (Schilling et al., 1996), Fg16NF and Fg16NR (Nicholson et al., 1998), FaF and FaR (Doohan et al., 1998), FP82F and FP82R (Parry and Nicholson, 1996), Tri1 F and Ttri2 R (Kulik, 2008), 198F2 and 198R1 (Nicholson et al., 2004), and CLOX1 and CLOX2 (Mule et al., 2004), respectively. Preliminary studies showed that the primer pair Tri1 F and Tri2 R described by Kulik (2008) as specific for *F. tricinctum* also generated products with the genomic DNA of *F. acuminatum,* and thus could not distinguish between the two species. Therefore, to confirm identity of the species, in addition to the expected positive signal in the qPCR assay using the aforementioned primer pair, separation was based on size and shape of micro- and macroconidia, morphological characteristics of conidiogenous cells, colony characteristics and growth rate as described above.

The identity of selected *F. solani* isolates was confirmed by the results of the translation elongation factor 1-alpha (*tef1*) gene sequences. For this purpose the *tef1* gene was amplified using primer pairs EF1 and EF2 described by O'Donnell et al. (1998). The PCR reactions were performed with a peQ STAR Thermocycler (96 Universal Gradient). The PCR mixture contained 5 µl of reaction buffer (16 mM $(NH_4)_2SO_4$; 67 mM Tris-HCl; 0.01% (v/v) Tween-20, pH: 8.8 at 25°C), 2 µl of 2 mM of $MgCl_2$, 1.5 µl of 0.15 mM dNTP's (Bioline, Lukenwalde, Germany), 0.75 µl of each primer (0.3 µM), 0.05 µl of 0.25 Unit BIOTaq DNA polymerase (Bioline, Luckenwalde, Germany), 3 or 6 µl of diluted genomic DNA, and double distillated water to make total reaction volume of 25 µl.

Conditions for amplification were an initial denaturation step of 3 min at 95°C, followed by 30 cycles of 1 min at 94°C (denaturalization), 45 s at 59.1°C (annealing), 1 min at 72°C (extension) and a final extension cycle at 72°C for 5 min. The amplified DNA fragments were then purified using 70% isopropanol precipitation, rinsed with 70% (v/v) ethanol, air dried, re-suspended in double distillated water, and sequenced (LGC Genomics, Berlin, Germany) in both directions using the PCR primers. The chromatogram of *tef1* sequence for each *Fusarium* species was inspected visually and sequence reads edited when necessary. The sequences were then used as queries for Fusarium-ID v. 1.0 database (Geiser et al., 2004), and the *Fusarium* MLST database (http://www.cbs.knaw.nl/fusarium; O'Donnell et al., 2010). Positive identification rate for the isolates of *F. solani* was >99%. These sequences were submitted to the GenBank database with accession numbers KY128330 to KY128334.

2.2.3 Aggressiveness of selected *Fusarium* isolates

To evaluate aggressiveness of the most common species recovered from the sampled roots, 72 isolates of six *Fusarium* species were used in a greenhouse assay. Field pea variety Santana served as a model plant. One isolate of *F. oxysporum* and two isolates of *F. solani* from pea roots, and one isolate of *F. avenaceum* and three isolates of *F. equiseti* from wheat roots were additionally included in the experiment.

Each isolate was grown on half-strength PDA and SNA at 20°C under alternating cycles of 12 h BLB fluorescent light and 12 h darkness. Spore suspensions for inoculation were prepared by flooding the cultures with 15 ml sterile distilled water and dislodging the conidia with a disposable hockey stick cell spreader. Spores were quantified with Fuchs Rosenthal hemocytometer.

Pea seeds were surface sterilized in 70% ethanol for 5 min and rinsed with distilled water before planting four seeds into 500 ml pots filled with previously autoclaved sand. Following sowing the pots were inoculated with 2 x 10^4 spores g^{-1} substrate. Controls were left non-inoculated. The experiment was arranged in a completely randomized design with three replicates. The pots were kept in the greenhouse at 19/16°C day/night temperature and 16 h photoperiod (provided with 400 W high-pressure sodium lamps). Pots were watered daily with tap water and additionally fertilized with complex N:P:K fertilizer Wuxal Super (8:8:6 + microelements). A total of 100 mg of N l^{-1} of substrate was divided in two equal portions and added 10 and 15 days after sowing.

After 21 days, plants were removed from pots, and the roots were separated from the above ground biomass. Above ground plant parts of each pot were weighted and dried at 105 °C until constant weight. Roots were washed under running tap water, and root rot severity was assessed using a visual 0-8 score scale based on external and internal root tissue discoloration levels adopted from Pflughöft et al. (2008). The external disease severity was rated as follows: disease severity rating (DSR) 0 = no symptoms, 1 = streaks at the transition zone, epicotyl or hypocotyl, 2 = brown lesion cover up to 50% of root perimeter, 3 = brown-black lesion cover 51 to 99% of root perimeter, 4 = black lesion cover 100% of stem perimeter, 5 = black lesion spread up to 30 - 49% of the tap root, 6 = black lesions spread up to 50 to 70% of the tap root, 7 = black lesions spread > 70% of the tap root, 8 = dead plant. The roots were then cut transversally across the lesions and internal disease severity was rated, where 0 = no visible

symptoms, 1 = epidermis/rhizodermis is brown to black, 2 = brown discoloration of cortical tissues, 3 = cortical tissues is partially black, but the center and endodermis are still healthy, 4 = cortex tissue is completely black, 5 = cortex tissue begins to rot (bursting of epicotyl or rhizodermis on the root), 6 = cortex tissue is completely rotten, 7 = shedding of the cortex tissue and endodermis, and 8 = dead plant. A disease severity index (DI) between 0 and 100 was calculated using following formula:

$$DI = [\Sigma(SR \times NR) / Nt \times MR] \times 100$$

Where, SR = Mean external and internal disease severity rating (DSR), NR = Number of infected plants having that DSR, Nt = Total number of plants assessed, MR = Maximum rating scale number.

Four distinct aggressiveness classes were assigned based on the gradual increase of severity of symptoms following inoculation. When the DI of inoculated plants was in the same range as the DI of the un-inoculated control (DI = 0-15), the isolate was classified as non-aggressive (approximately correspond to DSR of 0 and 1); DI = 16-30 – weakly aggressive (DSR ≈ 2 and 3); DI = 31-70 – moderately aggressive (DSR ≈ 4 to 6); and DI = 71-100 – highly aggressive (DSR ≈ 7 and 8) (Figure 2.1).

Within each species of *Fusarium* tested up to ten different treatments were selected at random, and fungi were re-isolated using the protocol described above from the roots in order to confirm that infection was the result of the inoculated species.

Figure 2.1 Classification of isolates into four distinct aggressiveness classes based on the root rot severity symptoms: (A) non-aggressive (DI = 0-15), (B) weakly aggressive (DI = 16-30), (C) moderately aggressive (DI = 31-70) and (D) highly aggressive (DI = 71-100).

2.3 Data analysis

As most data were not normally distributed and some groups had unequal variances, nonparametric tests were used for the evaluation of the results. The differences between years, host and site effects on isolation frequencies of *Fusarium* species were tested using the non-parametric ranking procedure of the Kruskal-Wallis test in R statistical software (version 3.3.0, R Core Team, 2013) using the package agricolae (de Mendiburu, 2014). If significant treatment effects were observed ($P < 0.05$), mean ranking values were separated with the Kruskal multiple comparison test (Conover, 1999). As there was no significant difference in *Fusarium* community composition (ANOSIM analysis, see text below), data collected from south and central Germany were pooled and analyzed together. Species that occurred less than ten times in the survey were excluded from statistical analyses. The Benjamini and Hochberg (1995) stepwise adjustment of *P* values was used to control false discovery rate and reduce type I errors in a post hoc procedure.

To determine the relationship between sampling intensity and number of observed *Fusarium* spp., species richness rarefaction curves were constructed using iNEXT package in R statistical software (Chao et al., 2014; Hsieh et al., 2016). To test for differences in *Fusarium* community composition, one way analysis of similarities (ANOSIM) (Clarke and Gorley, 2001) was performed on pooled abundance data from both years, using the PRIMER v7 software for windows (Plymouth Routines in Multivariate Ecological Research, Plymouth Marine Laboratory, UK). Bray - Curtis dissimilarity matrices of the fourth square root transformed data were constructed and applied to compare ranked similarities for differences within and between previously defined groups using 10.000 random permutations. ANOSIM is a non-parametric (distribution free) method of multivariate data analysis employed to compare the variation in species composition and abundance among sampling units. This test compares the ranks of distances between groups with ranks of distances within groups, and it is calculated by permutations applied to the underlying similarity matrix. The resulting R values in theory lie within the range of -1 to +1 (for more information see Chapman and Underwood, 1999). R is approximately 0 if the null hypothesis is true (no separation of community structure is found), $R < 0.25$ are commonly interpreted as barely separable, $R > 0.5$ as separated but overlapping, and R values > 0.75 as well separated (Clarke and Warwick, 2001).

The contribution of each *Fusarium* species to average dissimilarity between the sites was analyzed using the SIMPER routine (Similarity Percentage analysis) in PAST (Paleontological Statistics) software for windows (Hammer et al., 2001). The Bray - Curtis similarities between samples were disaggregated by computing average dissimilarities between all pairs of inter group samples and then broken down into separate contributions from each species to dissimilarity.

For the greenhouse experiment, overall mean effects of *Fusarium* spp. and effects of single isolates on the pea biomass were expressed as percentage of change in fresh weight per plant (FW change) relative to non-inoculated control, using the following formula:

$$\text{FW change (\%)} = [(X_2 - X_1)/X_1] \times 100$$

where, X_2 = mean fresh weight per plant in g of inoculated treatment, X_1 = mean fresh weight per plant in g of the non-inoculated control.

Differences in root rot severity (disease index values) and effects on fresh weight of *Fusarium* spp. averaged over isolates were analyzed using the Kruskal-Wallis test followed by the Kruskal multiple comparison test as described above. Differences among single isolates were tested separately by comparing mean rank sums from inoculated treatments with non-inoculated controls using Dunn's multiple comparison test with one control (Pohlert, 2014). Effect of isolate was considered significant when $P < 0.05$. Simple linear regression analysis was performed for each *Fusarium* species to estimate yield losses associated with root rot severity (Fox and Weisberg, 2011).

2.4 Results

2.4.1 Composition of the fungal communities at genus level

In 2013, a total of 776 isolates were obtained from 760 legume roots. Out of these, 540 belonged to 30 fungal genera while 237 were not identified (unknown number of species or genera) (Table 2.2). *Fusarium, Penicillium* and *Trichoderma* were the most frequently isolated, followed by *Phoma, Didymella, Aspergillus, Cylindrocarpon,* and *Rhizopus.* Only *Fusarium* occurred at relatively high frequencies at all sites, while genera such as *Penicillium*, *Cylindrocarpon, Trichoderma, Phoma, Didymella* and *Aspergillus* were also frequently present, but with different isolation rates and not always in all environments. *Penicillium* dominated the roots of white clover, while *Phoma* was not isolated from vetch species in 2013 (data not shown). Most remaining genera were represented by a few isolates only (Table 2.2).

2.4.2 Host and effect of sampling time on *Fusarium* species

Out of 1480 roots analyzed across both years, a total of 670 isolates of *Fusarium* species were obtained. Of these, 388 isolates were obtained in 2013 with 273 isolated from subterranean clover (n = 400), 3 from summer vetch (n = 80), 29 from winter vetch (n = 80) and 83 from white clover (n = 200). In 2014, a total of 282 isolates were obtained with 147 from subterranean clover (n = 320), 124 from winter vetch (n = 280) and 11 from white clover (n = 120). Rarefaction analysis showed that host affected the *Fusarium* species richness as much as sampling intensity did. The extrapolation of the rarefaction curve for summer vetch reached asymptotic value already after 80 samples (only 3 isolates of 2 *Fusarium* spp. were obtained) suggesting that sample size was sufficient and likelihood of finding new species with increasing sampling effort was low. Roots of subterranean and white clover more frequently yielded isolates of different *Fusarium* species for the same number of samples compared to winter and summer vetch (Figure 2.2A).

Table 2.2. List of fungal genera isolated from leguminous cover crop and living mulch species in 2013. Frequencies (%) of isolation with the number of roots yielding isolates given in parentheses.

Fungal genus	Italy	Switzerland	Germany	Sweden	Overall
Fusarium spp.	60.0 (72)	67.5 (81)	21.7 (104)	50.0 (20)	36.4 (277)
Penicillium spp.	-	0.8 (1)	11.7 (56)	37.5 (15)	9.5 (72)
Trichoderma spp.	0.8 (3)	5.0 (6)	5.4 (26)	7.5 (3)	5.0 (38)
Aspergillus spp.	8.3 (10)	3.3 (4)	2.9 (14)	-	3.7 (28)
Cylindrocarpon spp.	3.3 (4)	3.3 (4)	2.5 (12)	-	2.6 (20)
Rhizopus spp.	2.5 (3)	7.5 (9)	1.5 (7)	2.5 (1)	2.6 (20)
Phoma spp.	5.0 (6)	8.3 (10)	0.2 (1)	-	2.2(17)
Colletotrichum spp.	-	9.2 (11)	0.4 (2)	-	1.7 (13)
Didymella spp.	2.5 (3)	4.2 (5)	0.4 (2)	-	1.3 10)
Myrothecium spp.	-	-	1.5 (7)	-	0.9 (7)
Chaetomium spp.	-	3.3 (4)	-	-	0.5 (4)
Alternaria spp.	0.8 (1)	-	1.3 (3)	-	0.5 (4)
Stemphylium spp.	-	2.5 (3)	-	-	0.4 (3)
Stenocarpella spp.	-	2.5 (3)	-	-	0.4 (3)
Mortierella spp.	-	-	1.3 (3)	-	0.4 (3)
Verticillium spp.	-	-	1.3 (3)	-	0.4 (3)
Pyrenochaeta spp.	-	-	-	5.0 (2)	0.3 (2)
Diplodia spp.	-	-	0.8 (2)	-	0.3 (2)
Sclerotinia spp.	-	0.8 (1)	-	-	0.1 (1)
Curvularia spp.	-	0.8 (1)	-	-	0.1 (1)
Chaetomella spp.	-	-	0.4 (1)	-	0.1 (1)
Arthrinium spp.	-	-	0.4 (1)	-	0.1 (1)
Ulocladium spp.	-	-	0.4 (1)	-	0.1 (1)
Gonatobotrys spp.	-	-	0.4 (1)	-	0.1 (1)
Stachybotryna spp.	-	-	-	2.5 (1)	0.1 (1)
Hainesia spp.	-	-	-	2.5 (1)	0.1 (1)

Fungal genus	Italy	Switzerland	Germany	Sweden	Overall
Phialophora spp.	-	-	-	2.5 (1)	0.1 (1)
Botryodiplodia spp.	-	-	0.4 (1)	-	0.1 (1)
Microdochium spp.	-	-	0.4 (1)	-	0.0 (1)
Ascochyta spp.	-	-	0.4 (1)	-	0.0 (1)
Sordaria spp.	-	0.8 (1)	-	-	0.0 (1)
Massarina spp.	-	0.8 (1)	-	-	0.0 (1)
Other filamentous fungi	55.8 (67)	25.0 (30)	26.9 (129)	27.5 (11)	31.2 (237)
Overall	140.8[1]	145.8	78.5	137.5	102.1
No. roots assessed	120	120	480	40	760
No. roots yielding no fungi	16	11	190	5	222

[1]Frequencies of isolation within column may add up to > 100% due to mixed infections.

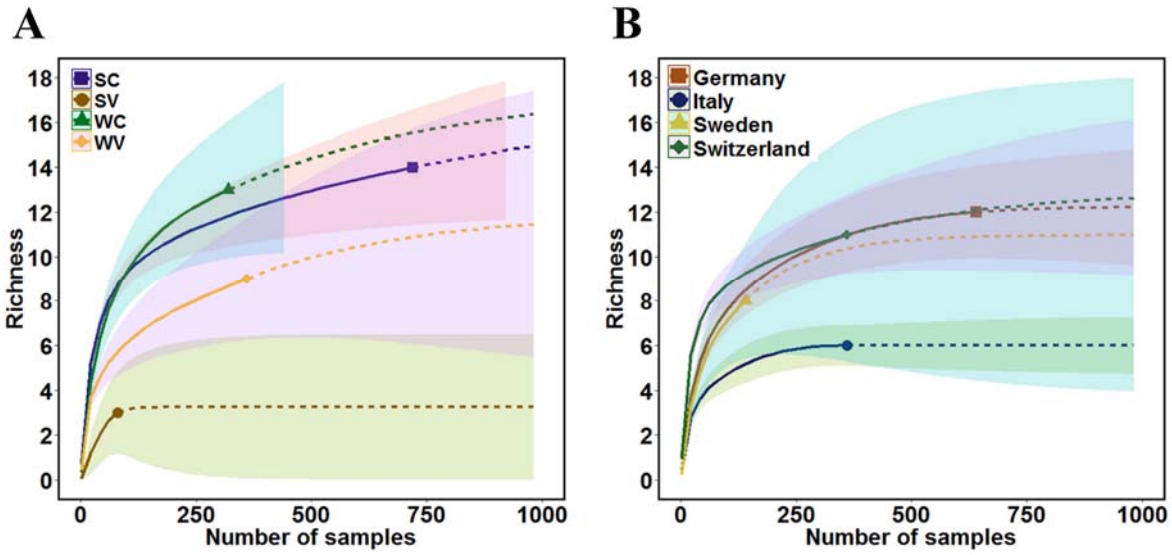

Figure 2.2. Sample based rarefaction (solid lines) and extrapolation (dashed lines) curves showing the relationship between sampling intensity and *Fusarium* species richness for each host plant (A), and each studied site (B). Gray-shaded regions represent the 95% confidence intervals obtained by a bootstrap method based on 1000 replications. SC = subterranean clover, SV = summer vetch, WC = white clover, WV = winter vetch.

Fusarium oxysporum and *F. avenaceum* were the most frequently isolated species in both, 2013 and 2014 (Figure 2.3). *F. oxysporum* was the only species occurring regularly at all sites and all plant hosts in both years. *F. avenaceum* was not detected in the roots of white clover from Sweden, or in subterranean clover from Italy in 2014 (Table 2.3).

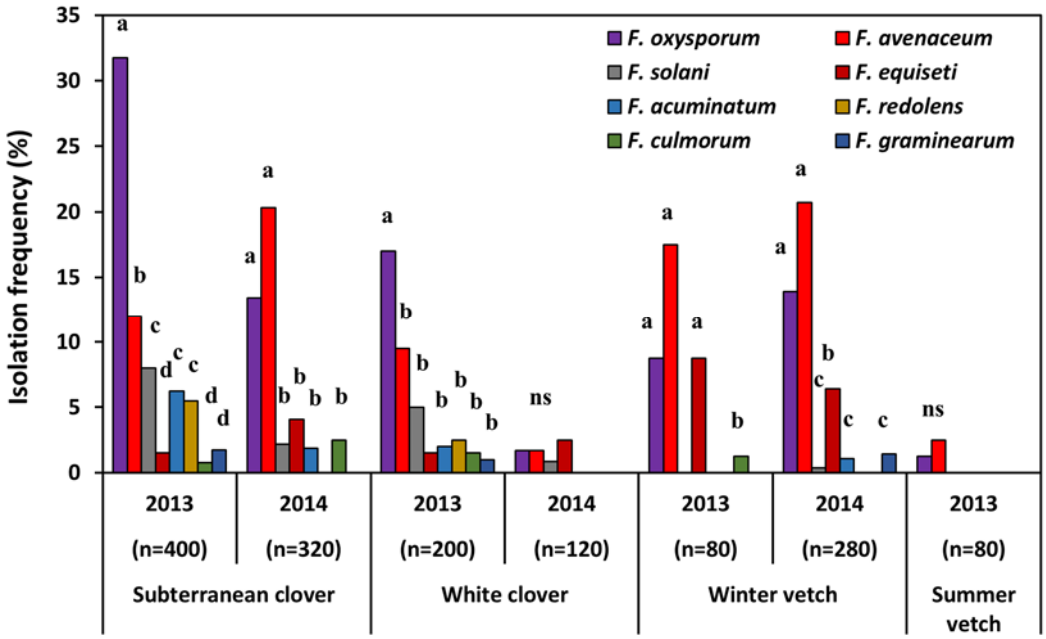

Figure 2.3. Isolation frequencies of *Fusarium* spp. recovered from the roots of subterranean clover, white clover, summer vetch and winter vetch in 2013 and 2014 averaged over sites. Means followed by different letters indicate significant differences in *Fusarium* spp. isolation rates within the host plant and respective year (Kruskal post hoc test, $P < 0.05$). Species that occurred less ten 10 times were excluded from analysis. n = number of assessed roots.

Isolation frequencies of *Fusarium* were significantly higher in 2013 (51.1%) than in 2014 (39.2%). This was mostly due to a decrease in frequency of *F. oxysporum* (22.2%, 2013, and 11.7%, 2014), *F. solani* (5.5%, 2013, 1.3%, 2014), *F. acuminatum* (3.8%, 2013, 1.3%, 2014) and *F. redolens* (3.6%, 2013, 0%, 2014) between the two years. These species all dominated the roots of subterranean clover (Figure 2.4A) In contrast, isolation rates for *F. avenaceum* (10.9%, 2013, 17.4%, 2014) and *F. equseti* (2.1%, 2013, 4.7%, 2014) increased between the two years, although these changes were not statistically significant. *F. avenaceum* was significantly more common on the subterranean clover and winter vetch, while *F. equiseti* was most frequent on winter vetch ($P < 0.05$) (Figure 2.4A). The remaining *Fusarium* species identified occurred only sporadically without apparent preference for any host.

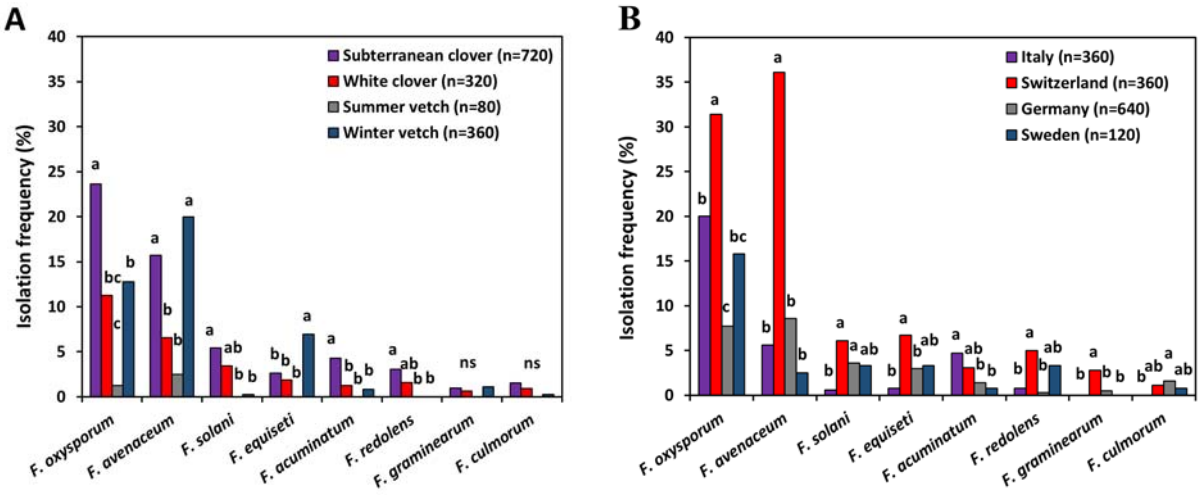

Figure 2.4. Isolation frequencies of eight most commonly isolated *Fusarium* species as affected by the host plant (A) and sampling site (B) averaged over the two sampling years. Different letters indicate significant differences according to Kruskal multiple comparison test ($P < 0.05$).

Table 2.3. Percentage of roots per site, year and host yielding *Fusarium* spp.

Fusarium spp.	Italy[1]			Switzerland			Germany						Sweden			Overall
	2013	2014	2014	2013	2014	2014	2013	2014	2013	2014	2013	2013	2013	2014	2014	
	SC[2]	SC	WV	SC	SC	WV	SC	SC	WC	WC	SV	WV	WC	WC	WV	
Number of roots assessed	**120**	**120**	**120**	**120**	**120**	**120**	**160**	**80**	**160**	**80**	**80**	**80**	**40**	**40**	**40**	**1480**
F. oxysporum	52.5	5.0	2.5	42.5	22.5	29.2	8.1	12.5	10.6	1.3	1.3	8.8	42.5	2.5	2.5	17.1
F. avenaceum	10.8	-	5.8	20.8	47.5	40.0	6.3	10.0	11.9	2.5	2.5	17.5	-	-	7.5	14.0
F. solani	0.8	0.8	-	17.5	-	0.8	6.3	7.5	4.4	-	-	-	7.5	2.5	-	3.4
F. equiseti	2.5	-	-	2.5	2.5	15.0	-	12.5	-	2.5	-	8.8	7.5	2.5	-	3.4
F. acuminatum	12.5	0.8	0.8	5.0	3.3	0.8	2.5	1.3	2.5	-	-	-	-	-	2.5	2.6
F. redolens	2.5	-	-	15.0	-	-	0.6	-	0.6	-	-	-	10.0	-	-	1.8
F. culmorum	-	-	-	-	3.3	-	1.9	5.0	1.3	-	-	1.3	2.5	-	-	1.0
F. graminearum	-	-	-	5.0	-	3.3	-	1.3	1.3	-	-	-	-	-	-	0.9
F. tricinctum	-	-	-	0.8	0.8	-		-	1.3	1.3	-	-	-	-	-	0.3
F. crookwalence	-	-	-	-	-	-	0.6	-	0.6	-	-	-	-	-	-	0.1
F. poae	-	-	-	-	0.8	-	-	-	-	1.3	-	-	-	-	-	0.1
F. sambucinum	-	-	-	-	-	-	-	3.8	-	1.3	-	-	-	-	-	0.3
F. sporotrichoides	-	-	-	0.8	-	-	-	-	-	-	-	-	-	-	-	0.1
F. torulosum	-	-	-	-	-	-	-	-	-	-	-	-	-	-	2.5	0.1
Total number of isolates	98	8	11	132	97	107	43	44	53	8	3	29	28	3	6	670

[1]Experimental fields of: Italy = University of Tuscia, Switzerland = Agroscope, South Germany = Technical University Munich, Central Germany = University of Kassel, Sweden = Swedish University of Agricultural Sciences. Site specific characteristics are given in Table 2; [2]SC = subterranean clover, SV = summer vetch, WC = white clover, WV = winter vetch

2.4.3 Site variations in populations of *Fusarium* spp.

Out of the 670 *Fusarium* isolates 336 (50.1%) originated from Switzerland (n = 360 roots), 180 (26.9%) from Germany (n = 640), 117 (17.5%) from Italy (n = 360), and 37 (5.5%) from Sweden (n = 120). Rarefaction analysis showed that Swiss and German sites harbored *Fusarium* communities of higher diversity compared to other sites. However, the extrapolated species rarefaction curves were fairly asymptotic for Italian, Swiss and German sites, while in contrast, the rarefaction curve for the Swedish site failed to reach an asymptote, indicating that additional species would likely be collected by further sampling (Figure 2.2B).

The one-way ANOSIM test showed that the *Fusarium* communities at the Swiss site were significantly different from those of the German, Italian, and Swedish sites ($P < 0.001$) with the latter three not significantly different from each other. However, a mid-range value of R (= 0.52) for the Swiss-Swedish comparison contrasted with much lower R values for the Swiss-German and Swiss-Italian comparisons (R = 0.10 and 0.26, respectively) implies that the separation was moderately strong for the former, and rather weak for the latter two comparisons. Lack of strong separation suggests that samples within the sites were just as similar in *Fusarium* community composition as samples between the sites (Table 2.4).

The most prevalent species were *F. oxysporum* and *F. avenaceum* accounting for 68.5% (461 isolates) of all fusaria. They were common at most of the sites, particularly in Switzerland ($P < 0.05$). In Sweden, *F. oxysporum* was dominating, while *F. avenaceum* occurred rarely. *Fusarium solani* and *F. equiseti* were more frequent at the German, Swedish and Swiss sites, and *F. acuminatum* was more common in Italy ($P < 0.05$). Also present but less frequent were *F. redolens, F. graminearum* mainly recovered from the Swiss site ($P < 0.05$), and *F. culmorum* was not isolated from Italy. Most remaining species found were represented by a few isolates only (Figure 2.4B).

Table 2.4. Differences among sites in the *Fusarium* community composition. R and p-values obtained for every pair of sampling site using one-way ANOSIM.

	Switzerland		**Germany**		**Sweden**	
	R value	***P* value**	**R value**	***P* value**	**R value**	***P* value**
Germany	0.1090	0.0001				
Sweden	0.5210	0.0001	-0.0180	0.6660		
Italy	0.2640	0.0001	0.0080	0.2000	0.0140	0.3130

The contribution of each species to dissimilarity among the sites was assessed using the dissimilarity breakdown method (Table 2.5). The dominant species *F. oxysporum* contributed the most to dissimilarity (mean = 35%) between examined sites with respect to abundance. *Fusarium avenaceum* was the second most important contributor accounting for 31% of the dissimilarity. This was due to its high frequency in Switzerland, contributing to dissimilarity between this site and Germany, Italy and Sweden with 38%, 39% and 39%, respectively. The frequencies of *F. solani, F. equiseti* and *F. acuminatum* account for another 22% of the differences. The former two species were relatively frequent in Switzerland, Germany and Sweden, but rarely occurred in Italy, contributing to dissimilarity between the sites with 9% and 7%, respectively. *F. acuminatum* contributed to dissimilarity with 6%, and showed particularly low frequencies at the Swedish site. The rest of the species contributed little to the dissimilarity because they were much less frequent.

Table 2.5. Results of the SIMPER routine: contribution (Cont %) of *Fusarium* spp. to average dissimilarities (Dissim) between examined sites based on abundance.

	Italy vs Sweden		Italy vs Switzerland		Germany vs Italy		(Germany vs Sweden		Germany vs Switzerland		Sweden vs Switzerland		Overall	
	Dissim	Cont %	Dissim	Cont %	Dissim.	Cont %	Dissim	Cont %	Dissim	Cont %	Dissim	Cont %	Cont %	Cumulative %
F. oxysporum	34.5	51.1	26.8	33.3	27.8	41.4	26.5	36.5	24.7	29.8	24.4	30.3	35.4	35.4
F. avenaceum	11.6	17.2	31.3	39.0	14.8	21.9	13.9	19.1	31.2	37.7	31.1	38.5	31.1	66.5
F. solani	4.3	6.4	5.0	6.2	7.1	10.5	9.2	12.6	7.0	8.4	6.2	7.6	8.7	75.2
F. equiseti	4.7	7.0	5.6	7.0	5.8	88.8	7.0	9.7	6.8	8.2	6.6	8.1	7.4	82.6
F. acuminatum	5.6	8.3	4.8	5.9	9.2	83.0	3.4	4.7	3.5	4.3	3.2	3.9	6.1	88.7
F. redolens	3.5	5.1	3.4	4.3	0.9	1.3	3.3	4.5	3.3	4.0	4.6	5.7	3.6	92.3
F. culmorum	0.6	0.9	0.7	0.9	3.5	5.1	3.7	5.1	2.1	2.5	1.1	1.3	2.9	95.2
F. graminearum	0.0	0.0	1.9	2.3	0.3	0.5	0.3	0.5	2.0	2.5	1.8	2.3	1.6	96.8
F. tricinctum	0.0	0.0	0.4	0.5	1.1	5.1	1.0	1.4	0.9	1.1	0.4	0.5	1.0	97.8
F. sambucinum	0.0	0.0	0.0	0.0	1.0	1.5	0.9	1.3	0.5	0.6	0.0	0.0	0.7	98.5
F. torulosum	2.8	4.1	0.0	0.0	0.0	0.0	2.7	3.7	0.0	0.0	0.9	1.2	0.7	99.1
F. poae	0.0	0.0	0.2	0.2	0.4	0.5	0.3	0.5	0.4	0.4	0.2	0.2	0.4	99.5
F. crookwellense	0.0	0.0	0.0	0.0	0.5	0.7	0.4	0.6	0.3	0.3	0.0	0.0	0.3	99.8
F. sporotrichoides	0.0	0.0	0.2	0.3	0.0	0.0	0.0	0.0	0.2	0.3	0.22	0.3	0.2	100.0

2.4.4 Aggressiveness of *Fusarium* species on pea

Fusarium avenaceum caused the most severe root rot symptoms on pea (mean DI = 82), followed by *F. oxysporum* (DI = 39), *F. solani* (DI = 34), and *F. tricinctum* (DI = 25). *Fusarium equiseti* and *F. acuminatum* caused little root damage and overall were not or only weakly aggressive, with mean disease indices of 16 and 13, respectively (Table 2.6).

Table 2.6. Mean root rot severity (DI), correlation between DI and fresh weights, and changes in fresh weights of three week old pea plants caused by six *Fusarium* species. Changes in fresh weights are expressed as percentage change in fresh weight of inoculated treatments relative to non-inoculated control.

Pathogen (n)[1]	Mean DI[2]	Mean FW change (%)	Correlation coefficient[4]	FW change (%)	
				Maximum	Minimum
F. oxysporum (16)	39.0 b[3]	-19.3 b	0.165**	-33.4	-10.2
F. avenaceum (16)	82.4 a	-74.0 a	0.910***	-100.0	-13.8
F. solani (23)	33.9 c	-3.3 c	0.080*	-39.2	+10.1
F. equiseti (4)	12.8 e	+5.0 d	0.003	-4.7	+14.1
F. acuminatum (8)	16.5 e	+7.6 d	0.056	-6.3	+22.0
F. tricinctum (2)	25.3 d	+1.0 cd	0.003	-2.5	+4.4
Control	6.3 e	0.0 cd	-	-	-

[1]n = total number of isolates tested; [2]Disease severity is expressed as Disease Index (DI), where DI = 0-15 – non-aggressive; DI = 16-30 - weak aggressiveness; DI = 31-70 - moderate aggressiveness; DI = 71-100 - high aggressiveness. Data presented are means across isolates for each *Fusarium* spp. tested; [3]Different letters indicate significant differences according to Kruskal multiple comparison test ($P < 0.05$); [4]***, **, *: $P < 0.001$, 0.01 and 0.05, respectively.

Overall, only *F. avenaceum* and *F. oxysporum* caused significant reductions in pea plant biomass (mean = 74.0% and 19.3%, respectively). Also, a highly significant linear relationship was found between root rot severity and biomass reduction especially for *F. avenaceum* ($R^2 = 0.91$, $P < 0.001$). Statistically significant but weak relationships between the two parameters were also observed for *F. oxysporum* and *F. solani*, while mean root rot severity for *F. equiseti, F. tricinctum* and *F. acuminatum* was low and could not be correlated with biomass reduction on the three-week old pea plants (Table 2.6).

Among individual isolates within *Fusarium* species, significant variation in aggressiveness occurred for both, root rot severity and reductions of biomass ($P < 0.05$). The majority of the *F. avenaceum* isolates were highly aggressive on pea causing severe root rot and damping-off symptoms. Out of the five isolates that produced moderate severity symptoms, three still

caused significant biomass reduction (FA3, FA4 and FA5) of more than 50% relative to non-inoculated control plants (Figure 2.5A and B).

Root rot severity following inoculation of the majority of *F. oxysporum* and *F. solani* isolates differed significantly from the non-inoculated controls. However, effects on plant growth varied considerably between and within these two species (Figure 2.5A). All *F. oxysporum* isolates caused biomass reductions, but compared to the corresponding control the difference was only statistically significant for isolate FO8. This isolate was recovered from subterranean clover roots, and caused 34% biomass reduction (Figure 2.5B). The lowest DI was observed for the isolate FO1 from pea, but biomass was still reduced by 17%. The effects of the *F. solani* isolates were much more variable. Eleven out of 26 isolates tested increased biomass of pea by up to 10%, despite low to moderate root disease severity. The most aggressive isolate of *F. solani* was also isolated from subterranean clover roots (FS22), and caused moderate disease severity with a biomass reduction of 39%. Both *F. solani* isolates isolated from pea (FS20 and FS24) caused high DI but had no effect on biomass (Figure 2.5A and B).

Two weakly pathogenic isolates of *F. tricinctum* had no significant effect on pea biomass. Isolates of *F. equiseti* and *F. acuminatum* were non- to weakly pathogenic, often causing increases in biomass, ranging from 9 to 20% (Figure 2.5A and B).

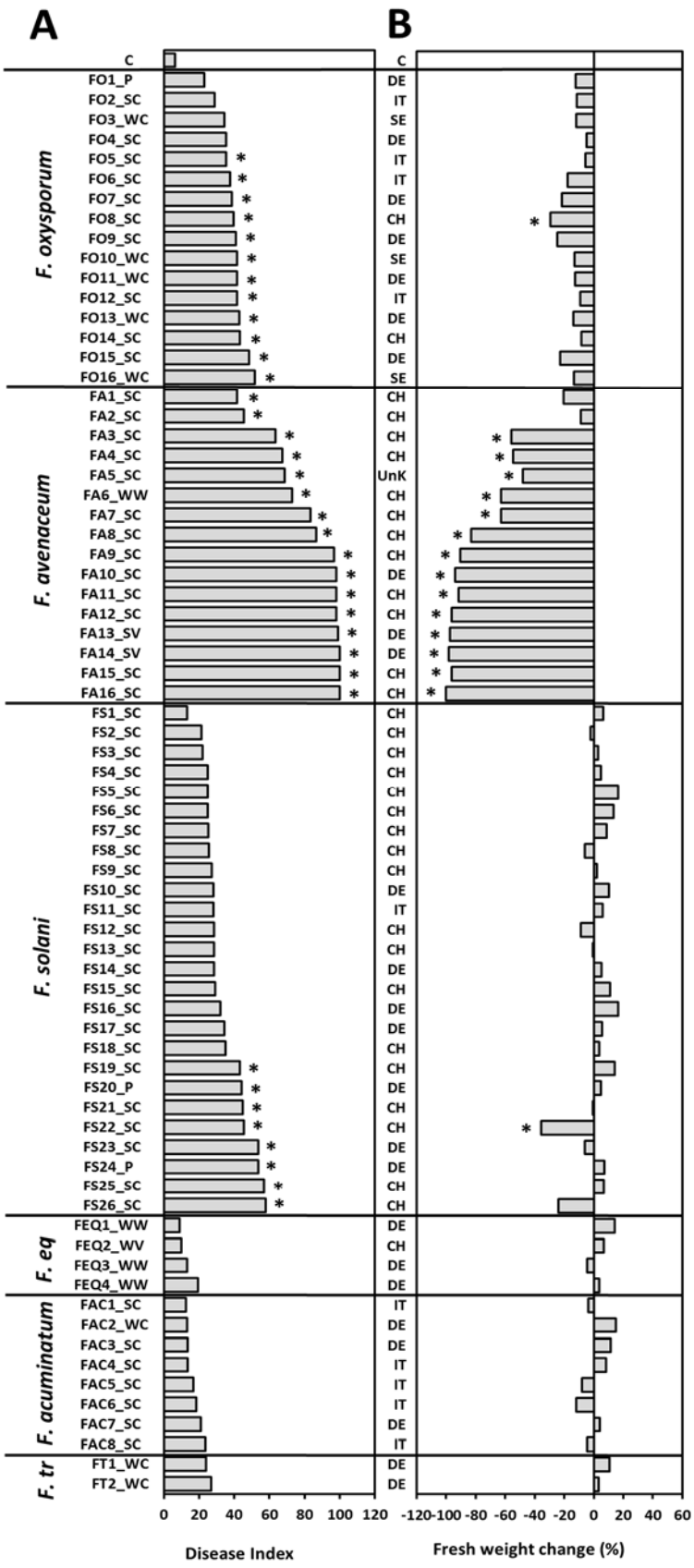

Figure 2.5. Effects of the *F. oxysporum, F. avenaceum, F. solani, F. equiseti (F. eq), F. acuminatum* and *F. tricinctum (F. tr)* isolates on disease severity (A), and fresh weight (B) of pea plants. Effects on the fresh weights are expressed as percentage change relative to non-inoculated control.

Disease severity is expressed as Disease Index (DI), where DI = 0-15 – non-aggressive; DI = 16-30 - weak aggressiveness; DI = 31-70 - moderate aggressiveness; DI = 71-100 - high aggressiveness. The letters in the suffix of each isolate ID number represent the host plant from which isolates were collected, where P = pea, SC = subterranean clover, WC = white clover, WV = winter vetch, SV = summer vetch, WW = winter wheat. Geographical origin of the isolates are denoted by the country of origin, where IT = Italy, CH = Switzerland, DE = Germany, SE = Sweden, and UnK = unknown. C = non-inoculated control. Asterisks next to the bars indicate significant difference from the non-inoculated control plants according to Dunn's multiple comparison test with one control ($P < 0.05$). Data presented are means of three replicate pots.

2.5 Discussion

Fusarium oxysporum and *F. avenaceum* were the most commonly detected *Fusarium* spp. accounting for 68.5% of total isolation percentage and frequently causing disease symptoms on pea, often reducing biomass. Each of these two *Fusarium* species was ubiquitous, occurring in most of the sites and on all four hosts. Less frequently found species were *F. solani, F. equiseti, F. acuminatum, F. redolens, F. culmorum, F. graminearum, F. tricinctum, F. crookwalence, F. poae, F. sambucinum, F. sporotichoides* and *F. torulosum*.

Fusarium oxysporum together with *F. solani* have previously been reported as commonly isolated fungi from diseased roots of red clover in Sweden (Rufelt, 1986; Lager and Gerhardson, 2002) and New Zealand (Nan, 1989). One or both species have been found in high frequencies in infected pea roots in Germany (Pflughöft, 2008), Sweden (Persson et al., 1997) and North Dakota, USA (Chittem et al., 2015). In addition, *F. oxysporum* has recently been reported to be the most commonly isolated *Fusarium* species from soybean roots in Iowa, USA (Arias et al., 2013b).

Members of *F. oxysporum* and *F. solani* represent a cosmopolitan species complex showing high levels of morphological and genetic variability within each group, consisting of saprophytic, endophytic and pathogenic strains (Leslie and Summerell, 2006). They can cause vascular wilts and root rots in over 100 plant species (Michielse and Rep, 2009; Kolattukudy and Gamble, 1995). However, despite this very broad host range of the species complex as whole, individual strains often infect only one or just a few plant species. Consequently, more than 70 host specific *formae speciales* for *F. oxysporum* (Lievens et al., 2008), and 11 host specific *formae speciales* for *F. solani* (Suga et al., 2002) have so far been described. Thus, detection of these species in the plant material is not proof of ongoing disease infection, and often additional screening tests are needed to confirm aggressiveness of recovered isolates.

The aggressiveness tests on pea in this study showed that all *F. oxysporum* and *F. solani* isolates tested are capable of causing root rot symptoms. However, only 1 out of 16 *F. oxysporum* isolates and 1 out of 26 *F. solani* caused significant biomass reductions, which is a more consistent indicator of aggressiveness than root rot severity alone. In addition, inoculation by 11 *F. solani* isolates resulted in even slightly positive changes in pea biomass. The most aggressive *F. oxysporum* isolate was obtained from subterranean clover and caused

a biomass reduction of 34%. Skovgaard (2002) observed a similar variability in aggressiveness among 28 *F. oxysporum* isolates from soil and pea plants collected from a wide range of locations in Denmark. The authors reported that out of these only seven strains were aggressive on pea, while the remaining were weakly to non-aggressive. Similar results were also observed by Chittem et al. (2015) who isolated *F. oxysporum* at high frequencies from diseased pea roots in North Dakota, but *in vitro* studies showed only weak pathogenicity of tested isolates to pea. Ondrej et al. (2008) and Arias et al. (2013a) reported similar results for the growth and yield of pea and soybeans after root inoculation by *F. solani* isolates.

Lack of external stress and the short time for colonization (3 weeks) of the root system by *F. oxysporum* and *F. solani* could be the reason for the low effects on peas we observed. Rush and Kraft (1986) observed that despite severe rot symptoms at the cotyledon attachment area even 35 days after emergence of peas, deterioration of roots and reduction of plant growth of plants inoculated with *F. solani* f.sp. *pisi* was still low. The same rot symptoms caused severe loss of roots and plant stunting 49 days after emergence (beginning of flowering). They concluded that one of the most important predictors of potential effects of *F. solani* f.sp. *pisi* is the timing of deterioration of roots and not the severity of symptoms. Thus, longer periods of host-pathogen interaction may be necessary before conclusions can be drawn. Nevertheless, in our study all of the *F. oxysporum* and *F. solani* isolates collected from the roots of subterranean clover and white clover readily infected pea, suggesting that the two fungi, especially *F. oxysporum* may be less host specific than reported previously (Suga et al., 2002; Lievens et al., 2008). We are currently extending our studies to investigate potential genotypic diversity in the population of recovered *F. solani* isolates (Chapter 3).

The high frequency of isolation of *F. avenaceum* found in our study, particularly from the roots of subterranean clover in Switzerland, is consistent with work of Wong et al. (1985) and Barbetti et al. (2007), who reported *F. avenaceum* as one of the most commonly isolated species from the roots of subterranean clover dominated pastures in Australia. Similarly, in a survey conducted during the early 1980s, Pegg and Parry (1983) reported *F. avenaceum* as the most frequently isolated fungi from diseased roots and stems of lucerne (*Medicago sativa*) in England. In our study, the fungus was isolated in high frequencies only from the samples collected at the Swiss site, thus our results are in contrast to that of Yli-Mattila et al. (2010), and Lager and Gerhardson (2002), who reported *F. avenaceum* as dominating pathogenic

fungi on red clover in Finland and red clover and white clover in organic fields in central Sweden.

The greenhouse assay in this study showed that *F. avenaceum* isolates caused the most severe root rot symptoms on pea. Only 5 isolates were moderately aggressive, while the other 11 isolates were highly aggressive on the spring pea variety Santana. Both, moderately and highly aggressive strains caused high reductions in fresh weights. The severe disease development observed for *F. avenaceum* isolates is consistent with the findings of Bacanovic et al. (2013) who reported high aggressiveness of *F. avenaceum* on spring pea variety Santana with reductions of fresh weight of up to 82%. Severe root rot observed in plants inoculated with *F. avenaceum* agrees with several other studies that also demonstrated high aggressiveness of the fungus on pea (Feng, 2010; Chittem et al., 2015).

Other economically important plants affected by *F. avenaceum* include a range of legume species such as lentil (Fletcher et al., 1991), soybean (Zhang et al., 2010), alfalfa (Couture et al., 2002), as well as canola (Chen et al., 2014), barley and oat (Nielsen et al., 2011). *F. avenaceum* together with *F. tricinctum, F. poae, F. culmorum* and *F. graminearum* form the complex causing *Fusarium* Head Blight (FHB) of small grain cereals in Europe. In recent years, in Northern Europe, *F. avenaceum* has become the dominating species of the complex (Uhlig et al., 2007). *F. avenaceum* also produces mycotoxins such as moniliformin, enniatin, and beauvericin (Morrison et al., 2002) that are harmful for animals and humans. As *F. avenaceum* is a generalist, there is a risk of building up inoculum that can lead to infection and mycotoxin contamination of subsequent crops. However, further work is needed to better understand *F. avenaceum* dynamics, epidemiology and impact on the yields of subsequent main crops under field conditions.

The isolates of *F. tricinctum, F. equiseti* and *F. acuminatum* caused some root discoloration on pea, but no reductions in fresh weights, some even increased biomass considerably. *Fusarium tricinctum* is generally considered a soil saprophyte and opportunistic pathogen in temperate regions. In wheat*, F. tricinctum* is part of the fungal complex causing Fusarium Head Blight (FHB) of small grain cereals in Europe and North America (Uhlig et al., 2007). The fungus has not been reported as part of the root rot complex of pea, however, a number of recent studies are suggesting that it could be an important pathogen of soybean (*Glycine max*) (Chitrampalam and Nelson, 2014). *Fusarium equiseti* and *F. acuminatum* are naturally

occurring endophytes and opportunistic pathogens in diverse ecosystems and are able to colonize the roots of various hosts (Leslie and Summerell, 2006). The presence of *F. equiseti* can inhibit *F. avenaceum* and *Peyronellaea pinodella* development in the root system of pea plants and suppress associated disease (Šišić et al., 2016, Chapter 4). The role of *F. acuminatum* in pea root health in unknown.

Several studies on the ecology and distribution of *Fusarium* spp. around the world suggest that climate is one of the major factors affecting the occurrence of these fungi in soils (Sanglang et al., 1995; Boohan et al., 2003). Although the sampling sites chosen for this study varied in their location specific climatic and soil conditions, the endophytic communities found at each site were dominated by similar *Fusarium* species. Comparison of the overall community structures yielded significant differences and separated the Swiss site from all others. However, only the separation of the Swiss and Swedish sites was supported by moderately high R values. In the comparisons of plant species within sampling sites, no clear separation between locations was observed. Thus, environment and host plant influenced the *Fusarium* community composition to some extent with the main effects being on the relative frequencies of fungal species found. The two most frequently isolated species from all four dissimilar climate areas, *F. oxysporum* and *F. avenaceum*, have obviously adapted to a broad range of environments allowing them an extensive distribution and colonization opportunities. Further research is, however, needed to identify precise combination of soil and environmental conditions that influence individual densities of *Fusarium* species at each studied site.

In conclusion, this study has provided new information about frequency, community structure, and pathogenicity of *Fusarium* spp. associated with the clover and vetch cover crop and living mulch species in Europe. Our results show that some of the major pathogens of various leguminous and non-leguminous crops regularly colonize the studied plant species and have the potential to cause yield losses of subsequent main legume grain crops such as pea. Thus, pathological risk assessment is needed to ensure the successful use of legume cover crops and living mulches in crop rotations. Nevertheless, whether the presence of highly aggressive strains will lead to infections in subsequent crops is likely to depend on additional environmental and soil factors that most likely can be influenced positively by farming practices aiming to improve diversity of soil biota and main crop biotic and abiotic stress resistance.

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CHAPTER 3: Phylogeny, ecology and pathogenicity of the *Fusarium solani* species complex in Europe

Manuscript in preparation

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Abstract

Members of the *Fusarium solani* species complex (FSSC) are best known as ubiquitous plant pathogens and soil saprophytes, that are also increasingly implicated as the causal agents of human and animal diseases. Comprising approximately 60 distinct phylogenetic species distributed among 3 major clades, the FSSC associated with legumes, particularly under European agro-climatic conditions, is still poorly documented. In the present study, a collection of 79 isolates belonging to FSSC recovered from several leguminous species grown across Europe was characterized by molecular genotyping (*tef1* and *rpb2* loci) and greenhouse aggressiveness assays. The isolates studied formed four lineages, all nested within FSSC clade 3. The majority of isolates, however were associated with two major lineages, the *F. solani* f. sp. *pisi* lineage mainly accommodating German and Swiss isolates, and the *Fusisporium* (*Fusarium*) *solani* lineage accommodating mainly Italian isolates. In subsequent aggressiveness tests on pea, that included a subset of 75 isolates, the majority of isolates caused root rot symptoms with weakly to moderately aggressive strains dominating the populations of tested *Fusarium* species. Aggressiveness was not correlated with isolate phylogenetic position, host plant or its geographic origin. In addition, 62 accessions belonging to 10 legume genera were evaluated for their potential to act as hosts for *F. solani* f. sp. *pisi*, the species mainly considered as a sole pathogen of pea. A total of 58 of the accessions were successfully colonized, with 25 of these being asymptomatic hosts.

3.1 Introduction

Fusarium solani (sexual morph *Haematonectria haematococca*; syn. *Nectria haematococca*) is a plurivorous filamentous fungus of significant agricultural importance, that has been accommodated as a single species in the section *Martiella* and *Ventricosum* within the genus *Fusarium* (Snyder and Hansen, 1941). Reevaluation of species taxonomy in the scope of the current molecular findings revealed that *F. solani* is a species complex (FSSC) which includes at least 60 phylogenetic species (Schroers et al., 2016). Members of the complex are worldwide spread fungi, well adapted to the soil environment, with considerable ecological plasticity, causing infections in humans, animals and plants (Leslie and Summerell, 2006; Zhang et al., 2006). Diversity, distribution and population density of particular strains is often determined by crop species, soil characteristics and climatic conditions (Aoki et al., 2012, 2003; O'Donnell, 2000).

The phytopathogenic strains within FSSC include some of the economically most important plant pathogens that have been associated with vascular wilts and root rots in over 100 agricultural crops (Kolattukudy and Gamble, 1995). However, despite this very broad host range of the species complex as a whole, individual strains are often associated with only one or just a few plant species. Consequently, the pathogenic strains have been divided into 12 *formae speciales* and two races on the basis of their aggressiveness to particular host (Toussoun and Snyder, 1961; Suga et al., 2000; Chung et al., 2011; Bueno et al., 2014).

Early studies on sexual reproduction of special forms and races showed that *F. solani* represents at least seven biological species classified as mating populations (MPs I-VII) with *Haematonectria haematococca* as sexual morph. The sexual morphs were found to be heterothallic and were somewhat correlated with the host range as sexual reproduction can occur only within each special form or race *i.e.* biological species (Matuo and Snyder, 1973). However, the designation *formae speciales* may lead to generalizations in behavior and incorrect assumptions concerning the aggressiveness of individual isolates. For example, studies on the host range of *F. solani* f. sp. *pisi* (*H. haematococca* MP VI), named by its specific pathogenicity on pea (*Pisum sativum* L.), revealed that the species was also pathogenic on chickpea (*Cicer arietinum*) as well on several other hosts (Westerlund et al., 1974; Matuo and Snyder, 1972). Similar results have also been reported for the host range and aggressiveness of *F. virguliforme* (formerly *F. solani* f. sp. *glycines*) and *F. solani* f. sp. *eumartii* (Romberg and

Davis, 2007; Kolander et al., 2012). Thus, the term *forma specialis* is often misleading and will most likely need to be reconsidered in the future.

Traditionally used methods for identifying special forms, which rely on morphological criteria, sexual compatibility, and aggressiveness tests, are time consuming, labor intensive and often inconclusive. This is because correct identification requires extensive knowledge of classical taxonomy and in case of aggressiveness tests, the environmental factors and genetic makeup of the host plant usually have significant influence on the bioassay outcome. Correct taxonomic identification of the species causing disease is, however, very important, because its epidemiology will have major implications on disease management strategies.

Molecular biological methods that utilize phylogenetic studies based on polymorphisms in DNA sequences of the translation elongation factor 1 alpha (*tef1*) and the second largest subunit of RNA polymerase II (*rpb2*) have shown to be sufficiently informative for reliable species recognition within FSSC and the genus *Fusarium* (Al-Hatmi et al., 2016). These protein coding gene regions have high phylogenetic utility because they show high levels of sequence polymorphism among closely related species, non-orthologous copies have not been detected, and universal primers have been designated making them alignable and comparable across the genus (Geiser et al., 2004).

In the current study, phylogenetic and pathogenic relationship among isolates of FSSC collected from infected pea, subterranean clover, white clover and winter vetch plants was investigated. The isolates originated from diverse agro-climatic regions and soil conditions based on their geographical origins and cultivation systems across Europe. The objectives of the study were to: (i) determine diversity and potential geographical patterns or host preference of FSSC isolates, (ii) compare aggressiveness and determine if FSSC isolates from non-pea hosts are capable of causing disease on pea under greenhouse conditions, and (iii) clarify the host range of *F. solani* f. sp. *pisi* among several leguminous species.

3.2 Material and methods

3.2.1 *Fusarium* isolates

A total of 79 FSSC isolates were collected for this study (Table 3.1). Among these, 18 isolates were recovered from pea (*Pisum sativum* L.), 39 from subterranean clover (*Trifolium subterraneum* L.), 3 from white clover (*T. repens* L.) and 14 from winter vetch (*Vicia villosa* R.). The isolates originated from Germany (n = 28), Switzerland (n = 24), Italy (n = 21) and Sweden (n = 1). Additionally, we included one isolate from faba bean (*V. faba* L.), and expanded the isolates associated with legumes with 2 isolates collected from compost and 2 (one of each) recovered from an infected hibiscus (*Hibiscus* sp.) and cherry tree (*Prunus sp.*), all from German environment (Table 3.1). All isolates were collected following the methods described by Baćanović (2015) in the period between 2009 and 2016, morphologically identified as *F. solani* and maintained as single-spore cultures at the Internal Culture Collection of the Ecological Plant Protection Department at University of Kassel. Four isolates of *F. redolens,* recovered from white clover grown in Sweden, were included as an outgroup in this study.

3.2.2 DNA extraction, PCR amplification and sequencing

Genomic DNA was extracted from cultures actively growing on half strength PDA agar plates (½ PDA; 19 g/l Difco PDA and 10 g/l agar) using the modified CTAB (cetyltrimethylammonium bromide*)* protocol described by Doyle and Doyle (1987). Fungal mycelium was collected into 1.5 ml Eppendorf tubes under a laminar flow hood, and fungal cells disrupted by grinding using sterile micro-pestles. The grounded mycelium was then suspended in 800 µl of CTAB extraction buffer (1.4 M NaCl, 20 mM EDTA, 2% CTAB, 100 mM Tris, pH 8.0), and incubated for 1h at 65°C in a thermomixer under constant agitation/shaking at 300 rpm. Subsequently, 600 µl of chloroform was added to each tube, mixed gently by inverting, and centrifuged for 5 min at 14000 rpm. The upper phase was then transferred into new 1.5 ml Eppendorf tubes containing 350 µl of isopropanol, precipitated for 15 min at room temperature and centrifuged for 10 min at 14000 rpm. The supernatant was discarded and the obtained DNA pellets were washed twice with 70% ethanol (with one centrifugation step in between), air dried, diluted 20 times in milli-Q water and stored at -20°C until use.

A portion of the translation-elongation factor 1 alpha (*tef1*) gene was amplified for all *Fusarium* strains using primer pairs EF1 and EF2 previously described by O'Donnell et al.

(1998). Based on the results of *tef1* phylogenetic positions, 28 strains were selected and the second-largest subunit of RNA polymerase II (*rpb2*) was amplified using primer pairs RPB2-5F2 (Sung et al., 2007) and fRPB2-7cR (Liu et al., 1999). Each polymerase chain reaction (PCR) had a total volume of 50 µl and contained 1 µl of diluted genomic DNA, 10× TrueStart Hot Start Taq Buffer [200 mM Tris-HCl (pH 8.3 at 25° C), 200 mM KCl, 50 mM $(NH_4)_2SO_4$], 2.5 mM $MgCl_2$, 0.2 mM of each of the dNTP, 0.4 mM of each primer, and 1 unit TrueStart Hot Start *Taq* DNA Polymerase (ThermoFisher Scientific, Darmstadt, Germany). The PCR reactions were performed in a Biometra TAdvanced Thermal Cycler (Applied Biosystems, Foster City, California, USA). Conditions for amplification for the *tef1* gene region were an initial denaturation step of 3 min at 95°C, followed by 30 cycles of denaturation (95°C for 30s), annealing (53°C for 30s) and elongation (72°C for 45s). The final elongation step was conducted at 72°C for 7 min. For the *rpb2* loci amplification consisted of 5 cycles of 45 s at 94°C, 45 s at 60°C and 2 min at 72°C, then 5 cycles with a 58°C annealing temperature and 30 cycles with a 54°C annealing temperature (Woudenberg et al., 2013).

Amplicons were purified using the DNA Clean & Concentrator kit (Zymo Research, Freiburg, Germany) according to the manufacturers instructions and sequenced in both directions either by Eurofins Genomics (Ebersberg, Germany) or by MacroGen (Amsterdam, Netherlands) using the above-mentioned primer pairs.

3.2.3 Phylogenetic analyses

Consensus sequences were initially assembled from forward and reverse sequence using MEGA v6 software (Tamura et al., 2013), and the sequences were used as queries for the Fusarium-ID v. 1.0 database (Geiser et al., 2004), and the *Fusarium* MLST databases (http://www.cbs.knaw.nl/fusarium; O'Donnell et al., 2010) to confirm identity of the isolates. The phylogenetic approach was then used to investigate the relationship between the tested strains and reference strains of FSSC retrieved from the GenBank. The sequences were aligned using MAFFT v.7 (http://mafft.cbrc.jp/alignment/server/index.html; Katoh and Standley, 2013) and adjusted manually with MEGA v6. Phylogenetic analyses, including the majority of known *Fusarium* species within the FSSC, were performed on single *tef1* data sets for all strains collected from this study, as well as for single *rpb2* and combined data sets of *tef1* and *rpb2* gene regions (28 selected strains). All the *Fusarium* strains used in the phylogenetic analyses including detailed information's for strains from this study and assigned GenBank accession

numbers are listed in Table 3.1 and Table 3.2. The best-fit model of evolution of phylogenetic relationship was determined by MEGA v6. A bootstrapped maximum-likelihood analysis was performed using the RAxML-VI-HPC v. 7.0.3 with non-parametric bootstrapping and 1000 replicates implemented on the Cipres portal (http://www.phylo.org/; Stamatakis et al., 2008). For the outgroup purposes, in addition to *F. redolens* isolates, *F. thapsinum* (H05-557S-1 DCPA and CBS 130176) was used to generate the phylogenetic trees.

3.2.4 Greenhouse experiments

Experiment 1. Aggressiveness of selected FSSC isolates to pea. To compare aggressiveness and to determine whether the FSSC strains from non-pea hosts are capable of causing disease on pea, a total of 75 isolates were tested in a greenhouse assay. The pathogenicity test included 48 isolates of *F. solani* f. sp. *pisi*, 24 isolates of *Fusisporium solani*, 2 isolates of *F. solani*, and 1 isolate of *F. keratoplasticum*. In this study, the isolates that formed distinct groups based on the phylogenetic analysis and showed no strong phylogenetic relationship to any of previously defined species within the FSSC were included in the *F. solani* group. Four isolates of *F. redolens* were also included in this experiment. The geographic origin and the host plants from which the isolates were collected are given in Table 3.1.

To prepare inoculum, each *Fusarium* isolate was cultured on ½ PDA at room temperature under alternating cycles of 12 h BLB fluorescent light and 12 h darkness. After 15 days, spores were washed with sterile distilled water and enumerated in the suspension with a Fuchs Rosenthal hemocytometer.

Seeds of field pea cv. Santana were surface sterilized in 70% ethanol for 5 min and rinsed with distilled water prior to planting. Four pea seeds (germination rate of 98 %) were then planted into 500 ml pots filled with autoclaved sand, and 2 x 10^4 spores g^{-1} substrate of the respective isolate was applied to each pot. Un-inoculated control plants were left untreated and watered with sterile distilled water only. Four replicate pots were sown per treatment and arranged in a completely randomized design. Experimental plants were kept in the greenhouse at 19°C day and 16°C night temperature. Natural day light was additionally supplemented with high-pressure sodium lamps (400 W) in order to provide a photoperiod of 16 h light day^{-1}. Plants were watered daily with tap water and additionally fertilized with complex N:P:K fertilizer

Wuxal Super (8:8:6 + microelements). A total of 120 mg of N l^{-1} of substrate was divided into four portions and given over the course of the experiment.

After 42 days of growing, plants were removed from pots, and the roots were separated from the above ground biomass. Above ground plant parts of each pot were weighted and dried at 105°C until constant weight was attained. Roots were washed under running tap water, and root rot severity was assessed assigning 0-8 disease severity ratings (DSR) based on external and internal root tissue discoloration levels as described in Chapter 2 (Pflughöft, 2008). Consequently, a disease severity index (DI) was calculated (see Chapter 2) and isolates were grouped into four distinct aggressiveness classes assigned relative to the un-inoculated control, where: DI = 0-36 – non-aggressive; DI = 37-55 – weakly aggressive; DI = 56-85 – moderately aggressive; and DI = 86-100 – highly aggressive. A threshold disease index of 36 for classifying the isolate non - aggressive was chosen because factors other than inoculation caused low levels of root discoloration in un-inoculated control plants and, up to this level there was no statistically significant difference in DI of inoculated treatments and un-inoculated control. Furthermore, the difference in DI value of the control treatment and the lowest threshold value for classifying isolates weakly aggressive was kept at the same distance level as in Chapter 2 (DI of +12). For example, in the current study mean DI of the un-inoculated control was 24 (±12) and the isolates were classified as non-aggressive when the DI of inoculated treatment was ≤ 36.

Twenty one different inoculation treatments were selected at random and the fungi were re-isolated from the surface sterilized roots (1% NaOCl, 3 roots per treatment) and identified morphologically to confirm recovery of the isolate.

Table 3.1. *Fusarium* isolates subjected to phylogenetic analysis and evaluated for aggressiveness to pea in greenhouse experiment 1.

Isolate ID[1]	Species[2]	Host/Substrate[3]	Geographical origin	Year	GenBank accession numbers[4]	
					tef1	*rpb2*
Fs1	*F. solani* f. sp. *pisi*	Pea (*Pisum sativum*)	Germany, Frankenhausen, Hessen	2013	KY556491	-
Fs2	*F. solani* f. sp. *pisi*	Pea	Germany, Frankenhausen, Hessen	2013	KY556463	-
Fs3	*F. solani* f. sp. *pisi*	Pea	Germany, Frankenhausen, Hessen	2013	KY556448	-
Fs4	*Fusisporium solani*	Pea	Germany, Frankenhausen, Hessen	2013	KY556500	KY556544
Fs5	*Fusisporium solani*	Pea	Germany, Frankenhausen, Hessen	2013	KY556511	-
Fs6	*F. solani* f. sp. *pisi*	Pea	Germany, Neu Eichenberg, Hessen	2013	KY556459	-
Fs7	*F. solani* f. sp. *pisi*	Pea	Germany, Neu Eichenberg, Hessen	2013	KY556466	-
Fs8	*F. solani* f. sp. *pisi*	Pea	Germany, Neu Eichenberg, Hessen	2013	KY556450	-
Fs9	*F. solani* f. sp. *pisi*	Pea	Germany, Frankenhausen, Hessen	2013	KY556451	-
Fs10	*F. solani* f. sp. *pisi*	Pea	Germany, n/a	2009	KY556452	-
Fs11	*F. solani* f. sp. *pisi*	Pea	Germany, Frankenhausen, Hessen	2013	KY556497	-
Fs12	*F. solani* f. sp. *pisi*	Pea	Germany, Frankenhausen, Hessen	2013	KY556447	-
Fs13	*F. solani* f. sp. *pisi*	Pea	Germany, Neu Eichenberg, Hessen	2013	KY556453	-
Fs14	*F. solani* f. sp. *pisi*	Pea	Germany, Frankenhausen, Hessen	2013	KY556449	-
Fs15	*F. solani* f. sp. *pisi*	Pea seed	Germany, Neu Eichenberg, Hessen	2012	KY556492	-
Fs16	*F. solani* f. sp. *pisi*	Pea seed	Germany, Neu Eichenberg, Hessen	2011	KY556493	-
Fs17	*F. solani* f. sp. *pisi*	Pea seed	Germany, Neu Eichenberg, Hessen	2011	KY556471	-
Fs18	*F. solani* f. sp. *pisi*	Pea seed	Germany, Neu Eichenberg, Hessen	2011	KY556458	KY556526
Fs19	*F. solani* f. sp. *pisi*	Subterranean clover (*Trifolium subterranean*)	Germany, Neu Eichenberg, Hessen	2013	KY556472	KY556535
Fs20	*F. solani* f. sp. *pisi*	Subterranean clover	Germany, Freising, Bavaria	2015	KY556488	KY556536
Fs21	*F. solani* f. sp. *pisi*	Subterranean clover	Germany, Neu Eichenberg, Hessen	2013	KY556454	KY556537
Fs22	*F. solani* f. sp. *pisi*	Subterranean clover	Germany, Neu Eichenberg, Hessen	2014	KY556473	KY556527
Fs23	*F. solani* f. sp. *pisi*	Subterranean clover	Germany, Neu Eichenberg, Hessen	2013	KY556455	KY556538
Fs24	*F. solani* f. sp. *pisi*	Subterranean clover	Germany, Neu Eichenberg, Hessen	2013	KY556474	-
Fs25	*F. solani* f. sp. *pisi*	Faba bean (*Vicia faba*)	Germany, Freising, Bavaria	2015	KY556460	-

Isolate ID[1]	Species[2]	Host/Substrate[3]	Geographical origin	Year	GenBank accession numbers[4]	
					tef1	*rpb2*
Fs26	*Fusisporium solani*	White clover (*Trifolium repens)*	Germany, Neu Eichenberg, Hessen	2014	KY556517	KY556542
Fs27	*Fusisporium solani*	White clover	Germany, Neu Eichenberg, Hessen	2014	KY556501	-
Fs28	*F. solani* f. sp. *pisi*	Compost	Germany, Hannover, Lower Saxony	2014	KY556475	KY556528
Fs29	*F. solani*	Compost	Germany, Hannover, Lower Saxony	2014	KY556524	KY556552
Fs30	*F. solani*	Hibiscus dying branch (*Hibiscus* sp.)	Germany, Witzenhausen, Hessen	2015	KY556525	KY556553
Fs31	*Fusiporium solani*	Cherry dying branch (*Prunus sp.*)	Germany, Witzenhausen, Hessen	2016	KY556520	KY556549
Fs32	*F. solani* f. sp. *pisi*	Subterranean clover	Switzerland, Reckenholz, Canton Zurich	2013	KY556476	-
Fs33	*F. solani* f. sp. *pisi*	Subterranean clover	Switzerland, Reckenholz, Canton Zurich	2013	KY556486	KY556529
Fs34	*F. solani* f. sp. *pisi*	Subterranean clover	Switzerland, Reckenholz, Canton Zurich	2013	KY556484	KY556530
Fs35	*F. solani* f. sp. *pisi*	Subterranean clover	Switzerland, Reckenholz, Canton Zurich	2013	KY556482	KY556539
Fs36	*F. solani* f. sp. *pisi*	Subterranean clover	Switzerland, Reckenholz, Canton Zurich	2013	KY556487	-
Fs37	*F. solani* f. sp. *pisi*	Subterranean clover	Switzerland, Reckenholz, Canton Zurich	2013	KY556495	-
Fs38	*F. solani* f. sp. *pisi*	Subterranean clover	Switzerland, Reckenholz, Canton Zurich	2015	KY556456	-
Fs39	*F. solani* f. sp. *pisi*	Subterranean clover	Switzerland, Reckenholz, Canton Zurich	2013	KY556477	-
Fs40	*F. solani* f. sp. *pisi*	Subterranean clover	Switzerland, Reckenholz, Canton Zurich	2013	KY556478	-
Fs41	*F. solani* f. sp. *pisi*	Subterranean clover	Switzerland, Reckenholz, Canton Zurich	2013	KY556464	-
Fs42	*F. solani* f. sp. *pisi*	Subterranean clover	Switzerland, Reckenholz, Canton Zurich	2013	KY556485	KY556531
Fs43	*F. solani* f. sp. *pisi*	Subterranean clover	Switzerland, Reckenholz, Canton Zurich	2013	KY556479	KY556532
Fs44	*F. solani* f. sp. *pisi*	Subterranean clover	Switzerland, Reckenholz, Canton Zurich	2013	KY556470	KY556533
Fs45	*Fusisporium solani*	Subterranean clover	Switzerland, Reckenholz, Canton Zurich	2013	KY556521	KY556543
Fs46	*F. solani* f. sp. *pisi*	Subterranean clover	Switzerland, Reckenholz, Canton Zurich	2015	KY556467	KY556534
Fs47	*F. solani* f. sp. *pisi*	Subterranean clover	Switzerland, Reckenholz, Canton Zurich	2015	KY556461	-
Fs48	*F. solani* f. sp. *pisi*	Winter vetch	Switzerland, Reckenholz, Canton Zurich	2015	KY556489	-
Fs49	*F. solani* f. sp. *pisi*	Winter vetch	Switzerland, Reckenholz, Canton Zurich	2015	KY556490	KY556540
Fs50	*F. solani* f. sp. *pisi*	Winter vetch	Switzerland, Reckenholz, Canton Zurich	2015	KY556480	-
Fs51	*F. solani* f. sp. *pisi*	Winter vetch	Switzerland, Reckenholz, Canton Zurich	2015	KY556468	-

Isolate ID[1]	Species[2]	Host/Substrate[3]	Geographical origin	Year	GenBank accession numbers[4]	
					tef1	*rpb2*
Fs52	*F. solani* f. sp. *pisi*	Winter vetch	Switzerland, Reckenholz, Canton Zurich	2015	KY556496	-
Fs53	*F. solani* f. sp. *pisi*	Winter vetch	Switzerland, Reckenholz, Canton Zurich	2015	KY556465	-
Fs54	*F. solani* f. sp. *pisi*	Winter vetch	Switzerland, Reckenholz, Canton Zurich	2015	KY556483	-
Fs55	*Fusisporium solani*	Subterranean clover	Italy, Localita' Riello, Viterbo	2015	KY556515	-
Fs56	*Fusisporium solani*	Subterranean clover	Italy, Localita' Riello, Viterbo	2015	KY556502	-
Fs57	*Fusisporium solani*	Subterranean clover	Italy, Localita' Riello, Viterbo	2015	KY556503	KY556545
Fs58	*Fusisporium solani*	Subterranean clover	Italy, Localita' Riello, Viterbo	2015	KY556498	KY556546
Fs59	*Fusisporium solani*	Subterranean clover	Italy, Localita' Riello, Viterbo	2015	KY556504	-
Fs60	*Fusisporium solani*	Subterranean clover	Italy, Localita' Riello, Viterbo	2015	KY556518	KY556547
Fs61	*Fusisporium solani*	Subterranean clover	Italy, Localita' Riello, Viterbo	2015	KY556505	-
Fs62	*Fusisporium solani*	Subterranean clover	Italy, Localita' Riello, Viterbo	2015	KY556509	-
Fs63	*Fusisporium solani*	Subterranean clover	Italy, Localita' Riello, Viterbo	2013	KY556519	-
Fs64	*Fusisporium solani*	Subterranean clover	Italy, Localita' Riello, Viterbo	2015	KY556510	-
Fs66	*F. solani* f. sp. *pisi*	Subterranean clover	Italy, Localita' Riello, Viterbo	2015	KY556469	-
Fs67	*Fusisporium solani*	Subterranean clover	Italy, Localita' Riello, Viterbo	2015	KY556499	-
Fs68	*Fusisporium solani*	Subterranean clover	Italy, Localita' Riello, Viterbo	2015	KY556506	-
Fs69	*Fusisporium solani*	Winter vetch	Italy, Localita' Riello, Viterbo	2015	KY556522	KY556550
FK70	*F. keratoplasticum*	Winter vetch	Italy, San Piero a Grado, Tuscany	2014	KY556523	-
Fs71	*Fusisporium solani*	Winter vetch	Italy, Localita' Riello, Viterbo	2015	KY556507	KY556548
Fs72	*Fusisporium solani*	Winter vetch	Italy, San Piero a Grado, Tuscany	2014	KY556512	KY556551
Fs73	*Fusisporium solani*	Winter vetch	Italy, San Piero a Grado, Tuscany	2015	KY556508	-
Fs74	*Fusisporium solani*	Winter vetch	Italy, San Piero a Grado, Tuscany	2015	KY556514	-
Fs75	*Fusisporium solani*	Winter vetch	Italy, San Piero a Grado, Tuscany	2015	KY556513	-
Fs76	*F. solani* f. sp. *pisi*	White clover	Sweden, n/a, Upsala	2014	KY556462	-
Fs77	*F. solani* f. sp. *pisi*	Subterranean clover	Germany, Neu Eichenberg, Hessen	2014	KY556457	KY556541
Fs78	*F. solani* f. sp. *pisi*	Subterranean clover	Switzerland, Reckenholz, Canton Zurich	2013	KY556494	-
Fs79	*Fusisporium solani*	Subterranean clover	Italy, Localita' Riello, Viterbo	2015	KY556516	-
Fs80	*F. solani* f. sp. *pisi*	Subterranean clover	Germany, Neu Eichenberg, Hessen	2014	KY556481	-
FR1	*F. redolens*	White clover	Sweden, n/a, Upsala	2014	KY556443	-

Isolate ID[1]	Species[2]	Host/Substrate[3]	Geographical origin	Year	GenBank accession numbers[4]	
					tef1	*rpb2*
FR2	*F. redolens*	White clover	Sweden, n/a, Upsala	2015	KY556444	-
FR3	*F. redolens*	White clover	Sweden, n/a, Upsala	2014	KY556445	-
FR4	*F. redolens*	White clover	Sweden, n/a, Upsala	2014	KY556446	-

[1]All isolates with exception of Fs77, Fs78, Fs79 and Fs80 were tested for aggressiveness on pea in greenhouse experiment 1; [2]Isolates that formed distinct groups based on the phylogenetic analysis and showed no strong phylogenetic relationship to any of the previously defined species within the FSSC were termed as *F. solani*; [3]Unless indicated differently isolates were collected from infected root system; [4]GenBank accession numbers for translation elongation factor 1-alpha (*tef1*) partial sequences and the second-largest subunit of RNA polymerase II (*rpb2*) gen region (selected isolates). n/a = not available.

Table 3.2. Reference strains sourced from the NCBI GenBank database used to examine phylogenetic relationships among collected isolates.

		GenBank accession numbers[1]	
Species	**Strain number**	***tef1***	***rpb2***
Fusarium solani	CBS 119996	HE647962.1	n/a
Fusarium petroliphilum	CBS 135955	n/a	KJ867426
Fusarium falciforme	CBS 138963	KT716213.1	n/a
Fusarium phaseoli	CBS 265.50	HE647964.1	n/a
Fusarium paranaense	CML 1988	KF597819.1	n/a
Fusarium solani f. sp. *piperis*	CML 2190	JX657675.1	n/a
Fusarium rectiphorum	FRC S1831	DQ247509.1	n/a
Fusarium haematococcum	FRC S1832	DQ247510.1	n/a
Fusarium kurunegalense	FRC S1833	DQ247511.1	n/a
Fusarium kelerajum	FRC S1837	DQ247516.1	n/a
Fusarium mahasenii	FRC S1845	DQ247513.1	n/a
Fusarium cf. *ensiforme*	FRC S1847	JF433028.1	n/a
Fusarium keratoplasticum	FRC S2477	KR673939.1	KR673969
Fusarium solani	FRC S485	DQ247312.1	n/a
Fusarium ambrosium	NRRL 20438	AF178332.1	n/a
Fusarium illudens	NRRL 22090	AF178326.1	JX171601
*Fusarium*sp. *cucurbitae* MPI	NRRL 22098	n/a	EU329489
Fusarium sp.	NRRL 22101	n/a	EU329490.1
Fusarium sp. *cucurbitae* MPV	NRRL 22141	n/a	EU329491
Fusarium solani f. sp. *mori*	NRRL 22157	AF178359.1	EU329493
Fusarium sp. *robiniae*	NRRL 22161	n/a	EU329494
Fusarium solani f. sp. *xanthoxyli*	NRRL 22163	AF178328.1	n/a
Neocosmospora vasinfecta	NRRL 22166	AF178350.1	EU329497
Fusarium sp.	NRRL 22178	n/a	EU329498
Fusarium sp.	NRRL 22230	n/a	EU329499
Fusarium phaseoli	NRRL 22276	n/a	JX171608
Fusarium sp.	NRRL 22278	n/a	EU329501
Fusarium ambrosium	NRRL 22354	n/a	EU329504
Fusarium kurunegalense	NRRL 22387	n/a	EU329505
Fusarium rectiphorus	NRRL 22396	n/a	EU329508
Fusarium sp. *batatas*	NRRL 22400	n/a	EU329509
Fusarium solani f. *batatas*	NRRL 22402	AF178344.1	n/a
Fusarium solani (FSSC6)	NRRL 22404	DQ247594.1	n/a
Fusarium sp.	NRRL 22436	n/a	EU329511
Fusarium.sp. *piperus*	NRRL 22570	n/a	EU329513
Fusarium sp.	NRRL 22579	n/a	EU329515
Fusarium sp. (FSSC 13)	NRRL 22586	AF178353.1	n/a
Fusarium plagianthi	NRRL 22632	AF178354.1	n/a
Fusarium sp. plagianthi	NRRL 22632	n/a	EU329519
Fusarium brasiliense	NRRL 22678	JQ670133.1	n/a
Fusarium tucumaniae	NRRL 22744	DQ247651.1	n/a
Fusarium solani f. sp. *pisi*	NRRL 22820	AF178355.1	EU329532

Species	Strain number	GenBank accession numbers[1]	
		tef1	***rpb2***
Fusarium virguliforme	NRRL 22825	n/a	GU170599
Fusarium solani	NRRL 25083	JF740714.1	n/a
Fusarium redolens	NRRL 25123	JF740748.1	n/a
Fusarium lichenicola	NRRL 28030	DQ246877.1	n/a
Fusarium brasiliense	NRRL 31757	n/a	EU329565
Fusarium sp. (FSSC 12d)	NRRL 32309	DQ246937.1	n/a
Fusarium lichenicola	NRRL 34123	n/a	EU329635
Fusarium virguliforme	NRRL 36899	FJ919494.1	n/a
Fusarium sp.	NRRL 45880	n/a	EU329640
Fusarium pseudensiforme	NRRL 46517	KC691555.1	KC691674
Fusarium solani (FSS5)	NRRL 46643	GU250544	GU250729
Fusarium euwallaceae	NRRL 54722	JQ038007.1	n/a
Fusarium petroliphilum	NRRL 54988	KC808210.1	n/a
Fusarium sp.	44a GJS 09-1459[2]	KT313606.1	n/a
Fusarium cf. *solani*	B8659[2]	HM852045.1	n/a
Fusarium solani f. sp. *cucurbitae*	Fsm731[2]	KC711041.1	n/a
Fusarium thapsinum	H05-557S-1 DCPA[2]	JX268965.1	n/a
Fusarium striatum	SQHI003[2]	KP715415.1	n/a

CBS = Centraalbureau voor Schimmelcultures—Fungal Biodiversity Center, Utrecht, The Netherlands; CML = Coleção Micológica de Lavras, Departamento de Fitopatologia, Universidade Federal de Lavras, Lavras, Minas Gerais, Brazil; FRC = Specimen number in the *Fusarium* Research Center, Pennsylvania State University; NRRL Agricultural Research Service Culture Collection, Peoria, Illinois USA;

[1]Reference strains GenBank accession numbers for translation elongation factor 1-alpha (*tef1*) partial sequences and the second-largest subunit of RNA polymerase II (*rpb2*) gen region. [2]Unknown culture collections; n/a = the sequences were either not available or not applicable to the current study.

Experiment 2. Host range of *F. solani f. sp. pisi*. To determine the host range and evaluate plant response to inoculation with *F. solani* f. sp. *pisi,* 60 accessions of 10 legume genera were tested in a greenhouse assay. This study was conducted over a set of four consecutive experiments. In each experiment, two field pea cultivars, cv. Santana and cv. EFB 33, were included as additional controls (Table 3.3).

The *F. solani* f. sp. *pisi* isolate (Fs21) classified as moderately aggressive to pea in experiment 1 was selected for the inoculation experiments. The inoculum was prepared by incubating the strain for 10 days in aerated malt extract broth (MEB, 17 g/l) at 20°C under constant agitation/shaking at 100 rpm. After 10 days of incubation, conidia were collected by filtration and enumerated in suspension as described above.

Preliminary studies on seed germination rates showed that the majority of accessions chosen for this experiment had a very low germination percentage. Thus, to ensure adequate seedling emergence, seeds of all plant accessions, with the exception of pea, were treated with 97% sulfuric acid for 4 min, rinsed in distilled water and germinated for 48h on wet filter paper in Petri dishes at room temperature. Pea seeds were treated with 70% ethanol prior to placing on wet filter paper. Single pre-germinated seeds were then transplanted into 200 ml pots filled with autoclaved sand. Each treatment consisted of 5 replicates with one germinated seed sown per pot. The experiment was arranged in a completely randomized design and the pots were inoculated 24h after transplanting with 2 x 10^4 spores g^{-1} substrate. Plants were kept in the greenhouse for five weeks under the conditions described for experiment 1. After five weeks of growing, plants were collected from each pot, and the biomass and disease severity (external root tissue discoloration levels only) were assessed as described above.

Cultural methods in combination with disease severity data were used to determine the host range of *F. solani* f. sp. *pisi* on tested plants. Three randomly selected roots from each treatment were surface-sterilized in 0.5% NaOCl for 10s, thoroughly washed in distilled water and placed on filter paper under a laminar flow hood for 1 h to dry. Subsequently, the roots were cut into approximately 1 cm long fragments and placed in Petri dishes containing ½ strength PDA medium and incubated under alternating cycles of 12 h BLB fluorescent light and 12 h darkness. After 10 to 15 days of incubation, fungal colonies developing from the root pieces were sub-cultured separately in Petri dishes containing PDA and SNA agar, incubated

as described previously, and identified based on cultural characteristics and microscopic examination of conidiogenous cells.

The response of the tested legume species to *F. solani* f. sp. *pisi* was determined according to criteria adopted in slightly modified form from Kolander et al. (2012). The accessions were considered symptomatic hosts if the inoculated isolate was re-isolated from surface sterilized roots, the average disease severity score was higher than 2 and significantly greater than in un-inoculated control plants. Accessions were also considered symptomatic if DSR < 2 but there was a significant reduction in mean biomass of inoculated plants compared to the corresponding control. Some of the control plants showed moderate symptoms on the roots (mean DSR > 2) caused by factors other than inoculation with *F. solani* f. sp. *pisi*, and in this case the host was considered susceptible if the final disease severity level of inoculated treatments was significantly higher than that of the corresponding control plants. The accessions were considered asymptomatic hosts if disease rating was less than or equal to 2, there was no significant reduction in biomass, and the fungus was re-isolated from the root parts following surface sterilization.

3.3 Data analysis

All statistical analyses were done using R statistical software (version 3.3.1, R Core Team 2013). For greenhouse experiment 1, the normality of data distribution and homogeneity of variances were tested by Shapiro-Wilks-W-Test and Levene's test, respectively. Prior to statistical analysis, disease severity index (DI) values were square root transformed. The data were first subjected to one way ANOVA to analyze differences in mean effects of different phylogenetic groups on root rot severity and plant biomass. Mean separations were made by Tukey HSD test. Differences among single isolates were tested separately by comparing means from inoculated treatments (each isolate) and un-inoculated control using Dunnett's t test (Hothorn et al., 2016). Treatments were considered significantly different if $P \leq 0.05$.

For greenhouse experiment 2, many groups had unequal variances, thus nonparametric tests were used for the evaluation of the results. Differences in root rot severity ratings and biomass of inoculated treatments and corresponding un-inoculated controls were compared with 2 by 2 comparisons using the non-parametric ranking procedure of the Dunn's test (Dinno, 2016).

3.4 Results

3.4.1 Phylogeny

Phylogenetic analyses inferred from the *tef1* and the *rpb2* sequences resolved the phylogenetic positions of the 83 isolates studied (including *F. redolens*) in relation to currently recognized monophyletic species in the FSSC complex. The *tef1* data set included 122 strains and consisted of 720 characters including alignment gaps, of which 220 characters were parsimony informative; the *rpb2* data set included 59 strains and consisted of 910 characters with alignment gaps, of which 168 were parsimony informative, and the concatenated *tef1* and *rpb2* gene sequence included 56 strains and comprised 1600 characters including alignment gaps, of which 262 were parsimony informative.

The isolates studied formed four different lineages, all nested within FSSC clade 3 (Figure 3.1). According to the single locus phylogenetic analysis, based on the *tef1* tree, 51 isolates were placed in two closely related subclades in the *F. solani* f. sp. *pisi* lineage: the first group of 27 isolates matched with *F. solani* f. sp. *pisi* (NRRL 22820) with 75% bootstrap value, and the second group of 24 isolates matched with *Fusarium solani* (FRC S485) with 65% bootstrap value. However, based on the tree generated from *rpb2* sequences that included representative isolates from both sub-clades, these topological differences did not receive any additional significant support either on *rpb2* or concatenated *tef1* - *rpb2* trees. The same strains that previously formed two sub-clades (*tef1* tree, Figure 3.1) were nested in one clade that matched *F. solani* f. sp. *pisi* (NRRL 22820) with some strains showing low intraspecific

variation (

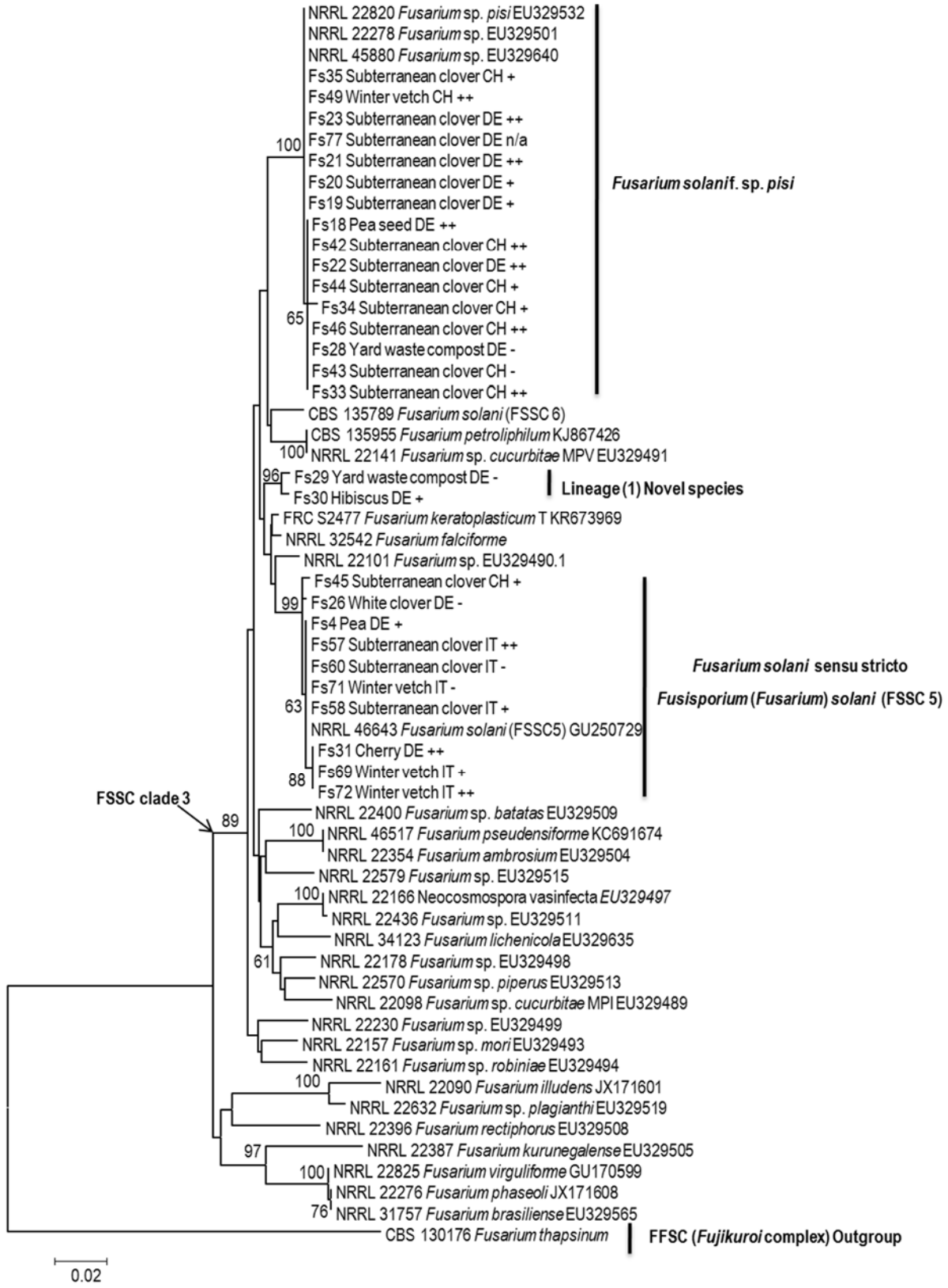

Figure 3.2 and Figure 3.3). Thus, based on the phylogenetic network obtained from concatenated gene trees, all 51 strains were assigned to a single species i.e. *F. solani* f. sp. *pisi*.

With the exception of Fs66 recovered from the roots of subterranean clover grown in Italy, all strains nested in *F. solani* f. sp. *pisi* lineage originated from different sources in Germany and Switzerland. This lineage also nested the one strain obtained from Sweden.

A group of 25 additionally identified isolates were placed in the *Fusarium solani* sensu stricto (FSSC 5) lineage, recently assigned epitype specimen and named *Fusisporium* (*Fusarium*) *solani* (Schroers et al., 2016). Likewise for *F. solani* f. sp. *pisi*, the relative distances using the single and the combined gene analyses (two locus tree) revealed similar intraspecific variations among the *Fusisporium* (*Fusarium*) *solani* isolates (Figure 3.1 - Figure 3.3). With few exceptions, this lineage accommodated mainly isolates obtained from Italy (Figure 3.1 - Figure 3.3).

The results of the *tef1* tree topology also showed one strain match (FK70) with *F. keratoplasticum* (FRC S2477, Figure 3.1), whereas the remaining two isolates Fs29 and Fs30, recovered from compost and hibiscus, respectively, did not cluster with any of the currently recognized FSSC species (Figure 3.1 - Figure 3.3). Based on the distances from nearest neighbor species and high bootstrap support values (≥96%) between sub-clusters using both, the single and the two genes phylogenetic analyses (*tef1* and the *rpb2* sequences), our results suggest that these isolates represent at least one new lineage within the FSSC clade 3.

All of the tested isolates were scattered within clade 3 without evidence of phylogenetic structure with respect to host. Four isolates of *F. redolens*, recovered from white clover grown in Sweden were placed in the *F. fujikuroi* species complex and used as an outgroup for species level resolution within the *tef1* locus.

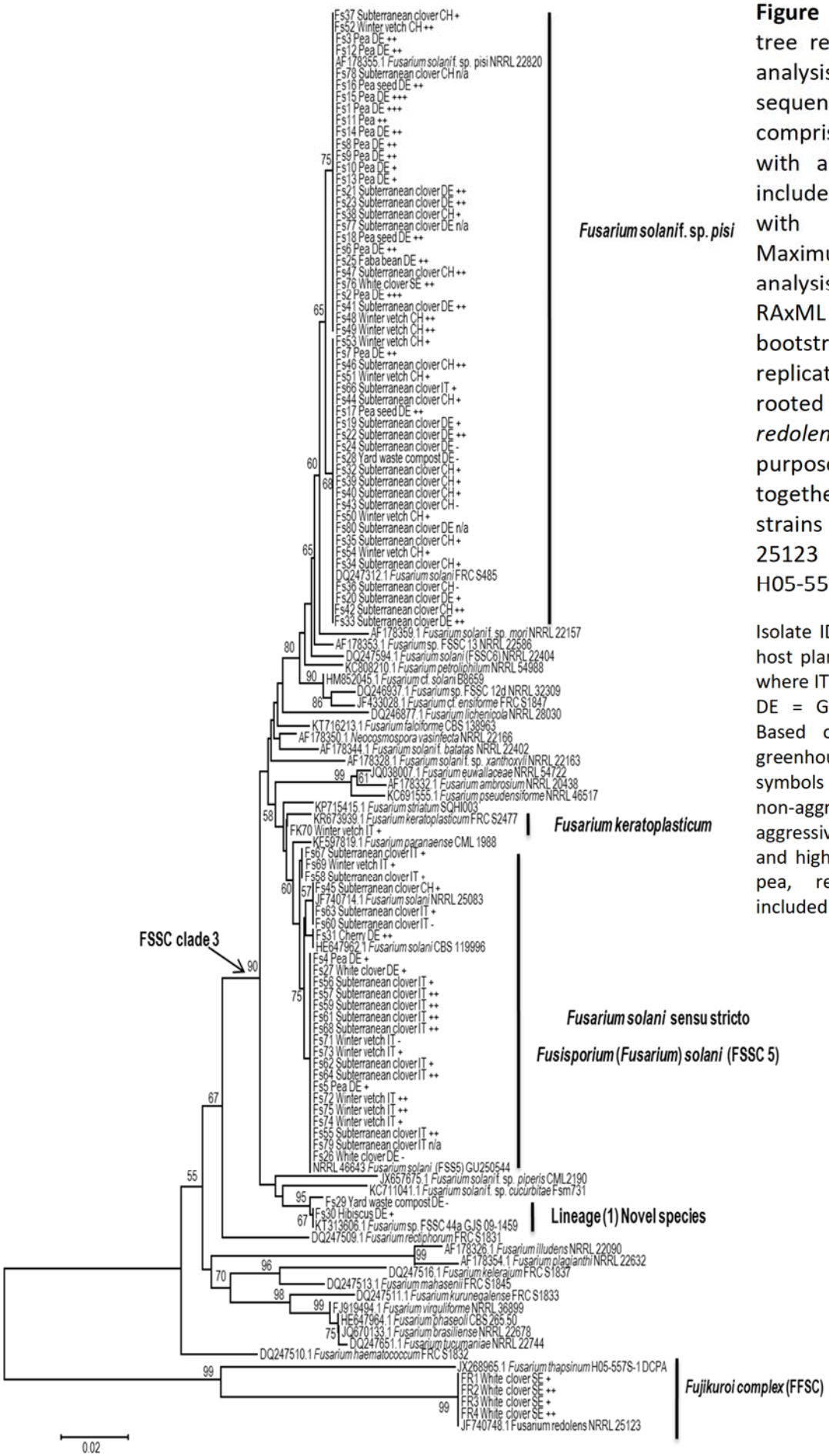

Figure 3.1. Phylogenetic tree resulting from RAxML analysis for the *tef1* gene sequences. The data set comprised 720 characters with alignment gaps, and included 122 sequences with reference strains. Maximum Likelihood analysis was performed by RAxML with non-parametric bootstrapping using 1000 replications. The tree was rooted with four strains of *F. redolens* collected for the purpose of this study together with reference strains *F. redolens* NRRL 25123 and *F. thapsinum* H05-557S-1DCPA.

Isolate ID number is followed by host plant and country of origin, where IT = Italy, CH = Switzerland, DE = Germany, SE = Sweden; Based on the results of the greenhouse experiment 1, the symbols -, +, ++, and +++ indicate non-aggressive, weakly aggressive, moderately aggressive and highly aggressive isolates on pea, respectively; n/a = not included in aggressiveness test.

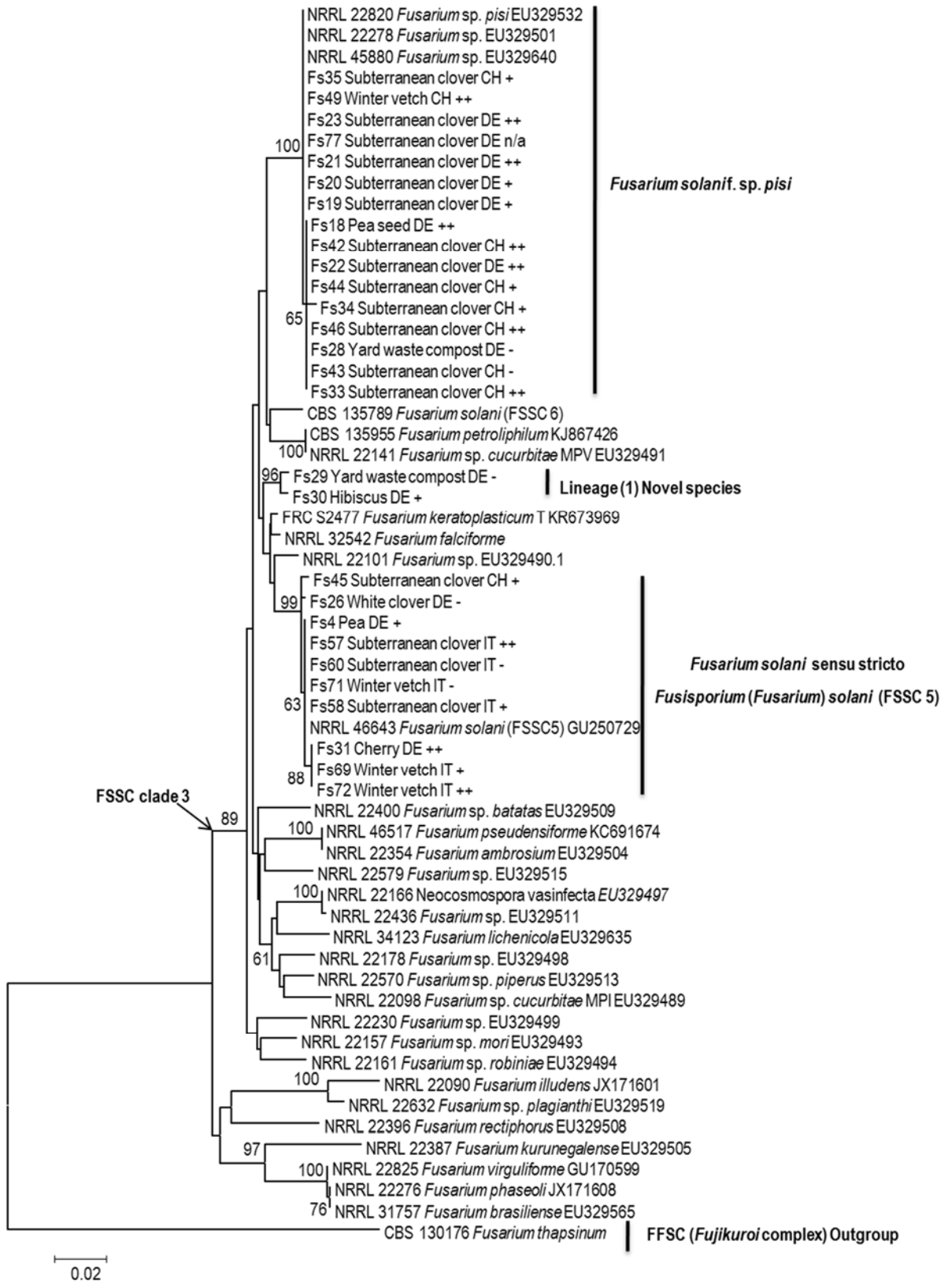

Figure 3.2. Phylogenetic tree resulting from RAxML analysis for the *rpb2* gene sequences. The data set comprised 910 characters with alignment gaps, and included 56 sequences with reference strains. Maximum Likelihood analysis was performed by RAxML with non-parametric bootstrapping using 1000 replications. The tree was rooted with one strain of *F. thapsinum* CBS 130176. Isolate abbreviations are provided in the caption of Figure 3.1.

Figure 3.3. Phylogenetic tree resulting from RAxML analysis for the combined *rpb2* and *tef1* gene sequences. The data set comprised 1600 characters with alignment gaps and included total of 56 sequences with reference strains. Maximum Likelihood analysis was performed by RAxML with non-parametric bootstrapping using 1000 replications. The tree was rooted with one strain of *F. thapsinum* CBS 130176. Isolate abbreviations are provided in the caption of Figure 3.1.

3.4.2 Greenhouse experiments

3.4.2.1 Experiment 1. Aggressiveness of selected FSSC isolates to pea

Root rot severity averaged over isolates varied significantly among different species within the FSSC. *Fusarium solani* f. sp. *pisi* (mean DI = 60.91) together with *F. redolens* (DI = 63.41) caused the highest overall disease severity, followed by *Fusisporium solani* (DI = 50.23) and *F. solani* group (DI = 39.65) (Tukey HSD, $P < 0.05$). There was no significant difference in mean fresh or dry plant biomass of inoculated treatments and un-inoculated control (data not shown).

Significant variations in aggressiveness were also observed among individual isolates of *F. solani* f. sp. *pisi, Fusisporium solani* and those included in the *F. solani* group (Figure 3.4). The majority of the isolates tested were pathogenic to pea. In general, weakly to moderately aggressive strains dominated the populations of the tested *Fusarium* species. Among the 48 *F. solani* f. sp. *pisi* isolates, 3 isolates recovered from subterranean clover roots (Fs24, Fs36, Fs43) and one isolate collected from compost (Fs28) did not differ significantly in root rot severity from the un-inoculated control and were classified as non-aggressive (4/48, 8%). Within the pathogenic isolates, 3 (6%) were highly aggressive, 24 (50%) moderately aggressive, and 17 (35%) were weakly aggressive. Similar results were also observed for the *Fusisporium solani* (Figure 3.4A). Among the 24 isolates tested, 3 (Fs26, Fs60 and Fs71) recovered from the roots of white clover, subterranean clover and winter vetch, respectively, were classified as non-aggressive. The remaining isolates induced mild to modest symptoms on pea roots and were differentiated into weakly (12/24, 50%) and moderately aggressive (9/24, 38%). The two *F. solani* isolates collected from compost (Fs29) and hibiscus (Fs30), placed into one separate clade (lineage 1, Figure 3.1) were rated as non- and weakly aggressive, respectively. The *F. keratoplasticum* isolate recovered from winter vetch in Italy (FK70 sorted in the *F. solani* group in Figure 3.4) was weakly aggressive. Among the *F. redolens* isolates, two were weakly and two moderately aggressive. Aggressiveness was not correlated with isolate phylogenetic position, host plant or geographic origin (Figure 3.1 and Figure 3.4).

In contrast to root rot severity, the effect of the tested isolates on pea biomass was much less pronounced. Significant reductions in fresh biomass compared to corresponding control were observed only in plants inoculated with *F. solani* f. sp. *pisi* isolates Fs1, Fs2, Fs17 and Fs18 recovered from pea roots, as well as for isolates Fs22 recovered from subterranean clover

roots (Figure 3.4B). In comparison to the un-inoculated control, dry plant biomass was significantly reduced only by isolate Fs1 (data not shown).

3.4.2.2 Experiment 2. Host range of F. solani f. sp. pisi

Fusarium solani f. sp. *pisi* was re-isolated from surface sterilized roots of 58 out of the 62 accessions tested. Among the 58 successfully colonized accessions, 33 (including the three *Pisum* cultivars) were symptomatic and 25 accessions were asymptomatic hosts of *F. solani* f. sp. *pisi* (Table 3.3). The fungus was not re-isolated from one *T. subterraneum* (acc. 1001) and three *T. repens* (acc. 1965, 1968 and 2010) accessions. As assessed by plating surface sterilized root segments, these accessions were considered as non-hosts for *F. solani* f. sp. *pisi.* In addition, several potentially pathogenic fungal species were isolated from the un-inoculated roots of one *Pisum* cultivar, seven *Lathyrus*, four *T. subterraneum* and one *T. repens* accessions. These included mostly *F. avenaceum* and *F. oxysporum*, a several other *Fusarium* spp. and in one case *Peyronellaea pinodella* (Table 3.3).

Inoculation with *F. solani* f. sp. *pisi* resulted in significantly different levels of root rot disease severity among the tested legumes (Table 3.3). The highest overall disease severity rating (DSR) was observed on *Trigonella foenum-graceum* (mean DSR = 7.8), followed by *Scorpiurus muricatus* (DSR = 5.4), *Pisum* cultivars (DSR = 5.1), *Melilotus albus* (DSR = 4.8) *Crotalaria ochroleuca* (DSR = 4.0) and *Lathyrus* accessions (DSR = 3.4). Inoculated *Vicia, Medicago, Trifolium* and *Galega* accessions showed lower overall disease symptoms, with mean severity ratings of 2.1, 2.0, 1.6 and 1.4, respectively. *Lotus pedunculatus* did not show any symptoms of fungal infection (Table 3.3).

Responses of individual accessions within *Lathyrus, Medicago, Trifolium,* and *Vicia* genera to inoculation with *F. solani* f. sp. *pisi* varied greatly (Table 3.3). For *Lathyrus*, with the exception of *L. aphaca* (L045), all accessions developed disease severity rating >2. Mean root rot severity ranged between 2.2 for *L. gorgoni* acc. L1663 and 5.2 for *L. sativus* acc. L1668. Responses of *Medicago accessions* were similar. Inoculated *M. arabica* (acc. 1735 and 211) and *M. polymorpha* (acc. 365) did not develop significant disease symptoms (DSR < 2), whereas *M. arabica* acc. 624 and *M. orbicularis* acc. 44 and 46 had DSR of 3.4, 2.8 and 2.3, respectively. Significant variations in response to *F. solani* f. sp. *pisi* were also observed for *Trifolium* accessions. Out of the 25 accessions tested, only 7 were found to be susceptible (DSR > 2),

with 5 belonging to *T. subterraneum* (DSR between 3.2 and 4.3), one to *T. diffusum* (acc. 906, DSR = 3.2) and one to *T. palestinum* (acc. 910, DSR = 4.0). The remaining 18 accessions had no symptoms or developed low levels of disease (DSR < 2). These included all *T. repens* accessions (n = 12), two *T. subterraneum* and one of *T. angustifolium, T. arvense* and *T. campestre*. Among the 14 *Vicia* accessions, six were considered susceptible based on the disease severity symptoms whereas five did not develop disease symptoms higher than 2. For susceptible accessions, the mean DSR ranged between 2.2 for *V. articulata* acc. 924 and 3.6 for *V. sativa* accessions 1576 and 1577. *V. sativa* acc. 1576 and *V. villosa* subsp. *varia* acc. 1644 were also classified as symptomatic host in the absence of visible disease symptoms because of a significant loss of biomass. Low variability in response to *F. solani* f. sp. *pisi* was found among the tested *Pisum* cultivars. Winter pea cv. EFB 33 (DSR = 4.1) was generally less susceptible to root rot compared to spring pea cv. Santana and cv. IPR83 (both DSR = 5.6) (Table 3.3).

Compared to the corresponding controls, fresh or dry plant biomass of inoculated treatments was significantly reduced only in *Pisum sativum* cv. IPR83, *Lathyrus incospicuus* acc. L1672, *Trifolium difusum* acc. 906, *T. palestinum* acc. 910, *Vicia articulata* acc. 924, *V. sativa* acc. 1576 and 1590, *V. villosa* subsp. *varia* acc. 1644, *Scorpiurus muricatus* acc. 69 and *Trigonella foenum-graceum* acc. 409. Additionally, for some accessions inoculation with *F. solani* f. sp. *pisi* resulted even in significant biomass increases (Table 3.3).

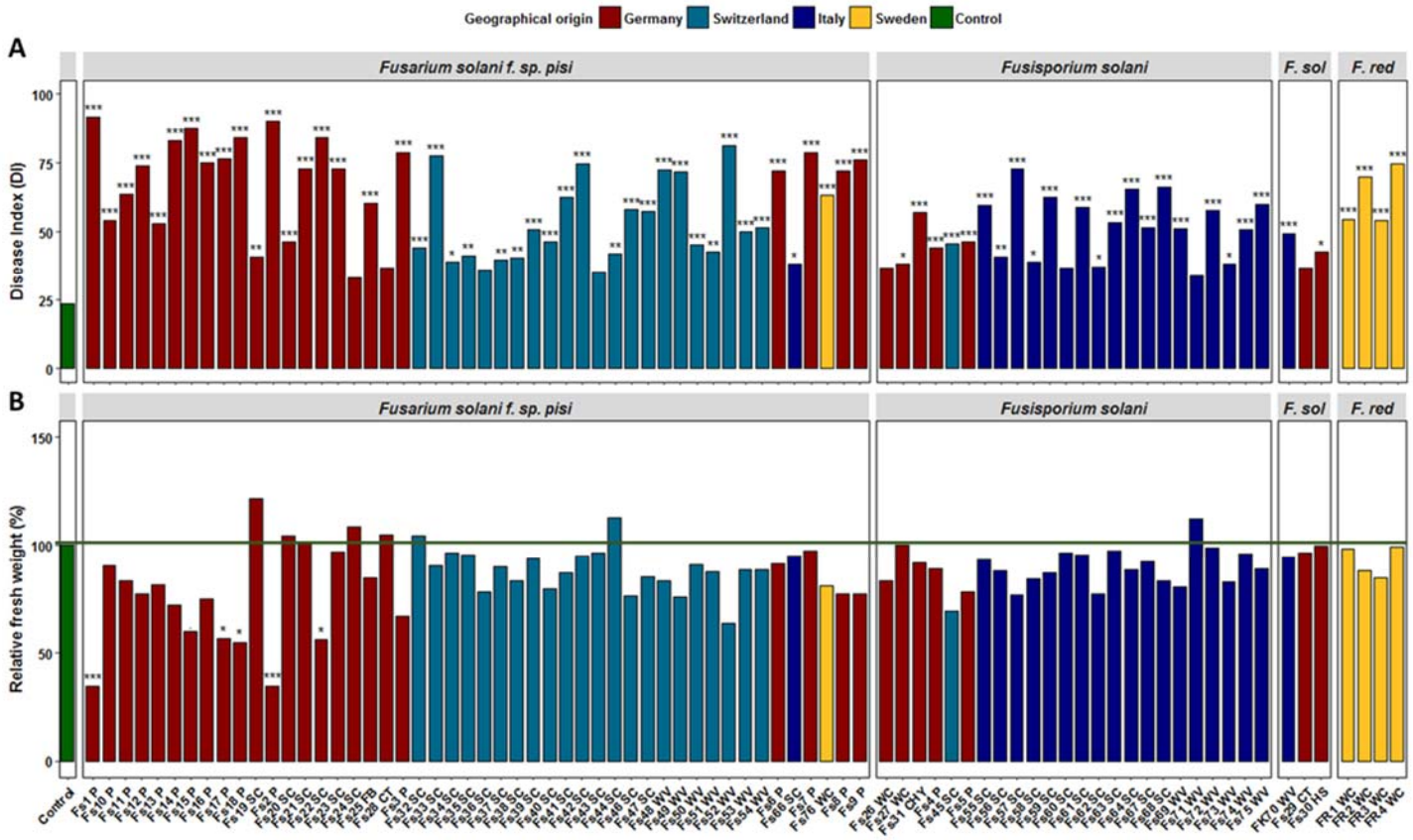

Figure 3.4. Effects of *Fusarium solani* f.sp. *pisi*, *Fusisporium solani, F. solani* (*F.sol*) and *F. redolens* (*F. red*) isolates on root rot disease severity (A) and fresh plant biomass (B) of pea. The isolates that formed distinct groups based on the phylogenetic analysis and showed no strong phylogenetic relationship to any of previously defined species within the FSSC along with one isolate of *F. keratoplasticum* were included in *F. solani* group. Effects on the fresh plant biomass of inoculated plants are given relative to the un-inoculated control treatment performance which was set at 100 %. The letters in the suffix of each isolate ID number represent the host plant from which isolates were collected, where P = pea, SC = subterranean clover, WC = white clover, WV = winter vetch, FB = faba bean, HS = hibiscus, CHY = cherry, CT = compost. Geographical origins of the isolates are denoted by the country of origin, where IT = Italy, CH = Switzerland, DE = Germany, SE = Sweden. C = un-inoculated control. Asterisks above to the bars indicate significant difference from the un-inoculated control plants according to Dunnett's t test. Symbols ∗∗∗, ∗∗, and ∗ indicate significance levels of *P* <0.001, <0.01, and <0.05, respectively. Data presented are means of four replicate pots.

Table 3.3. Plant species and accessions tested for susceptibility to *F. solani* f. sp. *pisi*, and the symptomatic(S), asymptomatic (AS) or non-host (NH) classification based on their response to infections measured 35 days after inoculation under controlled conditions.

Legume host	Common name	Code	DSR[1] control	DSR inoculated	FW[2] control (g)	FW[3] change g and (%)	DW[2] control (g)	DW[3] change g and (%)	Host plant[4]
PEA									
Pisum sativum L. ssp. *sativum* convar. *speciosum*	Field pea	IPR83	3.0 FA	5.6**	3.91	-0.53* (-13.5)	0.48	-0.07 (-13.7)	S
P. sativum L. ssp. *sativum* convar. *speciosum*	Field pea	EFB 33	0.3	4.1***	4.71	-0.22 (-4.8)	0.63	+0.03 (4.8)	S
P. sativum L. ssp. *sativum* convar. *sativum*	Field pea	Santana	1.4	5.6***	3.21	+0.09 (2.8)	0.37	+0.05 (13.6)	S
VETCHLINGS									
Lathyrus aphaca	Yellow vetchling	L045	0.4 FA	1.8	1.00	-0.07 (-6.8)	0.14	-0.01 (-7.9)	AS
L. dymenum	Spanish vetchling	L1662	0.2 FA+PP	3.4**	2.09	-0.13 (-6.2)	0.26	-0.03 (-9.8)	S
L. dymenum	Spanish vetchling	L1660	0.8 FC	3.4**	2.59	+0.06 (2.1)	0.31	+0.01 (3.9)	S
L. gorgoni	Orange vetchling	L1663	0.6	2.2**	1.23	-0.31 (-25.4)	0.15	-0.04 (-27.4)	S
L. inconspicuus	Inconspicuous vetchling	L1672	1.2 FG	3.3*	0.95	-0.38* (-40.6)	0.12	-0.05* (-42.9)	S
VETCHLINGS	Winged vetchling	L1683	3.0 FA	4.4	3.05	+0.37 (12.2)	0.40	>+0.01 (0.1)	AS
L. ochrus	Winged vetchling	L1684	1.4 FA	3.6**	4.67	-0.82 (-17.5)	0.52	-0.11 (-21.0)	S
L. sativus	Chickling vetch	L1668	2.4 FT	5.2*	1.95	-0.14 (-7.4)	0.24	-0.06 (-23.6)	S
L. sylvestris	Flat vetchling	L1695	1.2	3.4*	2.99	-0.03 (-1.0)	0.35	-0.01 (-2.1)	S

Legume host	Common name	Code	DSR[1] control	DSR inoculated	FW[2] control (g)	FW[3] change g and (%)	DW[2] control (g)	DW[3] change g and (%)	Host plant[4]
MEDICS									
Medicago arabica	Spotted medick	1735	0.0	0.4	2.23	-0.07 (-3.2)	0.40	+0.02 (4.7)	AS
M. arabica	Spotted medick	211	0.1	1.3	1.78	-0.07 (-4.2)	0.27	+0.03 (9.8)	AS
M. arabica	Spotted medick	624	0.0	3.4*	2.34	-0.11 (-4.6)	0.37	+0.04 (11.8)	S
M. orbicularis	Button medick	44	0.0	2.8***	0.74	+0.32 (43.4)	0.11	+0.08** (65.7)	S
M. orbicularis	Button medick	46	0.1	2.3**	0.99	+0.02 (2.2)	0.15	+0.04 (28.3)	S
M. polymorpha	Burr medic	365	0.0	1.6	1.81	-0.10 (-5.2)	0.28	-0.04 (-13.6)	AS
CLOVERS									
Trifolium angustifolium	Narrowleaf crimson clover	20	0.2	1.6	0.68	+0.08 (12.2)	0.09	+0.01* (14.8)	AS
T. arvense	Haresfoot clover	1928	0.0	0.1	0.26	+0.22* (82.1)	0.06	+0.05** (89.9)	AS
T. campestre	Hop trefoil	1	0.0	0.2	0.53	+0.04 (7.6)	0.07	+0.02 (29.8)	AS
T. diffusum	Diffuse clover	906	0.2	3.2**	1.09	-0.52** (-47.8)	0.12	-0.05** (-43.5)	S
T. palaestinum	Palestine clover	910	0.2	4.0*	0.60	-0.13 (-21.4)	0.14	-0.05** (-37.5)	S
T. repens	White clover	1935	0.0	1.0	0.68	-0.03 (-4.3)	0.06	>+0.01 (0.2)	AS
T. repens	White clover	1936	0.0	0.5	1.04	-0.18 (-17.1)	0.13	-0.04 (-34.6)	AS

Legume host	Common name	Code	DSR[1] control	DSR inoculated	FW[2] control (g)	FW[3] change g and (%)	DW[2] control (g)	DW[3] change g and (%)	Host plant[4]
T. repens	White clover	1937	0.5	1.3	0.85	-0.07 (-8.2)	0.11	0.0 (0.0)	AS
T. repens	White clover	1954	0.0	0.4	0.77	-0.01 (-1.1)	0.09	-0.01 (-7.5)	NH
T. repens	White clover	1959	0.0 FO	0.6	0.86	+0.14 (16.3)	0.12	+0.02 (15.0)	AS
T. repens	White clover	1960	0.2	1.0	0.55	-0.01 (-1.6)	0.07	>-0.01 (-0.6)	AS
T. repens	White clover	1965	0.0	0.2	0.52	-0.12 (-23.5)	0.08	-0.02 (-23.5)	NH
T. repens	White clover	1968	0.0	0.4	0.54	+0.01 (2.0)	0.08	>-0.01 (-1.1)	NH
T. repens	White clover	1976	0.0	1.0	1.06	-0.24 (-22.3)	0.11	-0.02 (-16.2)	AS
T. repens	White clover	1977	0.0	0.5	0.76	-0.13 (-17.6)	0.09	-0.02 (-28.6)	AS
T. repens	White clover	2001	0.0	0.8	0.66	-0.20 (-29.6)	0.08	-0.01 (-8.6)	AS
T. repens	White clover	2010	0.0	1.0	0.72	+0.64* (88.5)	0.08	+0.05 (67.1)	AS
T. subterraneum	Subterranean clover	1001	1.4 FO	1.8	2.20	-0.21 (-9.4)	0.36	-0.03 (-7.6)	NH
T. subterraneum	Subterranean clover	1021	2.0 FO+FA	3.0	1.32	-0.15 (-11.0)	0.17	-0.03 (-18.3)	AS
T. subterraneum	Subterranean clover	1040	0.0	3.3**	1.01	-0.38 (-37.3)	0.12	-0.05 (-39.4)	S
T. subterraneum	Subterranean clover	1042	1.6 FO	3.2**	1.25	-0.15 (-11.6)	0.18	-0.03 (-16.4)	S
T. subterraneum	Subterranean clover	1065	3.6 FO	3.4	1.91	-0.14 (-7.3)	0.26	-0.02 (-6.6)	AS

Legume host	Common name	Code	DSR[1] control	DSR inoculated	FW[2] control (g)	FW[3] change g and (%)	DW[2] control (g)	DW[3] change g and (%)	Host plant[4]
T. subterraneum	Subterranean clover	1067	0.0	3.4**	1.09	-0.35 (-32.1)	0.13	-0.03 (-26.9)	S
T. subterraneum	Subterranean clover	1068	0.0	4.3**	0.93	-0.53 (-56.8)	0.10	-0.06 (-56.7)	S
T. subterraneum	Subterranean clover	Campeda	0.5	3.6**	0.54	+0.02 (3.2)	0.08	-0.01 (-8.2)	S
VETCH									
Vicia articulata	Bard vetch	924	0.4	2.2**	2.90	-0.32* (-11.0)	0.43	-0.10** (-24.0)	S
V. benghalensis	Purple vetch	1517	0.4	1.8	2.09	+0.20 (9.8)	0.31	+0.11** (34.2)	AS
V. ervilia	Bitter vetch	1527	0.0	3.4**	1.50	+0.06 (4.0)	0.19	+0.02 (10.8)	S
V. fulgens	Scarlet vetch	1532	0.0	1.0	1.85	-0.41 (-22.3)	0.39	-0.13* (-32.5)	S
V. hirsuta	Tiny vetch	1536	0.0	0.6	0.84	+0.08 (9.2)	0.12	+0.02 (14.5)	AS
V. sativa	Common vetch	1576	0.8	3.6**	2.96	-0.51** (-17.4)	0.40	-0.02 (-5.0)	S
V. sativa	Common vetch	1577	1.2	3.6**	2.34	+0.34 (14.5)	0.37	+0.02 (6.0)	S
V. sativa	Common vetch	1579	0.6	3.0**	2.03	+0.75** (37.1)	0.28	+0.13** (44.7)	S
V. sativa	Common vetch	1581	1.0	3.2**	2.82	+0.09 (3.1)	0.43	+0.03 (7.0)	S
V. sativa	Common vetch	1590	0.2	3.0**	2.82	-0.82** (-29.0)	0.44	-0.09* (-19.9)	S
V. villosa	Common vetch	1641	0.0	0.6	2.98	-0.30 (-10.2)	0.47	-0.07 (-14.0)	AS

Legume host	Common name	Code	DSR[1] control	DSR inoculated	FW[2] control (g)	FW[3] change g and (%)	DW[2] control (g)	DW[3] change g and (%)	Host plant[4]
V. villosa	Winter vetch	1642	0.0	0.2	2.22	+0.35* (15.6)	0.29	+0.17** (58.2)	AS
V. villosa	Winter vetch	1643	1.8	1.3	1.95	+0.71* (36.6)	0.33	+0.06 (18.2)	AS
V.villosa subsp. *varia*	Winter vetch	1644	0.2	1.4	2.99	-1.30** (-43.4)	0.46	-0.19** (-42.4)	S
MELILOT									
Melilotus albus	White melilot	1933	0.1	4.8***	0.82	-0.14 (-17.5)	0.08	+0.01 (16.6)	S
BIRDS FOOTTREFOIL									
Lotus pedunculatus	Marsh birds foot trefoil	1927	0.0	0.0	1.15	-0.10 (-9.1)	0.15	+0.01 (7.6)	AS
RATTLEPOD									
Crotalaria ochroleuca	Slender leaf rattlebox	n/a	0.6	4.0**	1.54	-0.56 (-36.7)	0.17	-0.07 (-37.3)	S
GOATS RUE									
Galega officinalis	Goats rue	162	0.2	1.4	0.88	+0.09 (10.5)	0.12	+0.01 (10.2)	S
SCORPIONS TAIL									
Scorpiurus muricatus	Prickly scorpions tail	69	0.8	5.4**	0.59	-0.30** (-50.9)	0.04	-0.02* (-39.1)	S
FENUGREEK									
Trigonella foenum-graecum	Fenugreek	409	2.0	7.8**	0.92	-0.72* (-78.3)	0.09	-0.07* (-73.2)	S

[1]DSR = disease severity rating of un-inoculated control plants, and additionally re-isolated fungi from surface sterilized roots, where FA = *Fusarium avenaceum*, FO = *F. oxysporum*, FC = *F. culmorum*, FG = *F. graminearum*, FT = *F. tricinctum* and PP = *Peyronellaea pinodella*; [2]FW/DW = fresh/dry plant biomass of un-inoculated plants; [3]FW/DW change = expressed as gram change in the biomass of the inoculated plants compared to corresponding un-inoculated control, and in parenthesis the biomass of the inoculated plants was expressed as a percentage change of the biomass of corresponding un-inoculated control plants.; [4]Symptomatic (S), asymptomatic (AS) and non-host (NH) accessions; With exception of *Pisum sativum* accession IPR83 provided by Instituto Agronomico do Parana (IAPAR), Brasil, all of the accessions were provided by Technical University of Munich,

Germany. Data were pairwise (2 by 2) analyzed by comparing inoculated treatments and corresponding un-inoculated controls using Dunn's test. The symbols ***, **, and * indicate significance levels of P <0.001, <0.01, and <0.05, respectively.

3.5 Discussion

The incidence of *Fusarium* species in general and the FSSC in particular associated with legumes grown under diverse European agro-climatic conditions is still poorly documented. In the current study, the single gene phylogeny inferred from *tef1* gene sequences that included 79 isolates, as well the single *rpb2* and the concatenated *tef1 – rpb2* phylogenetic tree toplogies inferred for a selected subset of 28 isolates placed the examined strains in four different lineages, all nested within the FSSC clade 3 (O'Donnell, 2000). The majority of isolates, however were associated with two major lineages, the *F. solani* f. sp. *pisi* lineage mainly accommodating German and Swiss isolates, and the *Fusisporium* (*Fusarium*) *solani* lineage accommodating mainly Italian isolates. The subsequent aggressiveness tests on pea, that included a subset of 75 isolates, confirmed the pathogenicity of most of the FSSC isolates tested. Aggressiveness was not correlated with isolate phylogenetic position, host plant or its geographic origin.

About two-thirds of the identified strains examined here (n = 51) belonged to *Fusarium solani* f. sp. *pisi*, suggesting a significant pathogenic potential of this species and/or a common prevalence in different host plants. Previous studies have established *F. solani* f. sp. *pisi* primarily as causal agent of pea root rot and one of the main reasons for declining pea production worldwide (Kraft, 1984; Persson et al., 1997). The aggressiveness tests on pea in this study showed that the most aggressive isolates belonged to *F. solani* f. sp. *pisi*, however the tested population was dominated by weakly and moderately aggressive strains. Furthermore, this study has shown that pathogenic isolates of *F. solani* f. sp*. pisi* can be found in a variety of habitats under diverse agro-ecological conditions, and that the fungus is, in addition to pea, able to colonize roots of various hosts such as subterranean clover, white clover, winter vetch and faba bean under field conditions. The results of the greenhouse assay also suggest the presence of non-pathogenic strains within the population of *F. solani* f. sp. *pisi*.

The ability of *F. solani* f. sp. *pisi* to occupy diverse ecological niches and the significant variation in aggressiveness of individual isolates observed in this study is consistent with previous work of VanEtten (1978) who found consistent differences in symptom severity on pea plants following inoculation with 152 *F. solani* f. sp. *pisi* isolates collected from diverse habitats and geographical locations. While some isolates were found to be highly aggressive to pea, the

authors also reported the presence of non-pathogenic strains. Additional studies on the aggressiveness factors of *F. solani* f. sp. *pisi* revealed that a number of enzymes released from the fungi have major influence on their ability to cause disease. The pathogens capacity to degrade the phytoalexin pisatin through its ability to produce enzyme pisatin demethylase is one of the main components determining its aggressiveness to pea. All naturally occurring isolates without this ability were essentially non-pathogenic (Hadwiger, 2008).

Out of the 26 additionally identified isolates, 25 belonged to *Fusisporum solani* (*Fusarium solani* sensu stricto '5'), the species mainly considered as causal agent of dry rot of potatoes and as an opportunistic human pathogen (Schroers et al., 2016). This fungus has not been reported as part of the root rot complex of pea. As for *Fusarium solani* f. sp. *pisi*, our results suggest lack of host specificity and the ability of *Fusisporium solani* isolates to colonize various hosts under field conditions, as well their potential to cause significant symptoms on pea. In addition, while the population of *Fusarium solani* f. sp. *pisi* isolates mainly originated from German and Swiss environments, in this study the Italian isolates were mainly comprised in the *Fusisporium solani* lineage. While these results indicate certain biogeographic patterns of the FSSC species distribution with respect to the studied hosts, further research with more intensive sampling is needed to draw conclusions.

One additionally identified isolate belonged to *F. keratoplasticum.* The species is mainly associated with human eye infections (Short et al., 2013), however our data suggest considerable ecological plasticity of the fungus, pathogenic potential on pea plants and point to soil and plant debris potential environmental sources of human infections. The two isolates (Fs29 and Fs30 recovered from compost and hibiscus, respectively) included in the *F. solani* group on the other hand, represent at least one novel phylogenetic lineage in the complex. Insights into their significance will require additional studies to fully understand their ecological importance. Nevertheless, our data suggest the presence of additional species that might be of importance for pea root health. A comprehensive survey is currently undergoing that includes more than 100 pea and faba bean growers throughout Germany, in order to better understand the epidemiology, impact on yield and the role of FSSC as causal agents of pea and faba bean root rot.

The greenhouse data on the host range of *Fusarium solani* f. sp. *pisi* further support our observations that the species is not explicitly adapted to a particular host. Data from the single

isolate inoculation already indicate that the host range should be expanded to include 33 symptomatic and 25 asymptomatic hosts. According to the criteria used in our study, only one subterranean clover and three white clover accessions tested could be classified as non-hosts for *F. solani* f. sp. *pisi* isolates Fs21. The multiple additional hosts for *F. solani* f. sp. *pisi* observed in this study agree with previous studies that demonstrated a broader host range for several other special forms. Studies on the host range of *F. solani* f. sp. *eumartii*, named by its specific pathogenicity to potato (*Solanum tuberosum*), revealed that the species, in addition to potato, was pathogenic to pepper, eggplant, and tomato (Romberg and Davis, 2007). Similarly, *F. solani* f. sp. *phaseoli* (currently *F. phaseoli* comb. nov., Aoki et al. 2003) generally considered as a typical root rot pathogen of bean (*Phaseolus vulgaris* L.), has been, in addition to bean, associated with at least 4 other host plants (Farr and Rossman 2016). In an extensive survey of *F. solani* f. sp. *glycines* associated with sudden death syndrome of soybean, Aoki et al. (2003) found that the disease is in fact caused by two phylogenetically and morphologically distinct species, *F. virguliforme* (formerly *F. solani* f. sp. *glycines*) and *F. tucumaniae* in North and South America, respectively. More recent studies conducted by Kolander et al. (2012) showed that aggressiveness and host range of *F. virguliforme* is more diverse and expands to various legumes such as alfalfa, red clover, white clover, pea and bean. In addition, the authors demonstrated the ability of *F. virguliforme* to asymptomatically infect a range of plants, and thus the potential of the fungus to efficiently spread along crop rotations. Whether such problems exist in other special forms within the complex remains to be investigated, nevertheless the concept of *formae speciales* will likely need further revisions.

In conclusion, to the best of our knowlegde, this is the first detailed study on the epidemiology and diversity of the *Fusarium solani* species complex associated with the examined host plants in Europe. The new information generated has provided a valuable insights into the significance of individual isolates and along with a limited number of recent studies, suggest a wider host range and substantial ecological plasticity of individual strains within the distinct lineages of FSSC. Further research should aim to determine environmental and other biotic and abiotic relevant factors that potentially influence individual densities of different lineages with respect to examined agro-climatic zone.

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CHAPTER 4: Endophytic *Fusarium equiseti* stimulates plant growth and reduces root rot disease of pea (*Pisum sativum* L.) caused by *Fusarium avenaceum* and *Peyronellaea pinodella*

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Abstract

Endophytic root colonizing fungi are an intriguing group of microorganisms that have the ability to form mutualistic associations with plants. Many endophytes confer benefits to their hosts such as plant growth promotion and disease suppression. Their potential to promote agro-ecosystem efficiency through beneficial impacts on their hosts are of great interest for agriculture and may contribute to reduced needs for agrochemicals. We investigated the ability of three *Fusarium equiseti* (Fe) isolates to endophytically colonize pea roots and the influence of endophytic development on plant growth, pathogen proliferation and root rot disease caused by *F. avenaceum* (Fa) and *Peyronellaea pinodella* (Pp). Fe was inoculated following sowing, while Fa and Pp were either inoculated simultaneously with Fe or 5 days after Fe. When only Fe was inoculated, two of the isolates significantly promoted plant growth at the end of the 4 week experiment. Simultaneous inoculation of Fe with Fa or pre-inoculation of pea plants for 5 days with any one of the three *F. equiseti* isolates resulted in disease suppression and significant reduction of Fa population, particularly in the root cortex. However, Fe isolates significantly reduced disease and root cortex colonization rates of Pp only in the plants inoculated with Fe 5 days before the pathogen. This study shows that *F. equiseti* can promote pea growth and has the ability to alter the interaction pea - Fa/Pp, consequently leading to reduced root rot disease severity.

Keywords: mutualism**,** plant growth promotion, biological control**,** root endophytes, root pathogens

4.1 Introduction

The term endophyte encompasses those organisms that infect host tissue without causing visible disease symptoms at the moment of sample collection, and whose colonization can be demonstrated to be internal (Stone et al., 2000; Sieber, 2002; Schulz and Boyle, 2005). A vast diversity of endophytic fungi has been found to colonize roots of all agricultural and natural hosts studied to date (Lee et al., 2009; Kobayashi and Palumbo, 2000). The composition of endophyte communities that inhabit a particular host is influenced by the genetic make-up of the fungal species, host plant, and environmental conditions (Schulz et al., 2006). Root endophytes are well adapted to the soil environment with considerable ecological plasticity and a rather dynamic lifestyle. Many endophytes are also latent pathogens that may cause disease when host plants are under stress (Schulz and Boyle, 2005). Others in contrast, provide multiple benefits such as protection against root pathogens (Hyakumachi and Kubota, 2004), promotion of plant growth (You et al., 2012; Hyakumachi and Kubota, 2004), and improvement of tolerance and/or adaptability to environmental stresses (Rodriguez and Redman, 2008; Rodriguez et al., 2004). It is therefore crucial to differentiate beneficial from non-beneficial organisms as the former can be very efficient biocontrol agents, and are of great interest in non-chemical disease control.

Fusarium equiseti (Corda) Saccardo, a naturally occurring endophyte in diverse ecosystems, is able to colonize the roots of various hosts (Leslie and Summerell, 2006). The fungus has been reported to lack strict host adaptation (Macia-Vicente et al., 2009a), to promote plant growth (Saldajeno and Hyakumachi, 2011), to suppress several soil-borne pathogens (Macia-Vicente et al., 2008b; Horinouchi et al., 2007), to produce toxins antagonistic to nematodes (Nitao et al., 2001), and to control the parasitic plant *Striga hermonthica* (Kirk, 1993). The ability of *F. equiseti* to efficiently colonize roots of various hosts in diverse ecosystems suggests its capacity to sustain stable populations within roots through competitive displacement of pathogenic and other rhizosphere inhabiting organisms, and thus its biocontrol potential for improving plant growth and health.

The necrotrophic soil-borne pathogens, *Fusarium avenaceum* (Fries) Saccardo and *Peyronellaea pinodella* (L.K. Jones) Aveskamp, Gruyter & Verkley (syn: *Phoma pinodella*; *Phoma medicaginis* var. *pinodella*) pose significant challenges to growers and compromise sustainability of pea production worldwide. Recently, especially the pathogen *F. avenaceum*

has become increasingly problematic in Europe, USA and Canada (Feng, 2010; Baćanović, 2015; Chittem et al., 2015). Together with *P. pinodella* it has been reported to dominate the pea root rot complex in Germany (Pflughöft et al., 2012; Baćanović, 2015). Both pathogens infect at least 18 species in 14 plant genera, including important legumes such as lucerne, clover, beans, lentil and soybean (Farr and Rossmann, 2016). Despite high levels of morphological and genotypic diversity in populations of *F. avenaceum* (Yli-Mattila et al., 1996; Satyaprasad et al., 2000) and *P. pinodella* (Boerema et al., 2004), they do not segregate into host-specific pathotypes, a trait that would limit pathogen movement among different crop species. Therefore, persistence of strains pathogenic to pea is expected even under diverse crop rotations, making both pathogens difficult to control.

In the present study we investigated interactions between endophytic *F. equiseti* and two major root rot pathogens of pea during their development in the root system. The following questions were addressed: (i) what is the effect of root colonization with *F. equiseti* on plant growth, and on disease development in the presence or absence of pathogens, and (ii) is there an influence of endophytic colonization of *F. equiseti* on colonization and proliferation of *F. avenaceum* and *P. pinodella* in the rhizodermis and within the root cortex of pea plants.

4.2 Material and methods

4.2.1 Fungal isolates

Three strains of the endophytic fungus *F. equiseti*, Fe1, Fe2 and Fe3, and one strain of *Fusarium avenaceum* from the University of Kassel Culture Collection (Culture ID: Fe1: ART.Vv1.4/14, Fe2: KU.Ww3.7/14, Fe3: KU.Ww5.7/14, and Fa: ART.Sc5.7/13) were selected for inoculation experiments. *Fusarium equiseti* isolates have been characterized in previous study to have the capacity to colonize roots and the potential to promote growth of pea plants (unpublished data). *Fusarium equiseti*, Fe1, was isolated from asymptomatic roots of winter vetch (*Vicia villosa*) grown near Zurich (Switzerland); isolates Fe2 and Fe3 originated from the roots of winter wheat (*Triticum aestivum*) grown in Neu Eichenberg (Hessen, Germany).

The strain of the root rot pathogen *F. avenaceum* (Fa) was isolated from the roots of subterranean clover (*Trifoilium subterraneum*) grown near Zurich, Switzerland, while *Peyronellaea pinodella* (Pp) (Culture ID: 94.14.11) was originally isolated from diseased pea (*Pisum sativum* L.) plants, and was kindly provided by Dr. B. Tivoli, the National Institute of Agricultural Research (INRA) France.

Fusarium equiseti cultures for inoculation were grown on Synthetic Nutrient-Poor Agar (SNA; 1 g/l KH_2PO_4, 1 g/l KNO_3, 0.5 g/l $MgSO_4 \times 7\ H_2O$, 0.5 g/l KCl, 0.2 g/l sucrose, 0.2 g/l glucose, and 20 g/l agar) supplemented with sterilized filter paper (1 x 1 cm) to induce sporulation. *Peyronellaea pinodella* was incubated on Coon´s medium (4 g/l maltose, 2 g/l KNO_3, 1.20 g/l $MgSO_4$ x 7 H_2O, 2.68 g/l KH_2PO_4, and 20 g/l agar). Both fungi were incubated at 20 °C under alternating cycles of 12 h blacklight blue (BLB) fluorescent light (F40; range 315-400 nm with the peak at 365 nm) and 12 h darkness. After 14 days of incubation, spore suspensions for inoculation were prepared by flooding the cultures with 15 ml sterile H_2O_{dest} and dislodging the conidia with a disposable hockey stick. *Fusarium avenaceum* was incubated for 10 days in aerated malt extract broth (MEB, 17 g/l) at 20°C under constant agitation/shaking at 100 rpm. After 10 days of incubation, conidia were collected by filtrating the culture media through cheesecloth to separate the mycelium.

4.2.2 Experimental set up and growth conditions

Preliminary studies had shown that the Fa and Pp isolates used in this survey were highly aggressive to pea (Šišić et al., 2015). We therefore hypothesized that *F. equiseti* would be more efficient in suppressing the disease if given an advantage over the pathogens. Thus, the concentration of spores, as determined by a haemocytometer, was adjusted to two different levels: *F. equiseti* isolates were inoculated with $2x10^4$ spores g^{-1} substrate, whereas for *F. avenaceum* and *P. pinodella* spore concentration was adjusted to $1x10^4$ spores g^{-1} substrate. Furthermore, to generate differently timed treatments, *F. equiseti* was inoculated directly following sowing, while *F. avenaceum* and *P. pinodella* were either inoculated simultaneously with *F. equiseti* (**d**ays **a**fter **e**ndophyte **i**noculation, daei0) or 5 days after *F. equiseti* (daei5). The experiment was conducted under greenhouse conditions with 19°C day and 16°C night temperature, and the field pea variety Santana serving as a model host plant. Natural day light was additionally supplemented with high-pressure sodium lamps (400 W) in order to provide photoperiod of 16 h light day^{-1}. Pea seeds were surface sterilized in 70% ethanol for 5 min and rinsed with H_2O_{dest} before planting four seeds (germination rate of 98%) per 500 ml pot filled with previously autoclaved sand. The experiment was arranged in a completely randomized design with four replicates. Plants were watered daily with tap water. In addition, pots were fertilized with complex N:P:K fertilizer Wuxal Super (8:8:6 + microelements). A total of 100 mg of N/l of substrate was divided in two equal portions and added 8 and 19 days after sowing. The above inoculation treatments were arranged in a full factorial design giving a total of 20 treatments: Fe isolates alone (Fe1, Fe2 Fe3 alone), Fa and Pp alone following sowing, Fa or Pp alone five days after sowing, co-inoculations of Fa or Pp with Fe isolates at daei0 and at daei5, and un-inoculated control.

4.2.3 Evaluation of plant growth and disease

After 28 days of growing, plants were removed from pots, and the roots were separated from the aboveground biomass. Roots were washed under running tap water, and root rot severity was assessed using a visual 0-8 score scale based on external and internal root tissue discoloration levels adopted from Pflughöft et al. (2008). Above ground plant parts of each pot were weighted and dried at 105°C until constant weight was attained.

4.2.4 Evaluation of fungal root colonization by cultural methods

Six randomly selected plants per treatment were used to verify fungal root colonization by cultural techniques, and the remaining material was stored at −20°C. For each treatment half of the roots were left unsterilized (colonization of rhizodermis) while the other half were surface sterilized for 10 seconds in 1% NaOCl (colonization of inner root cortex), rinsed in distilled water and placed on filter paper under a laminar flow hood for 1 h to dry. Each root was then further divided into two halves: upper (epicotyl to 2 cm below seed) and lower (middle part to root apex), and five ca. 1 cm long root fragments from each half were plated on Petri dishes containing Coons medium, incubated as described above, and examined after 7 days for fungal growth. Fungal colonies developing from the root pieces were sub-cultured separately in Petri dishes containing half strength potato dextrose agar (½ PDA, 19 g/l Difco PDA and 10 g/l agar), Coons medium, and SNA and identified by microscopic examination based on the taxonomic keys of Leslie and Summerell (2006), Boerema (2004) and Watanabe (2002). The number of root segments colonized by Fe, pathogens and other filamentous fungi was recorded and percentages were calculated as follows: % of colonized root fragments = (Number of root pieces yielding the respective fungi / Total number of root pieces) x 100.

4.3 Data analysis

Standard statistical methods were applied to the data sets using the R statistical software (version 3.2.2, R Core Team, 2013) using the package agricolae. Prior to statistical analysis a disease index (DI) was calculated as a weighted mean using the following formula: DI = (Score x Number of plants with the sore) x 100) / (Total number of plants x 8).

One way ANOVA was conducted to analyze effects of Fe isolates alone or in combination with pathogens within corresponding inoculation period on plant growth. Prior to ANOVA, data were checked for normality using Shapiro-Wilks-W-Test; Levene's test was used to test the homogeneity of variance. Mean separations were made by Fisher's LSD test at $P < 0.05$. The data on root rot severity (disease index values) and percent of colonized root fragments were analyzed using the non-parametric ranking procedure of the Kruskal-Wallis test. The co-inoculated treatments were also divided into subsets according to pathogen inoculation period. When significant treatment effects were observed ($P < 0.05$), mean ranking values were separated using the Kruskal post-hoc test (Conover, 1999). The Benjamini and Hochberg (1995) stepwise adjustment of P values was used to control false discovery rates and reduce type I errors in both post hoc procedures.

4.4 Results

4.4.1 Root colonization by *F. equiseti* and other filamentous fungi (in the absence of pathogen)

All three *F. equiseti* isolates completely colonized the rhizodermis as shown by 100% isolation frequencies. However, the isolates of *F. equiseti* differed in the extent at which the cortex root tissue was colonized. All Fe isolates were isolated at significantly lower frequencies ($P < 0.05$) from the root cortex compared to the rhizodermis, as assessed by plating surface sterilized root segments. Fe1 and Fe2 moderately colonized the cortex, emerging from 56.7% and 46.7% root segments, respectively, whereas, the isolate Fe3 colonized the cortex tissue less extensively, emerging from 16.7% of the root segments (Table 4.1).

Table 4.1. Extent of colonization of the rhizodermis (Rhd) and the root cortical tissue (Ctx) by *F. equiseti* isolates and other filamentous fungi as determined by the percentage of colonized non-sterilized (rhizodermis) and surface sterilized (cortex) root segments (n = 30). **Asterisks indicate significant differences in fungal colonization rates between the rhizodermis and the cortex tissue within fungal species ($P < 0.05$).**

	Fusarium equiseti		*Penicillium* spp.		*Cladosporium* spp.		*Fusarium solani*		*Cephalotrichum* spp.	
	Rhd	**Ctx**	**Rhd**	**Ctx**	**Rhd**	**Ctx**	**Rhd**	**Ctx**	**Rhd**	**Ctx**
Control[1]	-	-	93.3	36.7*	6.7	26.7*	0.2	-	-	-
Fe1	100.0	43.3*	46.7	10.0*	3.3	16.7*	-	-	-	-
Fe2	100.0	53.3*	3.3	-	-	13.3*	-	-	6.7	-
Fe3	100.0	16.7*	10.0	6.7	3.3	13.3*	-	-	-	-

[1]Un-inoculated control plant roots

The rhizodermis of un-inoculated control plant roots was colonized at high frequencies (93.3%) by *Penicillium* spp. and at lower rates by *Cladosporium* spp. and non-pathogenic *F. solani*. Colonization rates of the root cortex tissue by *Penicillium* spp. was significantly lower (36.7%) compared to the rhizodermis ($P < 0.05$). In contrast, *Cladosporium* spp. were isolated at significantly higher frequencies from the root cortex than from the rhizodermis, as assessed by plating surface sterilized root segments (Table 4.1).

Following inoculation with Fe, both, *Penicillium* spp. and *Cladosporium* spp. root colonization rates decreased significantly ($P < 0.05$). Similarly to the un-inoculated control plants, in the presence of Fe, less extensive colonization of the cortex tissue compared to rhizodermis was observed for *Penicillium* spp. whereas the opposite trend was observed for *Cladosporium* spp. ($P < 0.05$) (Table 4.1). In the treatment with Fe2, two of the 30 root segments (6.7%) were also colonized by *Cephalotrichum* spp. (Table 4.1).

4.4.2 Effect of *F. equiseti* on plant growth

Inoculation of roots with *F. equiseti* isolates, Fe1 and Fe3 significantly increased plant growth of pea plants compared to the un-inoculated control. In contrast, isolate Fe2 had no effect on plant biomass (Figure 4.1a). In comparison to the un-inoculated control, *F. equiseti* isolates alone did not cause disease symptoms (Figure 4.1b).

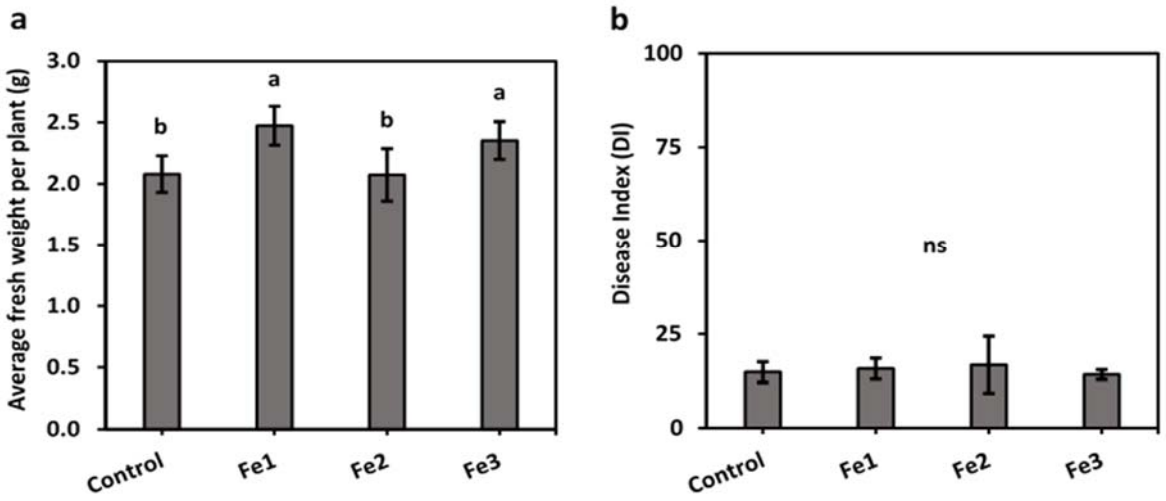

Figure 4.1. Effect of root inoculation with *Fusarium equiseti* isolates on average plant fresh weight (a), and disease severity of pea plants (b). **Disease severity is expressed as Disease Index (DI), where DI = 0-15 – non-aggressive; DI = 16-30 - weak aggressiveness; DI = 31-70 - moderate aggressiveness; DI = 71-100 - high aggressiveness. Error bars represent mean ± standard deviation. Different letters indicate significant differences and *ns* nonsignificant differences between the means according to Fisher's LSD test ($P < 0.05$). Data presented are means of four replicate pots.**

4.4.3 Effect of *F. equiseti* isolates on plant growth in the presence of pathogens

The detrimental effects of Fa on plant growth between the two inoculation times differed significantly ($P < 0.05$) (Figure 4.2). Compared to the un-inoculated control plants with a fresh weight of 2.0 g (Figure 4.1a), Fa reduced pea biomass by 83% (Figure 4.2a) when inoculated

alone at sowing, while reduction was only 14% when inoculated five days after sowing (Figure 4.2b and Figure 4.1 for un-inoculated control).

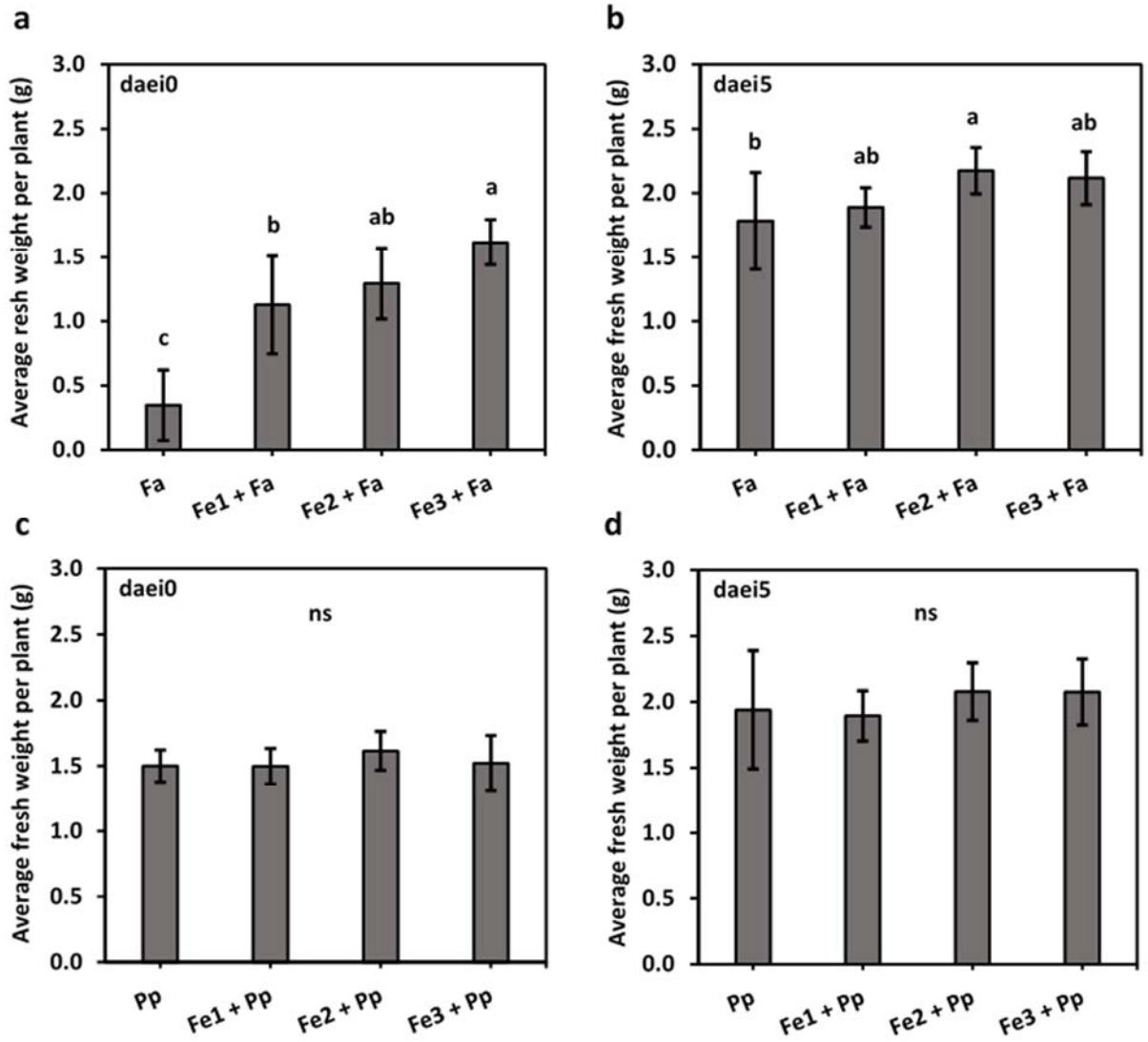

Figure 4.2. Effect of inoculation of *F. equiseti* (Fe) isolates on the growth of plants co-inoculated with the pathogens, *F. avenaceum* (Fa) and *P. pinodella* (Pp), either simultaneous (daei0, a, c), or pathogens were inoculated 5 days after Fe (daei5, b, d). Error bars represent mean ± standard deviation. Different letters indicate significant differences and *ns* nonsignificant differences between the means according to Fisher's LSD test ($P < 0.05$). Data presented are means of four replicate pots.

The plants simultaneously co-inoculated with either of Fe1, Fe2 or Fe3 and Fa following sowing had significantly higher biomass ($P < 0.05$) compared to the control plants inoculated with Fa alone. Total recorded biomass was the highest in the plants treated with Fe3 which was 4.6 fold higher than that of the pathogen inoculated control plants ($P < 0.05$). Plants in the treatment with isolates Fe2 and Fe1 had 3.7 and 3.2 fold higher biomass, respectively in

comparison to corresponding control plants inoculated with the pathogen alone (Figure 4.2a). When Fa was inoculated at daei5, only plants in the treatment with Fe2 had significantly higher biomass (22%) compared to the Fa control, showing that the relative effect of Fe isolates on growth enhancement was more pronounced when Fa was inoculated at daei0, when detrimental effect of Fa was higher (Figure 4.2b).

In contrast to treatments with Fa, no significant differences were observed for the different inoculation times with Pp, although at daei0 inoculation with Pp alone decreased pea biomass by 28%, whereas when inoculated at daei5 there was no effect of Pp on biomass compared to the un-inoculated control. Similarly, none of the *F. equiseti* isolates had significant effect on biomass in the Pp co-inoculated treatments regardless of the time at which the pathogen was inoculated (Figure 4.2c and d).

4.4.4 Effect of inoculation with *F. equiseti* on suppression of pea root rot

In comparison to control plants inoculated with Fa alone, the co-inoculation of the pathogen with Fe1, Fe2 and Fe3 following sowing (daei0) significantly decreased root rot symptoms caused by Fa, and even more when Fa was inoculated five days after Fe (daei5, Figure 4.4A). At both times at which the pathogen was co-inoculated, the effects of Fe isolates followed the same pattern with Fe3 providing greatest disease reduction and being the most effective in controlling the disease ($P < 0.05$) (Table 4.2).

In contrast, effects of Fe isolates on symptom suppression in the treatment with Pp differed significantly with respect to the time at which the pathogen was inoculated. No significant differences were found among treatments when the pathogen was inoculated at daei0. However, larger differences in root rot symptoms were observed when Pp was inoculated at daei5 (Figure 4.4B). Five day pre-inoculation of pea plants with all Fe isolates (daei5) resulted in significant ($P < 0.05$) reduction of root rot symptoms. As for Fa, isolate Fe3 provided the highest protection (Table 4.2).

Table 4.2. Root rot disease severity and corresponding disease reductions provided by *F. equiseti* isolates following inoculation with *F. avenaceum* and *P. pinodella* at two different times.

	F. avenaceum				***P. pinodella***			
	daei0[1]		**daei5**		**daei0**		**daei5**	
	DI[2]	**% reduction**	**DI**	**% reduction**	**DI**	**% reduction**	**DI**	**% reduction**
Control[3]	94.4 ± 2.9 a[4]	0.0	61.0 ± 12.2 a	0.0	68.8 ± 4.6 ns	0.0	62.3 ± 6.9 a	0.0
Fe1	74.9 ± 6.0 b	20.7	40.4 ± 5.1 b	33.8	66.8 ± 6.7	2.8	38.7 ± 5.5 bc	37.9
Fe2	63.3 ± 4.9 c	33.0	31.2 ±3.8 c	48.8	64.8 ± 5.8	5.7	40.3 ± 2.7 b	35.3
Fe3	54.7 ± 3.8 d	42.1	21.5 ± 7.3 d	64.7	69.2 ± 3.9	-0.6	28.5 ± 6.0 c	54.2

[1]daei0 – simultaneous co-inoculation of pathogens and Fe immediately following sowing; daei5 – Fe was inoculated 5 days prior to a pathogen; [2]Disease severity is expressed as Disease Index (DI), where DI = 0-15 – non-aggressive; DI = 16-30 - weak aggressiveness; DI = 31-70 - moderate aggressiveness; DI = 71-100 - high aggressiveness; [3]Pathogen alone. DI for un-inoculated plants were always less than 20 (see Figure 4.1b); [4]Data within the same column followed by different letters are significantly different (Kruskal post hoc test, $P < 0.05$).

4.4.5 Root colonization of pea plants by *F. avenaceum* and *P. pinodella* in the presence of *F. equiseti*

In the absence of Fe, Fa colonized the whole root system as shown by 100% isolation frequencies (Figure 4.3a and b). A general decrease was found in overall root colonization rates of Fa in co-inoculated plants compared to corresponding control plants inoculated only with Fa (Figure 4.3a and b). When inoculated at daei0, Fa was significantly reduced by 20% in the rhizodermis (non-sterilized roots) of Fe2 co-inoculated plants, whereas when the pathogen was inoculated at daei5, colonization of the rhizodermis by Fa was reduced ($P < 0.05$) by 60%, 30% and 46.7% by Fe1, Fe2 and Fe3, respectively (Figure 4.3a). In the root cortex, Fa was significantly reduced by all three Fe isolates when co-inoculated following sowing (daei0, 20% to 46.7%), and by Fe2 and Fe3 when Fa was inoculated five days after sowing (daei5, 16.7% and 23.4%, respectively) as assessed by plating surface sterilized pea root segments (Figure 4.3b). The re-isolation frequencies of Pp from the roots of Fe co-inoculated plants followed a similar decrease pattern as for Fa. Looking at the whole root system (Figure 4.3c and d), the colonization of the rhizodermis tissue by Pp was significantly reduced by 60% and 50% in Fe1 and Fe2 co-inoculated treatments at daei0. When Pp was inoculated at daei5 colonization of the rhizodermis was significantly reduced ($P < 0.05$) by all three Fe isolates (70% each) compared to corresponding control plants infected with Pp alone (Figure 4.3c). Colonization of the root cortex by Pp of co-inoculated plants following sowing (daei0) was unaffected by the presence of Fe. When Pp was inoculated at daei5, colonization of the cortex tissue was found to be significantly reduced by 46.6%, 53.3% and 53.3% by Fe1, Fe2 and Fe3, respectively (Figure 4.3d). Effect of Fe isolates on root colonization by both pathogens was greatest on the lower root parts, while, with the exception of Fe2/Fa co-inoculated treatment at daei0, no significant effects were seen in the upper root segments (Table 4.3).

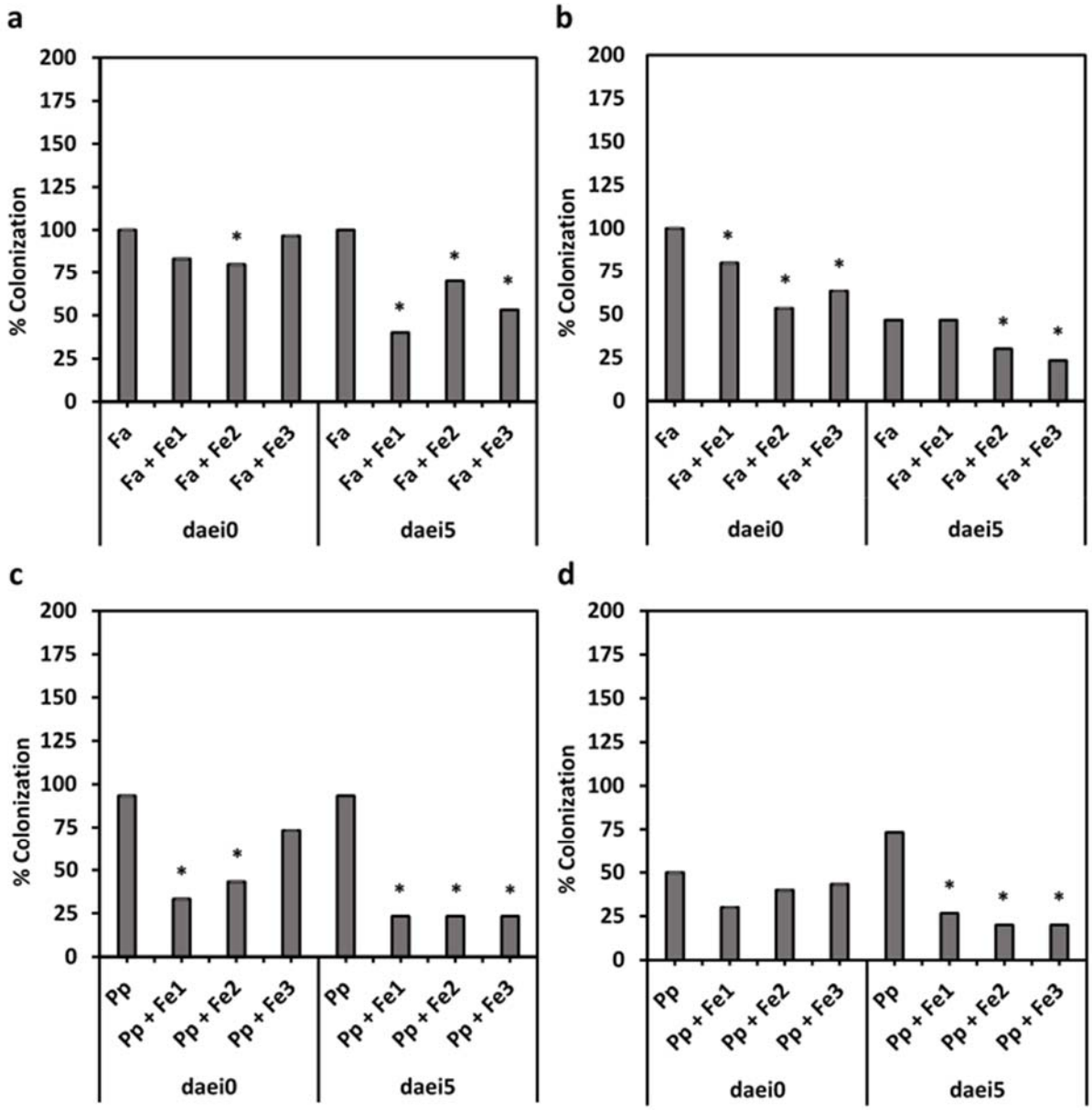

Figure 4.3. Colonization of root rhizodermis (a, c) and root cortex tissue (b, d) by *F. avenaceum* (Fa) and *P. pinodella* (Pp) inoculated alone or co-inoculated with *F. equiseti* immediately following sowing (daei0) or five days after sowing (daei5). Colonization rates were determined as the percentage of colonized non-sterilized (rhizodermis) and surface sterilized (cortex) root segments (n = 30). Asterisks above bars indicate significant differences in pathogen colonization rate with respect to the corresponding control plants inoculated with pathogen alone (Kruskal post hoc test, $P < 0.05$).

Table 4.3. Colonization (%) of the rhizodermis (non-sterilized pea root segments, n = 15) by *F. equiseti* and by *F. avenaceum* or *P. pinodella* at different root positions either co-inoculated together at sowing (daei0) or when *F. equiseti* was inoculated 5 days prior to pathogens (daei5).

	daei0				daei5				daei0				daei5			
	F. equiseti		*F. avenaceum*		*F. equiseti*		*F. avenaceum*		*F. equiseti*		*P. pinodella*		*F. equiseti*		*P. pinodella*	
Root position	**upper**	**lower**	**upper**	**lower**	**upper**	**lower**	**upper**	**lower**	**upper**	**lower**	**upper**	**lower**	**upper**	**lower**	**upper**	**lower**
Control	0.0 b[1]	0.0 b	100.0 a	100.0 a	0.0 b	0.0 b	100.0 a	100.0 a	0.0 b	0.0 b	100.0 a	86.7 a	0.0 b	0.0 b	100.0 a	86.7 a
Fe1	46.7 a	66.7 a	100.0 a	66.7 a	86.7 a	66.7 ab	53.3 ab	26.7 b	100.0 a	100.0 a	53.3b	13.3 c	100.0 a	100.0 a	33.3 b	13.3 b
Fe2	100.0 a	100.0 a	66.7 b	93.3 a	93.3 a	100.0 a	86.7 ab	53.3 b	100.0 a	100.0 a	60.0 b	26.7 c	100.0 a	100.0 a	40.0 b	6.7 c
Fe3	66.7 b	86.7 ab	100.0 a	93.3 a	86.7 ab	93.3 a	73.3 bc	33.3 c	100.0 a	100.0 a	80.0 b	66.7 b	100.0 a	93.3 a	40.0 b	6.7 c

[1]Different letters following percentages within the same row and inoculation treatment (daei0 or daei5) indicate significant differences in colonization rates between treatments (Kruskal post hoc test, $P < 0.05$).

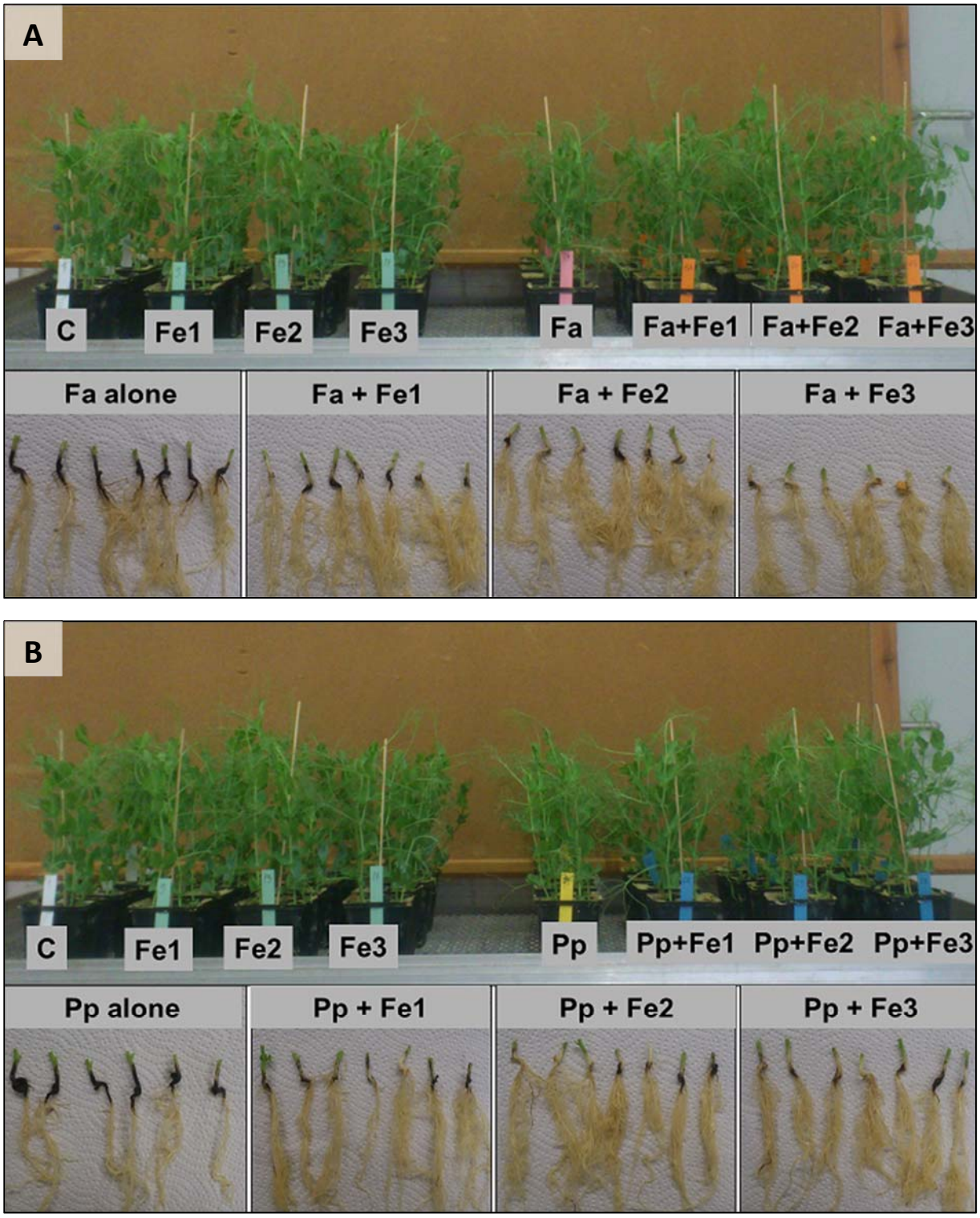

Figure 4.4. Effects of inoculation with *F. equiseti* isolates on plant growth and suppression of the root rot symptoms caused by *F. avenaceum* (A) and *P. pinodella* (B) inoculated five days after sowing (daei 5).

4.5 Discussion

The two *F. equiseti* isolates obtained from wheat roots, and one isolate obtained from winter vetch roots were all able to efficiently colonize the rhizodermis and root cortical tissue without necrotic lesion development, the trait which is characteristic for non-pathogenic (endophytic) plant-fungus interactions (Schardl et al., 2004; Benhamou et al., 2001; Rodriguez-Galvez and Mendgen, 1995). While Fe colonization often reduced symptom development and negative effects of the two pathogens tested, these interactions appeared to be isolate and pathogen specific.

Fusarium equiseti isolate Fe1 originally isolated from vetch and Fe3 originally isolated from wheat roots had significant plant growth promoting effects at the end of the 4 week experiment. In contrast, despite its capacity to efficiently colonize the root system, Fe2 (also isolated from wheat) had virtually no effect on pea growth. The nature of interaction and plant growth enhancement conferred by endophytic fungi are highly dependent on the host plant, fungal isolate, inoculation method, type of growing substrate and other *in vitro* conditions (Martínez-Medina et al., 2009; Forsyth et al., 2006; Larkin and Fravel, 1999; McAllister et al., 1997). For example, Saldajeno and Hyakumachi (2011) reported growth enhancement of cucumber plants after inoculation with *F. equiseti*, whereas, the colonization of barley roots by endophytic *F. equiseti* (Macia-Vicente et al., 2009b) did not lead to enhanced growth of the plants. Thus, some host-endophyte associations seem to be more beneficial to the hosts improving host fitness and growth.

Non-mycorrhizal fungal root endophytes may promote plant growth by a number of different mechanisms. These include production of plant growth regulators such as indole acetic acid and abscisic acid that may accelerate and modulate plant growth (Nassar et al., 2005; Arora et al., 2004), production of secondary metabolites *e.g.* fumonisin synthesis by some *Fusarium* spp. which can on the one hand act as potent mycotoxin, but on the other hand have been shown to improve development of maize roots (Yates et al., 1997), and improved availability of nutrients, particularly phosphorus uptake from the rhizosphere (Schulz and Boyle, 2005). Which of these mechanisms plays a role for the Fe-pea interactions studied here remains to be investigated.

Several studies have reported alleviation of disease through the inoculation of plants with endophytes (Wang et al., 2016; Waqas et al., 2015; Lee et al., 2009; Martínez-Medina et al., 2009; Kavroulakis et al., 2007; Arnold et al., 2003; Duijff et al., 1999). In the present study, we expected the endophyte to directly or indirectly interfere with the pathogen infection process and consequently stop or slow down disease development. Although we observed significant root rot disease suppression and reductions in root colonization rates of Fa and Pp in the plants treated with Fe compared with Fe untreated (pathogen infested) controls, the effect was highly dependent on the pathosystem, timing of pathogen inoculation and the Fe isolates used.

Simultaneous inoculation or pre-inoculation of pea plants with any one of the three *F. equiseti* isolates resulted not only in disease suppression, but often in improved plant growth and reduced colonization rates of *F. avenaceum.* A clear depression of *F. avenaceum* particularly in the root cortex of co-inoculated plants and in plants pre-inoculated with Fe, points to antagonistic effects and the capacity of Fe to interfere with early infection processes of Fa, and to limit the pathogen development in the rhizosphere and internal root tissue. In contrast to Fa, significant reductions of disease severity and decrease in root cortex colonization rates of Pp only occurred in the plants pre-inoculated with Fe five days prior to the pathogen. These results point to a higher competitive ability of Pp, and the need for a period of Fe establishment in the root system before it can compete with the pathogen. Several other studies have reported similar interactions among endophytes and pathogens in other pathosystems. Pre-inoculation with *F. equiseti* significantly suppressed associated diseases and reduced root populations of soil-borne pathogens, *Rhizoctonia solani* in cucumbers (Saldajeno and Hyakumachi, 2011) and *F. oxysporum* f. sp. *radicis-lycopersici* in tomatoes (Horinouchi et al., 2007), compared to simultaneous inoculations of the pathogen and the endophyte. Larkin and Fravel (1998), using different non-pathogenic *Fusarium* spp. isolates, inoculated 10 days ahead of the pathogen, showed significant reduction of Fusarium wilt disease on a variety of different crops, such as tomato, muskmelon, watermelon, basil and spinach. Nelson (1992) reported that pre-inoculation of tomato and cucumber with non-pathogenic *Fusarium* 2-7 days prior to pathogens reduced wilt symptoms and the multiplication of pathogens in the plants.

The isolates used in our experiment originated from different host plants, soil types and climatic regions. Nevertheless, all *F. equiseti* isolates had the potential to effectively suppress the development of the pathogens in the root and reduce associated disease symptoms. Isolate Fe3 appeared to be a highly efficient rhizodermal root colonizer and the strongest competitor, consequently providing up to 65 % disease reduction compared to pathogen treated control plants. Relatively high frequencies and stable populations of *F. equiseti* were found in natural vegetation under the environmental stress of sandy soils and salt marshes in desertified Mediterranean ecosystems (Macia-Vicente et al., 2008a). These Fe isolates readily colonized barley roots and conferred protection against the fungal root pathogen *Gaeumannomyces graminis* var. *tritici* (Macia-Vicente et al., 2008b). The ability of *F. equiseti* to sustain stable populations in diverse ecosystems and to colonize the roots of various host plants suggests that the mechanisms of action are not host specific. However, although similar results have been observed in the current study, the most efficient isolate, Fe3, had impaired colonization efficiency of the root cortical tissue. This suggests that the evaluated isolate may be a less prevalent endophyte with a strong competitive capability in the rhizosphere. Further research is therefore required to elucidate the exact mechanisms behind the observed growth promotion and disease suppression.

In our study, the Fe isolates, the pathogens, but also *Penicillium* spp. and *Cladosporium* spp. were found to occupy the same regions in the root. Whereas the fungi can infect and grow in pea roots treated with Fe, in comparison to the control, their root colonization ability was significantly reduced and mainly limited to the rhizodermis. This appears to point to competition. The mechanisms underlying such competitive interactions are still poorly understood. *In vitro* bioassays suggest that direct antagonism by the secretion of secondary metabolites toxic to pathogens, competition for nutrients in the rhizosphere, and competition for infection sites on the root are of importance (Schulz et al., 2006; Arora et al., 2004). However, we cannot exclude as a mode of action at least some degree of indirect antagonism mediated through activation of host defense mechanisms. Recent studies have reported that *F. equiseti* can induce changes in the root system such as abundant papillae formation and accumulation of phenolic compounds in the vacuoles (Macia-Vicente et al., 2009a), as well as physiological changes in the composition of plant extracts and root exudates (Horinouchi et al., 2007). Furthermore, Saldajeno and Hyakumachi (2011) showed that *F. equiseti* can induce

systemic resistance in cucumber plants against the pathogen *Colletotrichum orbicularis,* the causal agent of cucumber anthracnose.

In this study it was also observed that the un-inoculated control roots were colonized in high frequencies by *Penicillium* spp. whose colonization was mainly limited to the root rhizodermis. These observations indicate that the fungi are superficial root colonizers. The opposite trend observed for *Cladosporium* spp. whose isolation percentage was significantly higher from the root cortex tissue compared to the rhizodermis is probably due to biological activity of *Penicillium* spp. (Alam et al., 2011) which inhibited the emergence of *Cladosporium* spp. from non-sterilized root segments on artificial medium used, whereas the surface sterilization severely reduced *Penicillium* spp. allowing the *Cladosporium* spp. to emerge in higher rates from the cortex tissue.

In conclusion, our study clearly shows that some *F. equiseti* isolates can promote pea growth, influence the dynamics of the pathogen populations *in planta* and of root colonizers such as *Penicillium* spp. and *Cladosporium* spp., have the ability to alter the interactions pea - *F. avenaceum/P. pinodella,* by affecting their ability to colonize roots and consequently leading to reduced root rot disease severity. In recent studies of Baćanović (2015) and Pflughöft et al. (2012) foot and root rots have been reported as one of the main reasons for declining pea production in Germany, and *F. avenaceum* and *P. pinodella* were found to be the dominant causal agents of the disease. Our results highlight to the best of our knowledge for the first time endophytic behavior of *F. equiseti* and its ability to contribute to control of two major root rot pathogens of pea. Because *F. equiseti* is ecologically fit to sustain stable populations and function within diverse ecosystem conditions, the fungus has the potential to be developed into an effective biocontrol agent, which is of particular interest in non-chemical, long-term sustainable root disease management. Further research will focus on investigating the underlying interactions within these pathosystems and the potential for seed inoculation in order to understand the mechanisms involved with the aim to determine the full potential but also the limitations for biocontrol at the theoretical and practical level.

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CHAPTER 5: General discussion

Legumes can provide a range of ecological services to agroecosystems in addition to supplementary N when grown as cover crops, intercropped or under-sown (living mulch) with other main crops. However, on the one hand, there is a need to broaden the spectrum of legumes that can be used in crop rotations which are suitable for such agroecosystem intensification. Cover crops, for example, should be fast growing and highly productive to fit in the off-season between main crops, whereas living mulch should not be too competitive and should be complementary with the main crop by occupying the niches not used by the main crop. On the other hand, little is known about the disease resistance and thus potential pathological risks associated with legume rich crop rotations. As these often lead to soil-borne pathogen population build-up, the root health becomes particularly challenging in such legume intensive growing systems.

To the best of my knowledge, no work has been done on incidence of *Fusarium* spp. associated with roots of leguminous cover crop and living mulches, in particular subterranean clover, white clover, winter and summer vetch across the broad range of ago-climatic conditions in Europe. The prevalence of *F. avenaceum* and the ability of isolates of this species to cause severe root rot, indicate the possibility of the pathogen to emerge as a potential risk under intensive legume cropping practices. Other recent studies have also reported high incidence and shifts in the dominance of species within the *Fusarium* root rot complex of legumes. For example, declining importance of *F. solani* and the emerging importance of *F. avenaceum* in pea and *F. graminearum* in soybean root rot complex have been demonstrated in USA, Canada and Germany (Baćanović, 2015; Chittem et al., 2015; Arias et al., 2013a; b; Feng et al., 2010; Pflughöft, 2008). Furthermore, studies in Northern Europe have identified *F. avenaceum* as the dominating species of the *Fusarium* Head Blight (FHB) complex of small grain cereals (Uhlig et al., 2007). The fungus also causes economically important diseases on a range of different crops, such as soybean, maize, barley and oat (Zhang et al., 2010; Nielsen et al., 2011; Scauflaire et al., 2011), and its ability to asymptomatically colonize various weeds has also been demonstrated (Dastjerdi, 2014).

Climate has been reported as one of the major factors affecting the occurrence of *Fusarium* spp. (Sanglang et al., 1995; Boohan et al., 2003). In our study, *F. avenaceum* was prevalent at

Swiss, German and Italian sites, whereas in Sweden the fungus occurred rarely. However, more detailed sampling over the course of several years would be necessary to determine the temporal changes in the pathogen population and how these relate to the changes in crops over the course of rotations. Therefore, the precise combination of soil and environmental conditions that influence individual densities of *F. avenaceum* at each studied site, particularly the factors associated with low incidence of the fungus in Sweden remain to be elucidated.

In addition to being a well known wide host range pathogen, the ability of *F. avenaceum* to produce range of potent mycotoxins is of particular importance (Logrieco et al., 2002). While the mycotoxins produced by the fungus have been well characterized with respect to cereals (Bottalico and Perrone, 2002; Bottalico, 1998), little is known about *F. avenaceum* secondary metabolites with respect to legumes and in particular with the studied hosts under European agro-climatic conditions. Surveys from southern hemisphere countries, where subterranean clover and annual medics pastures play an important role in providing feed for grazing animals throughout the year, have shown that mycotoxins produced by *F. avenaceum* can cause severe feed refusal disorders in sheep (Tan et al., 2011; Lauren et al., 1992, 1988). Furthermore, existence of intra-specific genetic variability among isolates of *F. avenaceum* suggests that this morpho-species might also comprise a diverse complex of phylogenetic lineages that potentially reflect biogeographic patterns considering its cosmopolitan nature and diverse host range (Stakheev, 2016; Nalim, 2004). Therefore, the new information generated on the emerging importance and high pathogenic potential of *F. avenaceum*, warrant the need for further research that will examine the potential geographic exclusivity among different isolates collected with respect to mycotoxin producing profiles and the phylogenetic species recognition. These studies should also aim to precisely determine the role of the secondary metabolites in the long and short term strategies of the fungus in colonizing legumes as well as in occupying diverse agro-ecological niches.

Fusarium oxysporum was together with *F. avenaceum* the predominant species isolated from infected roots of studied legumes at all sites. However, more than 70 host specific *formae speciales* for *F. oxysporum* (Lievens et al., 2008) are recognized based on its specific pathogenicity to a particular host. Molecular phylogenetic analyses have further revealed a far more complex picture and showed that *F. oxysporum* comprises multiple cryptic species and represents an extremely diverse species complex that shows biogeographic distribution

patterns (O'Donnell et al., 1998a). In the current study, the prevalence of *F. oxysporum* in the roots of the studied legumes across the broad range of ago-climatic regions suggests that the species has adapted to a range of different environments allowing the fungus an extensive distribution and colonization opportunities. In addition, the high pathogenic variability of the tested population on pea is probably a reflection of diverse genotypes comprised in this species complex as a whole. However, further research is needed to draw conclusions on ecology and epidemiology of the collected *F. oxysporum* isolates, and to determine whether such biogeographically distinct patterns exist in the population of the recovered isolates.

In contrast to *F. avenaceum* and *F. oxysporum*, *F. solani,* a commonly associated species with legumes (Kolattukudy and Gamble, 1995) has been isolated in low frequencies from the roots of the studied hosts. Previously considered as a single morpho-species, the phylogenetic analysis based on the polymorphisms in DNA sequences showed that *Fusarium solani* is in fact a species complex composed of at least 60 phylogenetic species distributed among three major clades (clade 1, 2 and 3) (O'Donnell, 2000). The largest and most diverse group belongs to clade 3 (O'Donnell, 2000) that shows a cosmopolitan distribution and includes all seven mating populations (biological species) previously defined by Matuo and Snyder (1973). In the current work, the isolates studied showed different phylogenetic, pathogenic and ecological trends, supporting the current view that *F. solani* is a complex of morphologically cryptic species (Al-Hatmi et al., 2016; Schroers et al., 2016; O'Donnell, 2000). The substantial morphological and ecological diversity among the collected isolates was resolved with the two main lineages within the *F. solani* species complex (FSSC) clade 3, but with no clear phylogenetic structure that separated the isolates with respect to their hosts or aggressiveness to pea. The results obtained in the current work indicate certain geographical preference of the collected isolates, with the *F. solani* f. sp. *pisi* lineage mainly accommodating the German and the Swiss populations, whereas the *Fusisporium solani* lineage, with few notable exceptions, nested mainly Italian isolates. Additional research with more intensive sampling is required for solid conclusions, however.

The largest part of the identified strains was nested in the *F. solani* f. sp. *pisi* lineage (n = 51), the species named by its specific pathogenicity to pea. However, the field and the greenhouse data revealed that isolates of *F. solani* f. sp. *pisi* can occupy diverse ecological niches and that the species has a much wider host range than recognized previously. Multiple additional hosts

have also been demonstrated for several other special forms, such as *F. solani* f. sp. *glycines*, recently described as *F. tucumaniae* and *F. virguliforme* (Aoki et al., 2003), *F. solani* f. sp. *phaseoli* (currently *F. phaseoli,* Aoki et al. 2003), and *F. solani* f. sp. *eumartii* (Romberg and Davis, 2007). Although still widely used, the concept of *formae speciales* often leads to incorrect assumptions and generalizations about the behavior of individual isolates, and as it also represents an informal rank in taxonomic classification (Kistler, 2001) it is likely to be reconsidered in the near future.

Based on the results of the phylogenetic species recognition, additionally identified isolates were placed in *Fusarium solani* 5 (n = 25) recently described as *Fusisporium solani* by Schroers et al. (2016), and one strain matched with *F. keratoplasticum*, the two species commonly associated with dry rot of potatoes and human keratitis infections, respectively (Short et al., 2013; Schroers et al., 2016). The two isolates that formed a new lineage within FSSC clade 3 (lineage 1) showed different pathogenic and ecological trends. It will require additional studies in order to fully understand their ecological importance.

Using robust greenhouse and laboratory methodology, this work has also demonstrated the endophytic potential of *F. equiseti* to promote pea growth and the ability of this species to alter the interaction pea – *F. avenaceum*/*Peyronellaea pinodella*, consequently leading to reduced root rot disease severity. A number of studies have reported the potential of *F. quiseti* and other root endophytes to promote agro-ecosystem efficiency through their beneficial impacts on their hosts (Macia-Vicente et al., 2009b; Schulz and Boyle, 2005; Nitao et al., 2001; Kirk, 1993), a topic of great interest in non-chemical, long-term sustainable agriculture. However, while *F. avenaceum* and *P. pinodella* are well known pathogens of pea (Persson et al., 1997; Feng et al., 2010; Baćanović, 2015; Chittem et al., 2015), that are becoming increasingly problematic especially in Germany, the ecological roles of *F. equiseti* still remain controversial. In addition to being reported as potential biocontrol agent, *F. equiseti* is often recovered from diseased cereals and other crops, and consequently considered as a pathogen (Leslie and Summerell, 2006). However, according to Leslie and Summerell (2006), while it is not unusual to recover *F. equiseti* from diseased plants the completion of Koch's postulates is rarely reported. Thus, records of pathogenic potential of *F. equiseti* should be treated cautiously, as these are often based solely on the recovery of the fungus from the diseased tissue, and not on its ability to cause disease.

Given this background, in addition to the questions raised in chapter 2, we have also examined the possibility that the isolates of *F. equiseti* may not be as effective at causing significant root rot alone, but rather as a part of the root pathogen complex by gaining the advantage after the host resistance has been suppressed by other, more aggressive fungi. Taken together our overall results suggest beneficial activities of *F. equiseti* in the foot and root rot complex of pea. However, the mechanisms underlying these interactions are still to be elucidated. An overview of recent studies which have shed light on the potential underlying mechanisms suggest a combination of several mechanisms such as high competitive ability of *F. equiseti* in the rhizosphere, induction of physiological changes in the root system as well as activation of plant disease resistance through pathways still not well understood (Macia-Vicente et al., 2009a; Horinouchi et al., 2007). Furthermore, despite the potential of the fungus to be developed into an effective biocontrol agent, *F. equiseti* is a well-known species capable of producing various mycotoxins (Moss, 2002; Bottalico, 1998; Krogh et al., 1989) that may pose risks of contaminating the food and feed, and thus cause health threats to humans and animals. Therefore, further studies should also integrate mycotoxin risk assessments and how these interact and affect mycotoxin production by *F. avenaceum* as well as still poorly characterized secondary metabolites of *P. pinodella*.

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Summary

Modern cropping systems mainly rely on growing a narrow range of crop species and genotypes while the possibility of using novel crops, particularly leguminous species, which have the potential to play a major role in more diversified and sustainable food production systems has often been neglected. Growing legumes as cover crops, intercropped or under-sown with cereals can provide multiple beneficial services to agro-ecosystems. In addition to positive effects on yields, legumes in agricultural systems perform a range of ecosystem services beyond biological N fixation. An important concern is, however, that many of the species of interest share important soil-borne pathogens with some important main crops such as peas, beans, and cereals. While there is a need to broaden the spectrum of species that can be used as cover crops and living mulches in modern agriculture, little is known about their susceptibility to soil borne diseases.

The present work provided first documentation of diversity, geographical distribution, prevalence, and aggressiveness of *Fusarium* spp. associated with roots of subterranean clover, white clover, winter and summer vetch grown as cover crop and living mulch species across five European sites. Although the samples originated from Mediterranean to Nemoral environmental zones of Europe, no strong separation in *Fusarium* community structure was observed between the sites. At Swiss, Italian and German sites the most prevalent *Fusarium* spp. were *F. oxysporum* and *F. avenaceum*, whereas at Swedish site *F. oxysporum* dominated and *F. avenaceum* occurred rarely. Most remaining species, such as *F. solani*, *F. equiseti,* F. *redolens, F. graminearum* and *F. culmorum*, were represented by a few isolates only, and some absent in at least one of the environments. To determine the potential role of the studied legumes as alternate hosts of pathogens of importance to a main legume cash crop, 72 isolates of six *Fusarium* spp., were characterized for their aggressiveness on pea. The pathogenicity tests revealed high pathogenic potential of *F. avenaceum* and suggest that the species could emerge as potential risk in intensive legume cropping systems. The tested *F. oxysporum* and *F. solani* isolates mainly induced moderate severity symptoms on roots whereas, the isolates of *F. tricinctum*, *F. equiseti* and *F. acuminatum* caused some root discoloration on pea, but no reductions in fresh weights, some even increased biomass considerably.

Subsequently, a collection of 79 *F. solani* isolates was characterized by molecular genotyping (*tef1* and *rpb2* loci) and greenhouse aggressiveness bioassays. Previously recognized as a single morpho-species, *F. solani* includes multiple phylogenetic species and is a diverse species complex. The studied isolates formed four different lineages, all nested within *F. solani* species complex (FSSC) clade 3. The majority of isolates (76 isolates) however, were associated with two major lineages, the *F. solani* f. sp. *pisi* lineage mainly accommodating German and Swiss isolates, and *Fusisporium* (*Fusarium*) *solani* lineage accommodating mainly Italian isolates. Although the results obtained indicate certain geographical preference of collected isolates, additional research with more intensive sampling is required to make final conclusions. This study also revealed that pathogenic isolates of *F. solani* f. sp. *pisi,* the fungus named by its specific pathogenicity to pea, can be found in variety of habitats under diverse agro-ecological conditions. The fungus was, in addition to pea, able to colonize roots of various hosts such as subterranean clover, white clover, winter vetch and faba bean under field conditions. Greenhouse data on the host range of *F. solani* f. sp. *pisi* further supported this observation that the species is not explicitly adapted to a particular host. The results indicate that the host range should be expanded to include 33 symptomatic and 25 asymptomatic legume hosts. To what extent such problems exist in other special forms within the complex remains to be investigated, nevertheless the concept of *formae speciales* is often misleading and will most likely need to be reconsidered in the future.

Using robust greenhouse and laboratory methodology, this work has also demonstrated the potential of endophytic *F. equiseti* isolates to promote pea growth and alleviate detrimental effects of necrotrophic soil borne pathogens, *F. avenaceum* and *Peyronellaea pinodella*. Given the results of preliminary studies that showed high pathogenic potential of *F. avenaceum* and *P. pinodella* differently timed inoculation treatments were generated, where *F. equiseti* was inoculated following sowing, while *F. avenaceum* and *P. pinodella* were either inoculated simultaneously with *F. equiseti* or 5 days later. Simultaneous inoculation of *F. equiseti* with *F. avenaceum* or pre-inoculation of pea plants for 5 days with any one of the three *F. equiseti* isolates tested, resulted in disease suppression and significant reduction of *F. avenaceum* populations, particularly in the root cortex. In contrast, *F. equiseti* isolates significantly reduced disease and root cortex colonization rates of *P. pinodella* only in the plants inoculated with *F. equiseti* 5 days before the pathogen. Although, our overall results suggest beneficial activity of *F. equiseti* in the foot and root rot complex of pea, the underlying mechanisms are

still to be elucidated. Furthermore, despite the potential of the species to be developed into an effective biocontrol agent, the ability of *F. equiseti* to produce potent mycotoxins points to the need to assess mycotoxin risks and how these interact and affect mycotoxin production by *F. avenaceum* as well as still poorly characterized secondary metabolites of *P. pinodella*.

Zusammenfassung

Die heutigen Agrarsysteme basieren auf einem kleinen Sortiment an Kulturpflanzen und Genotypen. Bisher blieb die Möglichkeit der Verwendung neuartiger Nutzpflanzen, insbesondere Leguminosen, für eine diversifizierte, nachhaltige Fruchtfolge weitestgehend ungenutzt. Leguminosen als Haupt- und Zwischenfrucht oder als Untersaat im Getreide bewirken, neben der Ertragsteigerung durch die Stickstoffbindung der Leguminosen, eine Intensivierung der Ökosystemdienstleisungen in der Agrarbiozönose. Die komplementären Nutzpflanzen können jedoch auch als Wirte von den bodenbürtigen Krankheiten der Hauptfrüchte, Erbsen, Ackerbohnen und diverser Getreide, genutzt werden. Das Potential der neuen Kulturpflanzen für ein diverses Ökosystem wurde erkannt, allerding ist bisher noch wenig über deren Anfälligkeit gegenüber bodenbürtige Krankheiten bekannt.

Die vorliegende Studie dokumentiert als erste die Diversität, die geografische Verbreitung, das Auftreten und die Gefährlichkeit verschiedener *Fusarium*-Arten an Wurzeln diverser Zwischenfrüchte. Erdklee, Weißklee sowie Winter- und Sommerwicke wurden als Zwischenfrucht oder Untersaat an fünf Standorten, verteilt über Europa angebaut. Von den mediteranen bis zu den nemoralen Klimazonen konnten keine klare Abgrenzung der Artenzusammensetzung der Fusarien gesellschaften in den verschiedenen Umwelten fesstgestellt werden.

In der Schweiz, Italien und Deutschland waren die am häufigsten auftretenden *F. oxysporum* und *F. avenaceum*. In Schweden dominierte *F. oxysporum*, *F. avenaceum* trat selten auf. *F. solani, F. equiseti, F. redolens, F. graminearum* und *F. culmorum* traten nur vereinzelt in wenigen Isolaten auf und waren nicht in jeder Umwelt zu finden. Um das Gefährdunggspotential als Alternativ-/Zwischen-wirt der untersuchten Leguminosen festzustellen, wurden 72 Isolate der Hauptfrucht Erbse untersucht. Ein hohes pathogenes Potential zeigt *F. avenaceum*, welches ein potentielles Risiko für Leguminosereiche Fruchtfolgen darstellt. *F. oxysporum* und *F. solani*-Isolate riefen moderate Schadsymptome an der Wurzel auf. Isolate von *F. tricinctum, F. equiseti* und *F. acuminatum* verursachten Verfärbungen an der Erbsenwurzel jedoch keine reduktion der #oberirdisch Biofrischmasse, einige steigerten diese sogar erheblich.

Anschließend wurden 79 *F.solani*-Isolate molekulargenetisch identifiziert (*tef1* und *rpb2* loci) und in Töpfversuch im Gewächshaus auf das Gefährdungspotential untersucht. *F. solani* wurde bisher als eine einfache morpho-Art angesehen. Die Untersuchungen zeigten jedoch, dass diese mehrere phylogenetische Arten aufwiesen und somit einen eigenen diversen Artenkomplex bilden.

Die untersuchten Isolate wiesen vier unterschiedliche Stämme auf, die alle in dem *F.solani* Arten Komplex (FSAK) clade 3 enthalten sind. Der Großteil der Isolate (76 von 79) waren mit den 2 Hauptstämmen verbunden. *F. solani* f. sp. *pisi* -Linien wurde überwiegend in schweizer und deutschen Proben gefunden. *Fusisporium* (*Fusarium*) *solani*- Linien wurden vor allem in italienischen Isolaten gefunden. Obwohl die Ergebnisse gewisse territoriale Präferenzen suggerieren, sind weiter Untersuchungen, mit mehr Isolaten als bisher, nötig um eine abschließende Einschätzung zu treffen. Die Studie zeigte auch, dass die pathogenen Isolate von *F.solani* f. sp. *pisi*, welche nach Ihrem spezifischen Wirt Erbse bannt sind, in diversen Habitaten und unter verschiedenen ackerbaulichen Bedingungen gefunden werden kann. Der Schadpilz kann neben der Erbse auch die Wurzeln diverser Wirte wie Erd- und Weißklee, Winterwicke und Ackerbohne unter Feldbedingungen befallen.

Daten aus den Gewächshausversuchen unterstützen die Beobachtungen im Feld. Die Ergebnisse zeigen, dass der Umfang potentieller Wirte um 33 symptomatische und 25 asymptomattische Leguminosen erweitert werden muss. Inwieweit diese Problem in anderen Artenzusammensetzungen bestehen muss untersucht werden. Das Konzept der *formae species* ist oft irreführend und muss in Zukunft neu diskutiert werden.

Anhand robuster Gewächshaus- und Laborexperimenten, wurde das Potential endophytischer *F. equiseti*-Isolate, Schäden durch nekrotrophe bodenbürtige Pathogene wie *F. avenaceum* und *Peyronellaea pinodella* zu verringern gezeigt. Da in Vorstudien das hohe pathogene Potenial von *F. avenaceum* und *P. pinodella* gezeigt worden war, wurden unterschiedliche Zeitpunkte für die Inokulationen gewählt. Während *F. equiseti* immer zur Aussaat inokuliert wurde, wurden die Pathogene entweder gleichzeitig oder fünf Tage später inokuliert. Sowohl die gleichzeitige als auch die zeitversetzte Inokulation führte zu statistisch absicherbarer Krankheitsreduktion und Reduktionen der Besiedelung der Wurzelkortex mit *F. avenaceum* durch alle drei *F. equiseti* Isolate. Im Gegensatz dazu wurden Reduktionen der Infektionen und Besiedelung durch *P. pinodella* nur bei zeitgliecher Inokulation erreicht. Trotz der insgesamt

positiven Effekte von *F. equiseti* bedürfen die genauen Mechanismen der Krankheitsunterdrückung noch weiterer Untersuchungen. Da *F. equiseti* aber auch Mycotoxine produzieren kann, sollten diese Risiken genauer untersucht werden, bevor eine Weiterentwicklung von *F. equiseti* als Biokontrollorganismus ins Auge gefasst wird. Ebenfalls sollten potentielle Interaktionen mit Mycotoxinen von *F. avenceum* und anderen Sekundärmetaboliten von *P. pinodella* beachtet werden.

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