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Evaluation of resistance against fusarium root rot in peas

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Utvärdering av resistens mot fusariumrotröta i ärter

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Preface

To write this thesis has been rewarding in many ways. I hope the thesis will be a useful source of information on fusarium root rot resistance.

Firstly, I would like to thank my main supervisor Erland Liljeroth, Department of Plant Breeding, Swedish University of Agricultural Sciences, and co-supervisor Rolf Stegmark, Findus R&D, for valuable remarks during the process and for revising the manuscript.

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Abstract

Fusarium root rot, *Fusarium solani* f. sp. *pisi,* causes dark lesions and rots on the root- and foot region of peas. This paper provides a literature review, results from methodological development of resistance assessment, and results from resistance assessments. The main questions in this study were:

- What method can be used to evaluate resistance against fusarium root rot in peas?
- What is the level of resistance in a selection of cultivars?

The literature review aimed to cover the principles of fusarium root rot biology, mechanisms for pea resistance and the interplay between fusarium root rot and pea plant.

The virulence of 16 different isolates of *Fusarium solani* isolated from pea was assessed by measuring the symptoms on pea plants after inoculation. One of the most virulent isolates originated from a Scanian pea field, and was chosen for the following resistance assessments.

Two inoculation methods were evaluated by using them separately in the virulence assessment; soaking the seeds in spore solution for 24 h, and using the same method with addition of spore solution in the pots 4-5 days after sowing. There were no significant differences between the methods, and to just use the seed soaking method was chosen in the resistance assessments.

In the first resistance assessment, different spore batches were used for different groups of the included 41 accessions. This was due to difficulties with producing enough inoculum. A standard cultivar was incorporated in all accession groups. The standard cultivar provided the information that comparisons between spore batches wasn't adoptable, but it was possible to see which cultivars that were significantly more or less resistant than the standard cultivar.

In the second resistance assessment the same spore batch was used for all 44 accessions but without replicates. The accessions were ranked according to level of resistance.

Lesion length was found to be a superior measurement of resistance compared to using disease index in both resistance assessments, and is recommended for future high quality phenotyping.

Fourteen accessions were analysed with molecular markers for fusarium root rot resistance. The results of the marker study did not correlate with the results in the resistance assessments.

Contents

Vocabulary

Cytochrome P450	*"A generic term for a large number of related, but distinct, mixed-functions oxidative enzymes localized on the endoplasmic reticulum."* (Taiz & Zeiger 2010) Cytochrome P450 from *Fusarium solani* f. sp. *pisi* demethylate the pea defence compound pisatin.
Ergosterol	A sterol compound found in cell membranes of fungi.
PDA	Refer the name for six genes giving pisatin demethylating ability in *Fusarium solani* f. sp. *pisi* by production of a cytochrome enzyme (cytochrome P450). PDA can in this publication also refer to potato dextrose agar.
PEP	Refer the name for five pea pathogenic genes in *Fusarium solani* f. sp. *pisi*, associated with increased virulence.
Pisatin	A phytoalexin compound used as defence against pathogens by pea species.
QTL	Quantitative trait locus. "*The locus of a gene whose alleles have significant differential effects on the phenotype of a quantitative trait among individuals differing in genotype for the alleles"* (Hartl & Jones 2009).
Supernumerary chromosome	An extra chromosome that is not found in all representatives of the species. The DNA sequence of the chromosome should not be originating from the species itself (Covert 1998).

Background

Pea, Pisum sativum

Biology

The taxonomy of peas has received a lot of attention, followed by shifting nomenclature, as collocated by Smýkal et al. (2011). Maxted and Ambrose (2001) established three *Pisum* species:

- *P. sativum L.*
  -subspecies *sativum*
  (Including var. *sativum* and *var. arvense*)
  -subspecies *elatius* (Bieb.) Aschers. & Graebn
  (Including var. *elatius,* var. *brevipedunculatum* and var. *pumilo.*
- *P. fulvum* Sibth. & Sm.
- *P. abyssinicum* A. Br.

The garden pea belongs to *Pisum sativum* subsp. *sativum* (Laber 2014).

Pea (*Pisum sativum* L.) was likely domesticated in the Near East 7000-6000 BC (Zohary & Hopf 1973). The Middle East is believed to be the centre of origin. The *Pisum* genus is a relatively diverse genus which show rich features of introgression during the course of the domestication process (Smykal et al. 2011). All *Pisum* spp. are diploid, 2n=14 (Gritton 1980).

P. sativum is an annual plant (Laber 2014) and cleistogamic selfing (Cooper 1938) or crosspollinated (Harland 1948). During selfing fertilisation is completed 36-24h before the flower opens (Cooper 1938). Crosspollination is mediated by insects and have been reported to account for up to 60% of fertilizations, depending on genotype and presence of insects (Harland 1948). Cross pollination in commercial cultivars has been reported to occur to only a low extent, below 1% (Gritton 1980).

The seed of *Pisum sativum* consists of an embryo, two cotyledons and the seed coat. When the seed imbibes water at 25°C the radicles break the seed coat within 48 h and grow downwards along the gravitational axis. The first visible roots are formed on the seedling radicle, 1-2 cm from the seed. The germination is hypogeal, meaning that the cotyledons stay buried in the substrate during germination (Torrey & Zobel 1977 pp. 119-120). The radicle is developed into a tap root, which has the main part of the lateral roots located in the loose and well manured layer near the soil surface. The plant can be supplied with nitrogen by the nitrogen fixating bacterium *Rhizobium leguminosarium,* which is present in nodules on the pea roots. The aboveground stem is round to slightly rectangular, hollow and often prone to lodging. (Makasheva 1983).

Pea production system

This section will provide information on the production system for canning peas, with emphasis on the production system used by Findus in Sweden.

Agronomic practices

Garden pea can be sown either in spring/summer for harvest the same year, or in autumn for harvest the next year. Autumn sowing put more importance on the cultivar's cold hardiness features in sites with harsh winter climate (Hagedorn 1985, Åkerberg 1951). Sowing is also possible during winter on frozen ground (Nilsson J, personal communication). In Northern Europe sowing is done in spring, since the over wintering losses otherwise can be too costly. In Southern Europe peas are instead grown during autumn and winter, since the temperatures are too high during the summer (Laber 2014).

Soil samples can predict the infection pressure of aphanomyces root rot, a disease which can cause total crop failure. The soil samples are grown with pea plants for four weeks and the plant damage is then evaluated (Arvidsson A-K., personal communication). The result is part of the decision making on which fields to grow or not in Findus' production. Soil tests are preferably used also to determine the soil's nutritional status, and the amount of nutrients that need to be added to the field (Nilsson J., personal communication).

A plant density of 80-100 plants/m^2 with a drilling depth of 3-5 cm is advised (Laber 2014). The earlier sowing, the higher sowing density (Nilsson J., personal communication). Sowing is done when the soil temperature reaches at least 5-8 °C (Laber 2014). The sowing period extends over about three months. In Sweden seed treatments with Wakil XL[1] (Nilsson J., personal communication), Apron XL[2] (Bekämpningsmedelsregistret 2015a) and the biological seed dressing Cedress[3] (Bekämpningsmedelsregistret 2015b) are approved for use in processing peas.

Peas respond well to irrigation, but in Sweden that is generally not required except for on very light soils (Nilsson J., personal communication).

Pests and diseases of economic importance

Pests

The pea aphid *Acyrtosiphon pisum* is the most damaging pest in the Swedish production system (Nilsson J., personal communication). Other pests are e.g. the pea weevil *(Sitona lineatus*), pea moth *(Cydia nigricana)* and the pea midge *(Contarinia pisi)* (Andersson 2015). During favourable conditions the silver Y moth *(Autographa gamma)* can cause immense damage; mainly due to the difficulties of sorting out the larvae from the peas in the processing chain (Nilsson J., personal communication).

[1]Contains the active substances metalaxyl-M, fludioxinil and cymoxanil. The product has effect on downey mildew, *Pythium* spp and *Ascochyta* spp. (Syngenta 2013).

[2] Active substance: metalaxyl-M. Activity against seed and soil-borne fungal diseases: e.g. *Pythium* spp., *Phytophtora* spp., *Peronospora* spp., *Plasmopara* spp. and downy mildews. (Syngenta n.d. b)

[3] The active organism *Pseudomonas cloraphis* is active against *Acochyta* spp. in peas, and against Acrothecium rots in stored carrots. (Bioagri n.d.)

Diseases

Root pathogens

The aphanomyces root rot is a soilborne disease, and the most destructive root pathogen in Nordic pea production. Infestation may lead to total crop failure. To avoid infected fields is the currently available "control" method. Fields with high inoculum levels must be avoided for many years before growing peas there again (Bødker & Larsson 1993).

Climate change can cause crop diseases to gain importance (Berg 2012). An important and possibly upcoming disease is the fusarium root rot disease, *Fusarium solani* f. sp. *pisi*; one of the most commonly found root rot pathogens in Swedish pea production (Persson et al. 1997). Since *F. solani* f. sp. *pisi* thrive in temperatures at 25-30°C (Kraft 2001) the disease could be anticipated to increase in a warmer climate. During the unusually warm summer of 2014 severe plant damage was observed in some Scanian pea fields. *F. solani* f. sp. *pisi* was isolated from diseased plants. The pathogen was suspected to explain at least parts of the plant-losses (Stegmark R., personal communication).

An inventory on pea foot and root rot pathogens in Sweden and Denmark found *F. solani* to be commonly prevalent on pea plants around the time for flowering. In Denmark *F. solani* f. sp. *pisi* was the most prevalent pathogen. *P. medicaginis var. pinodella* was the most common pathogen in Sweden, see Figure 1 (Persson et al. 1997).

F. solani, F.oxysporum and Pythium spp. were the most frequently isolated pathogens from peas grown in Dutch soil samples. Both diseases were isolated from about 60% of the sampled fields. The soils originated from fields that had been cropped with pea at least once during the last decade (Oyarzun et al. 1993a). In contrast were grain peas in Germany found to have a higher incidence of fusariums such as *F. redolens* (61%) and *F. avenaceum* (50%) in comparison with *F. solani* (28%) and *F. oxysporum* (42%) (Pflughöft et al. 2012).

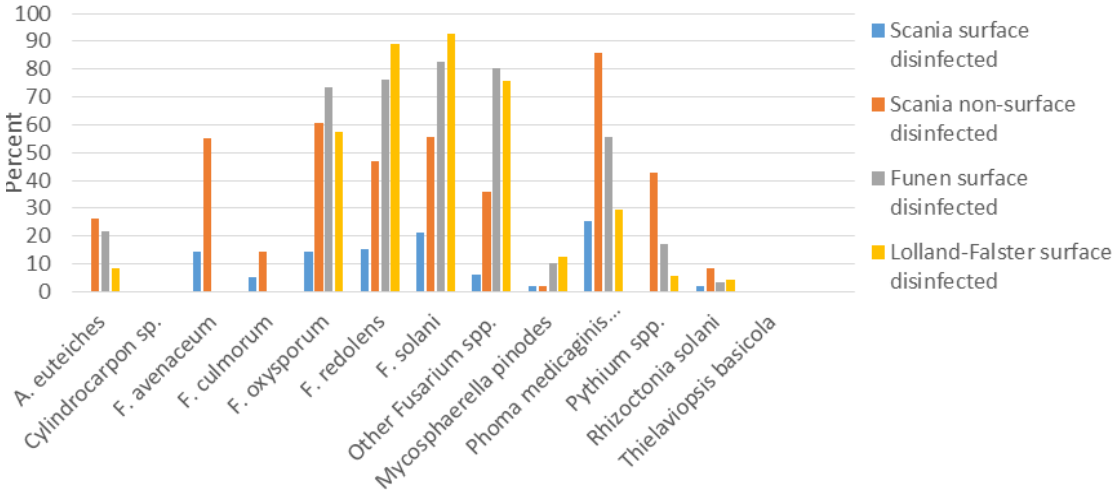


Figure 1. Inventory of pea foot ant root rot pathogens. The region Scania is located in the very Southern part of Sweden, Funen and Lolland-Falster are situated in Denmark. (modified from Persson et al. 1997)

Leaf- and stem pathogens

The vast majority of cultivars grown in Sweden nowadays have high levels of partial resistance against downy mildew, *Peronospora viciae* f. sp. *pisi*. Resistance breeding has

totally eliminated the need for fungicidal sprays in most Swedish canning pea production (Stegmark 1992, Stegmark R., personal communication).

Mycosphaerella pinodes and *Ascochyta pisi* both attack the overground parts of the plant. Together with *Phoma medicaginis* var. *pinodella* that also attacks the root system, these fungi belong to the so called Ascochyta complex (Bødker & Larsson 1995).

Seed health analyses 2010-2014

Many pathogens can be carried either in the seed tissue or on dust particles on the seed (Kraft 2001). The health of a particular seed batch can be tested by incubating seeds on agar nutrient media and evaluate the prevalence of different pathogens (ISTA 2008). The most prevalent seed borne pathogens in Findus pea production are provided in the seed health test results, Table 1. The tests were carried out by the seed division at the Swedish Board of Agriculture. The table comprises 76 seed samples from Swedish pea seed fields during the years 2011-2014. When the proportion of infected seeds of any pathogen has been below 1% but above 0% has this been described as <1% in the test protocols. The calculation for the true value in the table used the estimated average of 0,5% in those cases.

Table 1. Fungal taxa or species found on peas after seed health testing according to ISTA protocols 2010-2014. *Ascochyta. spp* has been increasingly distinguished into *A. pisi* and *A. pinodes* during the test span, why the data on these species should be higher, but have in part been masked by "A. spp."[4]

Disease	Diseased seeds (%)	Prevalence of disease in samples (%)
A. spp.	2,6	97
A. pisi	0,7	27
A. pinodes	0,5	24
Fusarium spp.	2	89
Botrytis spp.	2,3	71

Ascochyta diseases was the most prevalent disease causing pathogen taxa, present on at least 2,6% of the seeds and prevalent in 97% of the samples. *Fusarium* spp. followed with 2% of the seeds infected, prevalent in 89% of the seed samples.

Resistance of Pisum sativum

No commercial cultivars have complete resistance against fusarium root rot but numerous sources of resistance have been identified (Ondrej et al. 2008, Infantino et al. 2006, Grünwald et al. 2003, Kraft 2001, Kraft et al. 1994, Kraft & Roberts 1971, Hagedorn 1960). Breeding lines which combine a high degree of resistance with acceptable agronomic traits are available (Porter et al. 2014, Coyne et al. 2008).

Weeden & Porter (2007) mapped three QTLs linked to fusarium root rot resistance in two populations of recombinant inbred lines. The QTLs were found on chromosome II, IV and VII.

[4] The test provider on the Seed division, Swedish Board of Agriculture, explained that the species differentiation in the *Ascochyta* genus from *Ascochyta* spp. to either *A. pisi* or *A. pinodes* has improved over the length of the test period, why there is an inconsistency in the way these pathogens have been described in the test results. Any of the *A. pisi* or *A. pinodes* might have been used together with each other or with *Ascochyta* spp. in the individual seed health test result. The table must therefore be interpreted with this consideration. The seed division also explained that they haven't determined *Fusarium* spp to species level.

The QTL on chromosome II had the greatest effect on resistance. The QTL on chromosome VII had the weakest effect. The QTL on chromosome IV only gave effect if the QTL on chromosome II was present, indicating that this QTL could be part of a downstream biosynthetic pathway. The QTL region on chromosome II overlapped with the region of the A allele locus which is involved in the anthocyanin/polyphenol/flavanoid pathway, and the authors suggested that the effect of the QTL was due to the *A* gene (Weeden & Porter 2007).

Anthocyanins can be part of a resistance mechanism against fusarium root rot. Kraft (1977) found that the anthocyanin pigment delphinidin hindered conidial germination if the sugar availability was not too high.

The *A* allele was dominant and produced e.g. pigmented flowers and seed coats, while plants homozygous for the recessive a allele e.g. carried white flowers and had green seed coats. The *A* allele brought characteristics of low consumer acceptability such as dark colour, grassy taste and weak typical pea aroma (Taurick & McLellan 1986). The A allele was considered inappropriate to use in plant breeding although it has been shown to give resistance against the soil borne disease *Pythium ultimum* (Kraft 1977, Stasz et al. 1980).

If the QTL displaying the greatest effect was the *A* allele would this leave only the QTL with the weakest effect, on chromosome VII, available for breeding tailored at human consumption (Weeden & Porter 2007).

A QTL on the distal chromosome VII explains resistance to multiple diseases

In recombinant inbred lines from a cross between two commercial pea cultivars (one susceptible and one resistant) 39% of the variation in resistance could be explained by a QTL found at the distal end of chromosome VII. The microsatellite markers AA160 and AD53 in proximity of the QTL explained 27 and 50% of the genetic fusarium root rot resistance, respectively, when interaction between genetic and environmental factors were accounted for. These markers were recommended for marker-assisted selection (Feng et al. 2011).

A QTL in about the same location controlled resistance to *F. avenaceaum* (Li et al. 2012) and *A. euteiches* (Hamon et al. 2013). Unfortunately, it has not been established whether the QTL found in Weeden & Porter (2007), Feng et al. (2011), Li et al. (2012) and (Hamon et al. 2013) is the same. Resistance against *A. euteiches* and *F. solani* f. sp. *pisi* has earlier been shown to be positively correlated. The low correlation was weak but made significant thanks to a large dataset (Grünwald et al. 2003)

Fusarium root rot, Fusarium solani f. sp. pisi

Disease cycle and epidemiology

The fusarium root rot disease is soil borne and seed borne; seed borne probably because of dust particles on the seeds (Cook et al. 1968). The disease infects the germinating or growing pea plant through the epicotyl, hypocotyl, upper taproot (Kraft 2001) or through the root elongation zone slightly beyond the root cap (Gunawardena et al. 2005). Lesions are produced at the infection site and then spread through the root system (Kraft 2001, Gunawardena et al. 2005). The root vascular system may be discoloured in red, especially around the cotyledonary attachment area, but the discolouration doesn't progress in to the plant's aboveground parts (Kraft 2001), although *F. solani* has been isolated as far as up to the seventh node in plants (Persson et al. 1997). The disease symptoms above ground include stunting, chlorosis and necrosis of the lower foliage (Kraft 2001). Plants grown in soils with good structure and adequate water supply doesn't necessarily give aboveground symptoms of root rot diseases, even when severely infected (Bödker et al. 1993). *F. solani* f. sp. *pisi* produces resting structures in form of chlamydospores. These propagules occur in naturally infested soils (Nash et al. 1961).

Biology

Life cycle

Fusarium solani (Mart.) Sacc. f. sp. *pisi* (Jones) Snyd. et Hans. is the name on the ascomycete anamorph of the fungus causing foot and root rot on peas, chickpeas and ginseng. The teleomorph, *Nectria haematococca,* causes branch blight on mulberry (Matuo & Snyder 1973). *Fusarium solani* f. sp. *pisi* is reproductively isolated and is a distinct species, despite its denomination as a "forma specialis" (f. sp.) (O'Donnell 2000).

Infection process

The infection process has been studied by microscopy and ergosterol content analysis. Spore solution was poured over potted plantlets 7-14 days after sowing. Infection symptoms visible for the eye was observed on the seed coat 2-4 days after infection. The seed was the earliest point of attack for the pathogen. The seed coat got embedded in mycelia whereupon *F. solani* f. sp. *pisi* colonized the hypocotyl from the seed tissue about four days after primary infection. For a picture of hypocotyl infection, see Figure 2. After seven days lesions were found on the epicotyl, upper taproot and on lateral roots. These lesions increased and coalesced with time while lesions on lower taproot and lateral roots remained small, showing only minor traces of fungal infection. The ergosterol assay confirmed the results of the microscopic analysis and revealed that the fungi

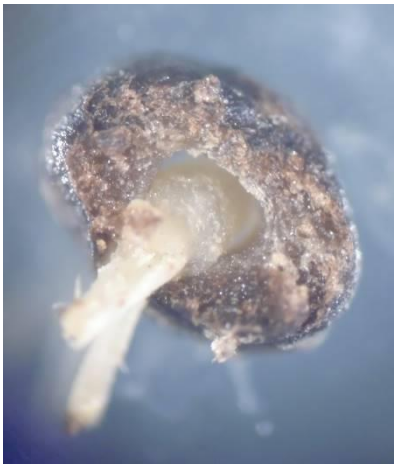


Figure 2. Hypocotyl infected with isolate 14, 21 days after inoculation. The cortex is mainly constituted by necrotic tissue, but the vascular tissue inside the pericycle has a healthy appearance. Photo: V. Tönnberg

infected the pea tissue in the following order: "*seed, hypocotyl, upper part of the taproot, and epicotyl.*" (Stahl et al. 1994).

Hyphae penetrated both the hypocotyl, epicotyl and taproot tissue directly through epidermis, without exhibiting appressoria, but one out of three fungal strains developed swollen hyphal tips during the infection process. The pea tissue was also invaded through stomata, but this infection gate was of minor importance in comparison to direct penetration (Stahl et al. 1994).

Hyphae that penetrated the epidermal cells of the epicotyl advanced through cortex intra- and intercellularly. Endodermis slowed down the infection process, but the presence of nearby mycelia caused the vascular parenchyma to disintegrate, leading to final colonization of the stele. Colonization of the cortex stopped 2-3 cm in above ground plant parts and from here *F. solani* f. sp. *pisi* colonized only the xylem cells. The plant reacted to the colonization of the xylem with cell wall deposits. The deposits successively lead to clogging of the xylem vessels. Unwounded and wounded leaves inoculated with spore solution were defended by a hypersensitivity reaction (Stahl et al. 1994).

Spore performance

In growing media *F. solani* developed abundant microconidia after 2-3 days in culture. The microconidia were formed on lateral, oftentimes branched, conidiophores which terminated with a single phialide, from which the microconidia were formed. These are non to 1-septate. Macroconidia developed after 4-6 days in cultivation (Booth 1971). Micro- and macroconidia are showed in Figure 3A-C.

Macroconidia needed carbon, nitrogen and some other factor present in yeast extract for germination. The unknown factor was fully replaceable with ethanol or acetoin, and partly replaceable with acetaldehyde or certain aminoacids. It was hypothesized that the required factor was an intermediate in the production of amino acids (Cochrane et al. 1963).

Figure 3A-C. (A) Microcondia, (B) Micro and macroconida with *Alternaria* spp. spores, (C) Germinating microconidia. Photos: V. Tönnberg.

Chlamydospores developed on low nutrient media after 7-14 days on short lateral hyphal branches or intercalary (Booth 1971). Chlamydospores are formed when the environment provides only low amounts of carbon, but contains enough mineral nutrients to support chlamydospore formation (Meyers & Cook 1972, Hsu & Lockwood 1973). Chlamydospores are formed to a higher extent from macroconidial germtubes when they are lysing. It has been proposed that nutrients exuded during lysing are used during the chlamydospore formation (Hsu & Lockwood 1973). Nutrient deprivation is known to induce autolysis in several fungi, including *F. solani* f. sp. *pisi* (Ko & Lockwood 1970). Germination of chlamydospores is promoted during nutrient rich soil conditions, as provided by leaching

exudate from imbibing and germinating pea seeds (Cook & Flentje 1967). Simultaneously, environments were both carbon and nitrogen are present in adequate amounts decrease the longevity of germinating chlamydospores (possessing germtubes) by inducing germtube lysis (Cook & Snyder 1965, Cook & Flentje 1967).

Hyphae of the closely related pathogen *F. solani* f. sp. *phaseoli* proliferated poorly in bean root tissue, in contrast with hypocotyl tissue (Christou & Snyder 1962). *F. solani* f. sp. *phaseoli* conidia were found to be produced en masse on sporodochia emerging from hyphae at soil level under light and high moisture (Nash et al. 1961). This might have implications also for *F. solani* f. sp. *pisi.*

Incidence and impact on pea production

A Canadian inventory estimated that 85% of the pea fields and 42% of the pea plants were affected by *F. solani* f. sp. *pisi* at harvest time. *F. solani* f. sp. *pisi* was by far the most common disease (Basu et al. 1973). This was confirmed by a later study were the fungi were isolated from plant samples (Tu 1986). The pea dry-weight harvest averaged 23% less for moderately diseased pea plants compared to healthy (Basu 1978) while severely diseased pea plants had a yield loss of in average 57%. The severely affected plants were characterized by *"six cm or more brown to black discoloration of the lower stem and tap root region, most lateral roots decayed, most leaves yellowed; plants often stunted, wilted, moribund or killed."* (Basu et al. 1976) Moderately affected plants had less symptoms than according to this description (Basu 1978).

Fusarium root rot is a serious pathogen also in the US pea production areas (Kraft 1994, Schroeder 1953). Recently *F. avenaceum* has been found as the most prevalent root rot pathogen in North Dakota (U.S), were the root rot disease previously has been thought to be caused by *F. solani* f. sp. *pisi* (Chittem et al. 2010). *F. avenaceum* has lately been recognized as the most prevalent root rot pathogen in Alberta (Canada) (Hwang et al. 2009)[5].

Preventative measures

Prognosis

To estimate the disease potential in a field before sowing could help to avoid severely infested fields and subsequent yield losses. Soil samples are used for estimation of disease potential for *A. euteiches* in Findus' pea production (Arvidsson A-K., communication). A similar approach has proven efficient for the root rot complex as a whole in the Netherlands. By growing peas in field sampled soil the degree of potential attack could be well quantified by a disease severity index. The contributions from the most prevalent diseases (*F. solani* f. sp. *pisi, A. euteiches, T. basicola*) were on the other hand not possible to distinguish between (Oyarzyn 1993b).

There have been an attempt to develop a molecular prognosis method by detection of the amount of virulence gene copies of PDA1 and PEP3 in soil samples. PDA1 and PEP3 together seemed to be important for high virulence. Before being practically applied the

[5] Additional information: Some level of resistance to F. avenaceum has been found in the Austrian winter pea types (Odom et al. 2014).

method would need to be complimented with validation from field results, and possibly also modifications of the model in form of soil predisposition to favour disease development (Etebu & Osborn 2011).

Agronomic practices

To favor pea development and avoid unnecessary disease some can measure be undertaken: Soil compaction should be avoided, sufficient moisture should be available for plant growth (Kraft 2001), sowing depth shouldn't be exaggerated (Lockwood 1962) and seeds should be of high quality (Short & Lacy 1974).

Crop rotation was recommended to be at least five years long to reduce disease severity (Kraft 2001). It was considered important to breed for cultivars with enhanced resistance (Feng et al. 2011). Use of waste lime (a waste product from the sugar industry) decreased disease severity in preliminary investigations with the related pathogen *F. avenaceum* (Chittem et al. 2010), and may be a method to evaluate for control of fusarium root rot as well.

Genetics of pathogenicity

Cell wall degrading enzymes

Cutinase activity as well as activity from other plant cell wall degrading enzymes produced by the fungi, such as pectinase and cellulase, may account for part of the virulence (Köller et al. 1982). The role of cutinases in the infection process of *F. solani* f. sp. *pisi* is unclear. Publications about the matter have been contradictory, as discussed by Stahl et al. (1994) and Etebu & Osborn (2011).

Small prefabricated amounts of cutinase, possibly already carried by the spore, resulted in low levels of cutin monomers when cutinase got in contact with the plant cuticle. The monomers trigged further synthesis of cutinase from *F. solani* f. sp. p*isi* (Woloshuk & Kolattukudy 1986).

Cutinase genes were expressed in spores of *F. solani* f. sp. *pisi* within 15 minutes from exposure to cutin monomers. Cutinase was synthesised after about 40 minutes. Cutinase may have aided in overcoming the plant cuticle barrier. Another function of the cutinase activity may have been to signal to the spore that it was contact with its' host (Woloshuk & Kolattukudy 1986).

Pisatin demethylation

The ability to demethylate the pea defence phytoalexin pisatin is linked to virulence. Pisatin is produced in planta in response to infection by *F. solani* f. sp. *pisi* (Pueppke & Van Etten 1974). Six genes with "pisatin demethylating ability (PDA)" *PDA1, PDA2, PDA3, PDA4, PDA5, PDA6-1*, *PDA6-2* and *PDA7* which all encode for pisatin demethylation have been identified (Kistler & VanEtten 1984, Mackintosh et al. 1989, Miao & VanEtten 1991, Funnell et al. 2002).

The role of cytochrome P450 in the pisatin demethylation has for long been recognised (Matthews & VanEtten 1983), and it has been confirmed that *PDA* genes codes for final gene products of cytochrome P450s (Maloney & VanEtten 1994).

PDA1 was seen as a requirement for virulence (Kistler & VanEtten 1984a, Kistler & VanEtten 1984b), and strains containing *PDA1* as only *PDA* gene were subjected to site-directed disruption of *PDA1.* Surprisingly, *F. solani* f. sp. *pisi* still not lost all virulence. This led to the conclusion that other genes but *PDA* genes must be involved in pathogenesis (Wasmann & VanEtten 1996).

Pea pathogenicity genes

Adjacent to the *PDA1* gene three additional ”pea pathogenic (PEP)” genes; *PEP1, PEP2* and *PEP5* were found. Both the *PDA*- and *PEP* genes were located on a supernumerary chromosome, which had previously been unknown. *PEP5* was the only *PEP* gene similar to a gene with known function and might encode a transporter protein (Han et al. 2001).

Highly virulent isolates contained homologues of *PEP1-5* and the two transcriptional units associated with these, named *PEP3* and *PEP4. PEP* gene homologues were found in isolates without the supernumerary chromosome, proving that homologues can be present also in the conventional chromosomal setup (Temporini & VanEtten 2002).

Virulence determinants

Derivatives from a substance group produced by *F. solani* f. sp. *pisi* called naphtazarin has showed influence on virulence type. Isolates which produced much of the plant toxins displayed higher virulence. Some derivatives were more toxic than other (Kern & Naef-Roth 1965)[6].

[6] Additional information: F. solani f. sp. pisi produced four phytotoxic naphtazarin derivates named fusarubin, javanicin, marticin and isomarticin in vitro, and the substances could be isolated from diseased pea tissue. Isolates that produced more toxins, and mostly isomarticin, in vitro were shown to be more virulent.

Interaction between pea and fusarium root rot

Factors affecting the disease

As with all biological systems many factors will affect the final disease severity. These factors include the following, as stated by Oyarzun et al. 1993b):"…

- *density and virulence of the pathogens*
- *competitiveness of the pathogens in relation to the other soil microflora*
- *susceptibility of the test plant*
- *physical and chemical characteristics of the soil*
- *environmental conditions"*

Pathogen virulence

Ondrej et al. (2008) emphasized how the virulence type of the isolate influences the outcome of any testing for resistance, with reference to the findings of Reinking (1950). In the work of Reinking some isolates infected the cotyledonary attachment area and lesions spread along the plant with extensive foot and root rots. Other isolates had less pronounced foot and root rots, and did not display the same pattern of spread from the cotyledonary attachment area (Reinking 1950). Different isolates' virulence differed significantly in several virulence assessments with different methodologies (Ondrej et al. 2008, Persson et al. 1997, Bolton et al. 1970, Reinking 1950).

Depending on inoculum density will it be more or less difficult to distinguish the virulence level from one isolate to another. Highly virulent isolates and lower inoculum concentrations was preferred for virulence assessments, and 10^5 spores/ml of *Fusarium solani* f. sp. *pisi* was preferred over 10^6 spores/ml with the seed soaking method (Ondrej et al. 2008).

Pathogen density

A field that had been cropped with pea in five subsequent years contained 2000-5000 cfu *Fusarium solani* f. sp. *pisi* /gram soil. Soil from adjacent shrubs, about 20 m from the field that had never been cropped with pea contained 100-300 cfu/gram soil (Kerr 1963). Soil rhizosphere contained up to 3300 cfu/gram soil, while root tissue contained up to 15000 cfu/gram tissue (Reyes 1980).

Inoculum placement had impact on disease development. There were no differences in disease symptoms when inoculum was placed in the upper 10 cm of a 30 cm container, or mixed throughout the container. Inoculum placed in the lower 10 cm failed to cause any measurable effect at 5000 cfu/g soil (Rush & Kraft 1986).

Soil microbial community

In field, *F. solani* f. sp. *pisi* appeared together with other soilborne diseases of peas as a complex (Persson et al. 1997, Oyarzun et al. 1994, Tu 1986, Basu et al. 1973,). When combined the different diseases can cause additive damages; as shown for *Pythium ultimum* (Kraft & Roberts 1969, Escobar et al. 1967). The damages can as well be

diminished during coexistence, as demonstrated for *Fusarium oxysporum* f. *pisi* (Ondrej et al. 2008, Buxton & Perry 1959).

Different soils influenced the potential for disease development. The number of *F. solani* f. sp. *pisi* propagules per soil unit was not correlated with disease severity when comparing different soils (Oyarzun et al. 1994). When surveying Dutch soils they varied from suppressive to conducive to F. solani f. sp. pisi, while mostly conducive to *A. euteiches* and suppressive to *Thielaviopsis basicola* (Oyarzyn et al. 1997).

Host susceptibility

Among the most common pea cultivars used as controls in resistance evaluations were 'Bolero' and 'Dark Skin Perfection' (Porter 2010, Rush & Kraft 1986, Kraft & Roberts 1971). The cultivars susceptibility is based on genetic factors, but it is important to bear in mind how resistance also depend on the physiological processes during different environmental conditions. Plant stress lead to increased susceptibility to *F. solani* f. sp. *pisi* (Kraft 2001, Kraft et al. 1981). Seeds with poor vigor; that e.g. have been stored during suboptimal conditions may be susceptible while high vigor seeds would be resistant (Kraft 1986). To soak seeds in water for 24h at 22°C decreased the incidence of seed and seedling rot (Short & Lacy 1976), while soaking seeds at 10, 15 or 30°C for 48h in general had no effect or increased the incidence of seed and seedling rot (Short & Lacy 1976). To soak the seeds for 48h compared with 24h reduced the pea germination capacity, disrupted the plant growth dynamics, and decreased the epicotyl length (Ondrej et al. 2008).

Sugars stimulated the growth of *F. solani* f. sp. *pisi* (Kraft 1977), sugars that may be released from seeds with high electrolyte loss (Kraft 1986). It was proposed that the amount of seed exudate directly correlated to disease incidence (Short & Lacy 1976a) because of the observed spermosphere effect that stimulated chlamydospore germination and infection (Short & Lacy 1974; 1976b). Germinated chlamydospores were as longest observed at a distance of 7 mm from germinating pea seeds (Short & Lacy 1976b).

Peas with the *A* gene for anthocyanin production contained the pigment delphinidin primarily in the testa. The compound was *"fungistatic to conidial germination of Fusarium solani* f. sp. *pisi"* , but the pathogen was able to germinate despite delphinidin presence when sufficient sugars were available (Kraft 1977).

Physical and chemical soil characteristics

Compacted soil decreased pea root growth (Kraft & Boge 2001) and gave increased disease symptoms of *F. solani* f. sp. *pisi* with increased compaction in clay soils (Vigier & Raghaven 1980).

Environmental conditions

F. solani f. sp. *pisi* gave more disease symptoms with higher temperatures, shown within the temperature range of 15-25°C (Gretenkort & Helsper 1993, Short & Lacy 1976b, Kraft & Roberts 1969). The optimal temperature range for *F. solani* f. sp. *pisi* was estimated to be between 25 and 30°C (Kraft 2001). High soil moist favored development of disease symptoms (Short & Lacy 1976b, Kraft & Roberts 1969).

Resistance assessment methods

In vitro

Early in vitro studies were performed on plants grown in soil amended with inoculum and nutrients (Reinking 1950, Hagedorn 1960).

Lockwood supplied spore suspension (10^6 conidia/ml) along seed rows 0-6 days after sowing and evaluated disease severity by indexing 15-20 days thereafter (Lockwood 1962).

Kraft counted the number of lesions on epicotyls eight days after sowing ($3*10^6$ macroconidia/g soil) (Kraft 1974). Kraft later used chlamydospores ($2\text{-}4*10^4$ cfu/g soil) as inoculum and assigned indexes to the disease symptoms eight days after sowing (Kraft 1975).

Whalley (1984) disinfected small seedlings and transferred them to a 0,1% water agar containing 10^6 conidia/ml. The seedlings were incubated in a 24°C growth chamber and were evaluated 14 days later.

Susceptible and resistant lines were distinguished after three days at 15°C by measuring the fungi's diameter on pea epicotyl callus in tissue culture, and after nine days of incubation by measuring the fungal cell membrane compound ergosterol on explants. Plantlets grown in cylinders with a spore-containing agar could be evaluated after six days, and ergosterol extraction gave accurate results after 16-26 days of incubation (Gretenkort & Helsper 1993).

A popular method soaked the seed in a conidial suspension for up to 24h prior to sowing. The method was originally developed by Dr. Simon Menzies, Plant Pathologist, DSIR, New Zealand (Kraft et al. 1994). The seedlings were evaluated for disease symptoms after approximately three weeks (Porter 2010, Ondrej et al. 2008, Grünwald et al. 2003).

In situ

Evaluations of resistance in situ were performed in already infested fields by evaluation of the chlorosis and necrosis on lower plant leaves together with stunting and plant death in heavily infested fields (Coyne et al. 2008, Coyne et al. 2015); or by estimation of the infested root tissue percentage (Tu 1991).

Short & Lacy (1976) sprayed a chlamydospore suspension onto the soil surface and incorporated the inoculum into the 7-10 cm upper soil layer. Neither the natural soil infestation of *F. solani* f. sp. *pisi* nor the establishment of the inoculum was determined, so it is unfortunately difficult to make predictions of the inoculum method's potential.

Method

Methodological investigations

Isolate collection

Seventeen Swedish isolates of *F. solani* were available for the study. A Czech isolate (number 18), known to be highly virulent (Ondrej et al. 2008) was also included.

Single spore cultures were made from isolate 1-17 by spreading spore suspensions on SNA-medium (Nirenberg 1976) and collecting one germinated spore from each isolate onto PDA-medium[7]. The morphology of the cultures was then studied on both PDA- and SNA-medium and identified as *F. solani* according to Nelson et al. (1983) by Lars Persson, Brandsberga Gård AB.

Inoculum production

For each isolate a eight to fourteen days old PDA-plate with single spore culture were put in 500 ml E-flasks with 200 ml Czapex-Dox medium[8] on a rotating shaker (40 rpm), Figure 4. The shaker was exposed to diffuse daylight and continuous lightning from a 400W metalhalide lamp. Due to problems with keeping the intended temperature of 26°C in the greenhouse chamber, the isolates were grown under slightly varying temperature conditions. Isolate 1-9 were grown at 26°C for three days. Isolate 10-17 were grown for three days at 26°C and two days at 24°C and with 16h added light per day. Isolate 18 was grown for three days at 24°C, with 16h added light per day. After the cultivation periods the cultures were sieved (mesh size ≈ 1 mm) and diluted to 10^5 spores/ml. All equipment in contact with the spore solutions were autoclaved between different isolates to exclude any possible cross contamination.

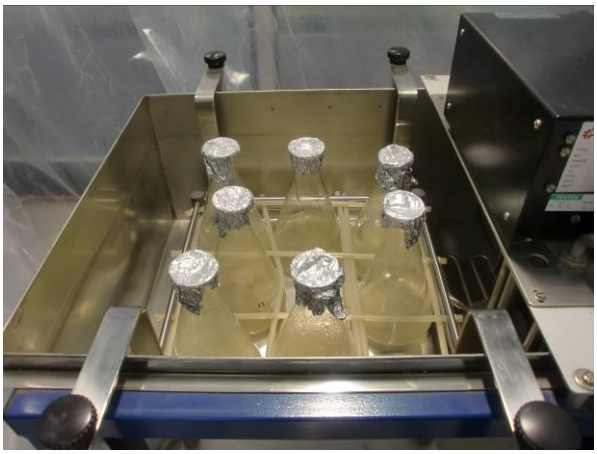


Figure 4. The rotating shaker set up.

Due to bacterial contamination during the making of the single spore cultures isolate no. three and no. thirteen were discarded: 16 isolates of different origin were used for the virulence assessment.

[7] Supplied by Merck.
[8] Supplied by Sigma Aldrich.

Table 2. **Origin of isolates used for inoculation in the virulence assessment**

Isolate	Origin
1	From pea trial field 2013
2	From pea trial field 2013
4	From pea trial field 2013
5	From Findus' greenhouses
6	From Findus' greenhouses
7	From Findus' greenhouses
8	From Findus' greenhouses
9	From Findus' greenhouses
10	From Findus' greenhouses
11	From Findus' greenhouses
12	From Findus' greenhouses
14	From soil test
15	From soil test
16	Archive isolate
17	Archive isolate
18	Czech Republic

Experimental setup

It was hypothesized that the isolates, see Table 2. could differ in virulence on hypocotyl and roots respectively[9]. To investigate this two inoculation methods (I & II) were compared on the cultivar 'Bolero'.

In method I the seeds were soaked in spore solution (10^5 spores/ml) for 24 h before sowing.

In method II the seeds were soaked as in method II, but spore solution was also added to the substrate at 50-100% of plant emergence to imitate a soil inoculum of 6500 spores/cm^3.[10] This corresponded in time to 4-5 days after sowing. The spore solution was obtained from the same spore suspension used for the soaking procedure, had been stored dark in 2°C for 5-6 days. The appearance of the propagules was examined for a few isolates at this point, and showed both non-germinated and germinated spores, which had grown up to a few millimeters.

Figure 5. What the experiment looked like in the green house.

For both methods, seeds from each replicate were inoculated separately, with a volume equivalent to 5 ml of inoculum, or for the control 5 ml Millipore water, per seed. Seeds that had swelled during the inoculation, 10-12 seeds per replicate, were sown in cartonnage pots (w 9cm, l 11cm, h 11cm), with 0,7 l coarse vermiculite (0-5mm). The pots

[9] See section *"Pathogen virulence"*

[10] For treatment II were the spore suspensions calculated to imitate the level of soil infestation used by Kraft & Boge (2001), with 6500 cfu/cm^3 vermiculite volume. (Kraft & Boge used chlamydospores in their study.)

were placed in 1 cm deep upside-down turned lids on top of a water holding cloth on the pot table.

The control for both treatments consisted of seeds soaked in millipore water. Directly sown seeds were included as a second control to assess the impact of water soaking. There were also water soaked seeds sown in a plastic container (w 30cm, l 40cm, h 10cm), in which the plants were used in a related assessment, see "Plate study". The experimental setup is compiled in Table 3.

Table 3. Experimental setup in the virulence assessment.

Activity	Treatment I	Treatment II	Control	Direct sowing
Initial number of seeds	26 seeds/isolate	26 seeds/isolate	120 seeds	120 seeds
No. of replicates/ mean no. of seeds per pot	2/13	2/13	8/15	8/15
Inoculation method	Seed soaking 24h (10^5 spores/ml)	Seed soaking 24h (10^5 spores/ml) + spore solution in pot substrate 4-5 days after sowing ($6,5*10^3$ spores/cm^3)	Seed soaking 24h (millipore water)	None
Growth conditions	24°C, with 20°C as night temperature 20 p.m. – 4 a.m. Metalhalide light, 400W, was added at daytime when the incoming sunlight <20 kilo lux. Water was supplied daily with sprinkler.			

Assessment of seed solute leakage

To assess the amount of leached solutes[11] from seeds belonging to different replicates the electrical conductivity was measured. The readings were performed after a modified ISTA protocol (ISTA 2015) with an electrical conductivity-reader (EC-93, Nieuwkoop B.V.) for all spore solutions. The seeds were weighed separately for each replicate and were then put to soak in the spore suspensions/millipore water. After 24h (+/- 15 min) electrical conductivity readings were taken again. Seeds that had not swelled after 24 h were surface dried and weighed. Their weight was subtracted from the initial sample and the comparable value for the electrical conductivity calculated as below:

$$\frac{Conductivity\ after\ 24h\ \left(\frac{\mu S}{cm}\right) - Conductivity\ of\ spore\ suspension\ \left(\frac{\mu S}{cm}\right)}{Fresh\ weight\ of\ primed\ seeds\ (g)}$$

Plate study

At the end of the experiment, lesions were cut out at the boundary area between healthy and decaying tissue on four arbitrarily chosen plants from each isolate and 28 out of the 120 plants grown in the additional plastic tray. The samples were placed on PDA-medium

[11] See section *"Host susceptibility".*

in room temperature. When visually distinct colonies had established, mycelia were transferred to PDA or SNA to enable further microscopic identification.

Plant evaluation

- Emerged plants (extension of plumule above soil surface) were counted at 7 and 14 days after sowing.
- Wilting plants were recorded 14 days after sowing.
- The vermiculite was washed of the roots 21 days after sowing.
  - Epicotyl length (length of the first node) and the longest coherent lesion running on the primary root/epicotyl was measured for every plant.
  - The individual plants were assigned an index, see Table 4, based on disease symptoms.
  - If sciarid flies had eaten on the diseased tissue the lesion length and index was still estimated, even if the estimate sometimes couldn't deliver the same accuracy as undamaged plants.
  - Fresh weight was measured for every replicate separately. Plants that obviously were about to die (attributed by e.g. wilting and stunting) were excluded from the plant evaluation.

Table 4. The disease index. The term "converted percentage" means a quantitative translation of the damage to a corresponding value within the interval. E.g.: A plant (with or without hypocotyl lesions) with epicotyl lesions around 20% of the perimeter would be assigned an index of 1,1.

Index interval	Description
0	No symptoms
0,05-1	Converted percentage of hypocotyl perimeter made up of lesions
1,05-1,5	Converted percentage of epicotyl perimeter made up of lesions
3	Lesions on secondary roots
4	Infection of secondary roots have reached root tips and/or infections in the lower parts of the root system.

Statistical treatment

The replicates' means of epicotyl length, longest coherent lesion and index were assessed for differences between methods and for interaction between method and isolate, with the GLM procedure in Minitab. The same measured parameters were assessed for differences between isolates with the proc mixed procedure in SAS and Tukey's test ($p<0,05$). The proc mixed procedure estimated the mean value of the observations adjusting for the fact that there were different numbers of observations in the pots.

The replicates' means were analyzed with the Pearson correlation coefficient in Minitab for correlation between the following parameters: epicotyl length, longest coherent lesion, index, plant fresh weight, emergence seven days after sowing, electrical conductivity in the inoculum and increase in conductivity during inoculation.

Resistance in pea accessions

Experiment I

Experimental setup

The inoculation method (I) that included only the seed soaking procedure was chosen for further testing together with isolate 14, originating from soil samples.

A total of 41 different cultivars and breeding lines, including nine cultivars treated with the fungicide Wakil XL[12] were part of the assessment. The nine treated cultivars were not available without seed treatment. Since Wakil XL was not reported to exert any effect on *Fusarium* spp. these accessions were included despite their treatment.

The experimental procedure was slightly adjusted from the virulence assessment. Seeds were allowed to soak in 2 ml 10^6ml spores/ml spore suspension per seed for 24h ± 15 minutes. Seeds that had swelled during inoculation were sown in four cartonnage pots (as previously described) per cultivar, 12-14 seeds/pot, sowing depth about 1 cm. The pots were placed in 2 cm deep upside-down turned lids on top of a water holding cloth on the pot table.

Temperature, light and water supply followed the same regime as in the methodological investigations.

"Radar", a commercially available pyrethrin spray was applied twice during the experiment to control sciarid flies.

Inoculum production

Because of difficulties of obtaining enough spore solution in one batch three batches were produced and inoculated with as many of the cultivars as possible, but separated in time. The environmental conditions therefore have differed slightly between the treatments as well. To partly account for these differences a standard cultivar "Bolero" was included in the experiments for all spore suspension batches.

The three different spore batches were grown on a rotating shaker (40 rpm) with 7-43 days old agar plates in E-flasks with Czapek Dox[13] medium. The shaker was exposed to diffuse daylight and 16 h added light per day from a 400W metalhalide lamp in 24°C. Isolate 18 was grown for three days at 24°C, with 16h added light per day. After the cultivation periods the cultures were sieved (mesh size ≈ 1 mm) and diluted to 10^5 spores/ml.

Plant evaluation

The plant evaluation procedure was the same as for the virulence assessment, except that the index was adjusted, see Table 5. The aim was to get a more descriptive index for higher disease severities.

[12] Wakil XL contains the active ingredients metalaxyl, fludioxinil and cymoxanil (Syngenta n.d) and is used as a seed dressing against downy mildew, *Pythium spp.,* and *Ascochyta spp* in peas (Syngenta 2013).
[13] Supplied by Sigma Aldrich.

Table 5. The disease index. The term ”converted percentage” means a quantitative translation of the damage to a corresponding value within the interval. E.g.: A plant (with or without hypocotyl lesions) with epicotyl lesions around 20% of the perimeter would be assigned an index of 1,1.

Index interval	Description
0	No symptoms
0,05-1	Converted percentage of hypocotyl perimeter made up of lesions
1,05-1,5	Converted percentage of epicotyl perimeter made up of lesions
3	Lesions on secondary roots
3,5	Lesions on side roots and in lower root system.
4,05-5 in classes of 0,05	5-100% total root lesions when there are lesions in the lower root system.

Statistical treatment

The replicates’ means of epicotyl length, longest coherent lesion and index were examined, for each batch respectively, for significant differences between accessions with Tukey’s test ($p<0,05$) in Minitab.

Experiment II

Experimental setup

A second assessment was performed with modifications from the first assessment. This experimental setup excluded the possibility to use analysis of variance on the result, but would make it possible to rank many cultivars with a limited amount of work.

The 41 different accessions used in the first assessment together with three additional breeding lines with high fusarium root rot resistance (Coyne et al. 2008) were immersed in the inoculum suspension for 24 hours ± 15 minutes. Accessions without seed treatment were immersed in 2 ml 10^5 spores/ml suspension[14] per seed, while treated seeds were immersed in 4 ml suspension/seed[15]. Seeds were sown in one vermiculite filled plastic pot (w 12,5; l 12,5 h 10 cm) per cultivar, at a density of 25 seeds/pot[16], see Figure 6.

Figure 6. Experimental setup in experiment II.

[14] The spore concentration was lowered with 10^1 compared to the virulence assessment and first resistance assessment.
[15] The amount of inoculum suspension was doubled to decrease possible phytotoxic effects for the treated accessions, which would dilute the released amount of active chemical substance.
[16] The seeds were spaced approximately 2,5 centimeters apart (c/c).

Temperature, light and water supply followed the same regime as in the methodological investigations and in experiment I.

To control sciarid fly larvae Vectobac (*Bacillus thuringiensis israeliensis* H-14) was applied with 30 ml of a 0,025% suspension[17] 4, 13 and 19 days after sowing. "Radar", a commercially available pyrethrin spray, was applied twice during the experiment against the adult flies, in combination with many yellow sticky traps. The previously upside-down turned lids that the pots had been standing in, were turned upright, and the water holding cloth on the pot table was removed to make the environment dryer and less suitable for the sciarid flies.

Inoculum production

Inoculum was produced under the same environmental conditions as described for the first assessment, but in 800 ml E-flasks containing 300 ml Czapek-Dox solution inoculated with a 27 days old PDA plate. The inoculum from four E-flasks was harvested and put together as one inoculum source after nine days, with an electrical conductivity of 5,2 μScm^{-1} . The concentration was adjusted from $6,4*10^6$ to $1*10^5$ spores/ml (lowered ten times compared to the first assessment) , which resulted in an Ec of 0,04 μScm^{-1} in the final inoculum suspension

Plant evaluation

Evaluation was carried out 21-22 days after sowing. The plants performances were measured with the following variables: epicotyl lesion length, hypocotyl lesion length, index and fresh weight. (Accession 32, 39, 41-44 were not measured for fresh weight). The aim on dividing the measurement on hypo- and epicotyl respectively was to investigate if the location and size of the symptom had impact on resistance.

Marker screening

'Bolero' and 13 other accessions were picked out for the study with DNA-markers.
A positive 'Carman' and negative 'Reward' control was included, as reported in Feng et al. (2011). The microsatellite markers AA160[18] and AD53[19] which explained 27 % and 51 % of the genetic resistance respectively in a study by Feng et al. (2011) were used in the screening.

Approximately 1 cm^2 leaf tissue from seedlings of the selected lines were put in microfuge tubes and immersed in liquid nitrogen. The tissue was grinded with a pestle before added 400µl of Edward's solution[20], and grinded further. The tubes were centrifuged for 1 min at 13000 rpm. The supernatant was transferred to another tube, added 300µl isopropanol and left in room temperature for two minutes. After centrifuging for 5 minutes at 13000

[17] Corresponding to 0,0003 % of active bacteria.
[18] AA160 forward primer sequence (5'-3'): AGATAGACATGAGAATGGTGGC, backward primer sequence (5'-3'): CTGCTCCAACAACACAAGATAAACA.
[19] AD53 forward primer sequence (5'-3'): CACCAGAAGGATGAGGAATAGT, , backward primer sequence (5'-3'): CATTGAGATTCTTGAAGGGAGT.
[20] Edward's solution recipe: 10 ml 1M Tris pH=7,5, 2,5 ml 5M NaCl, 2,5 ml 0,5M EDTA and 2,5 ml 10% SDS dissolved in 325 ml H_2O.

rpm the supernatant was discarded. The remaining pellet was diluted in 100µl millipore water to the template solution.

The extracted amount and quality of the template solution measurements was with Nanodrop ND-1000 Spectrophotometer. To check for degradation of DNA in the template solution2 µl template solution with 2 µl DreamTaq Green Buffer (10x) and 16 µl millipore water were put on a 1,5 % agarose gel with ethidium bromide[21]. The gels were run for 30 minutes with 90V before visualization with UV-light.

For the PCR reaction 2µl template solution was mixed with 40 µl DreamTaq Green Buffer (10x), 40 µl dNTP 2mM, 20 µl forward primer, 20 µl reverse primer, 4 µl DreamTaq Green DNA Polymerase 5U/ µl and 240 µl millipore water[22]. The PCR was initiated with 5 min with 98 °C, run 30 cycles with 20 s 98 °C, 30 s 60 °C and 30 s 72 °C, and ended with 5 min 72 °C.

The PCR product solutions, ladder[23] and a water sample were put on 1,5% agarose gels with added ethidium bromide. The gels were run for 30 min with 90V before visualization with UV-light.

[21] Agaros Standard provided by Saveen Werner AB. Ethidium bromide 0,07% provided by AppliChem.
[22] The primers were provided by Sigma-Aldrich. The buffer, dNTP and polymerase were provided by Thermo Fisher Scientific.
[23] Gene Ruler 1kb DNA Ladder, ready-to use, provided by Thermo Fischer Scientific.

Result

The statistical analysis was performed with the statistical software Minitab ® 16.1.1.0, or where mentioned with SAS® or Excel.

Methodological investigations

Infected plants showed symptoms in form of lesions, wilting of lower foliage, seeds with lesion spots, seeds that in some cases had developed root but not shoot and wilting plants with stunted growth, sometimes girdled with a moist brown foot rot progressing from the seed attachment area, see Figure 7A-D.

Figure 7A-D. Fusarium root rot symptoms. (A) Seed with dark spots and rotting seed. (B) Rotting seed with white mycelia and impaired shoot development. (C) Wilting shoot (D) root- and foot rot lesions.

Sciarid flies ate from the lesions caused by the fungal infection, and from both diseased and healthy fine roots. This might have affected the result of the assessment especially regarding fresh weight, and to a minor extent also the lesion length measurements. The most infected plants seemed to be most severely attacked.

Assessment of seed solute leakage

Seed solute leakage correlated with a number of other measured variables, see Table 6. Seed solute leakage was positively correlated (0,65) with emergence seven days after sowing, and to a lesser extent negatively correlated (0,41) with the initial electrical conductivity in the inoculum.

Effect of inoculation method

Incubated root samples of inoculated plants from all isolates showed outgrowth of *F. solani* on the plates from the diseased root tissue; occasionally, also *Fusarium avenaceum* and *Alternaria* spp were found. Incubated samples from uninoculated plants grown in a plastic tray displayed, with descending frequency, growth of *Alternaria* spp, *F. avenaceum, F. solani* and *Botrytis* spp., see Figure 8A-B, next page.

No significant differences (p>0,0,5) between inoculation method I and II were shown for epicotyl length, longest coherent lesion, index or emergence.

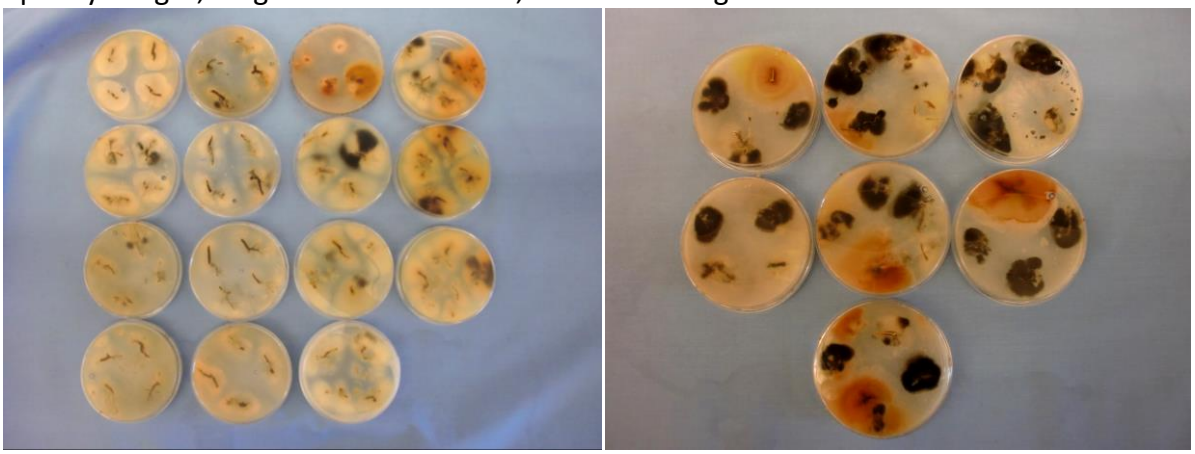


Figure 8A-B. (A) Samples from inoculated plants, one plate per isolate. (B) Samples from uninoculated plants. Cream white colonies belong to *F. solani*, black colonies belong to *Alternaria* spp., and orange colonies belong to *F. avenaceum.*

On the contrary, there was a significant interaction between isolate and treatment on fresh weight (p=0,029), while there was a tendency for treatment alone to effect fresh weight (p=0,060) (Tukey's test). This was valid when data from isolates with high impact on the result (isolates with less than four read pots due to disproportionate large attack from sciarid flies) were included in the data set. When such isolates (2, 4, 7) were excluded, the difference was no longer significant (p=0,079 and p=0,270).

Effect of isolates

Since diverging effects of the methods were weak was it decided to not include the methods as a variable when analyzing the isolate effect. All isolates were significantly separated from the untreated control (19) and the directly sown control (20) regarding index (Proc mix and Tukey's test p>0,05.). The control and directly sown control were consistently least diseased when measuring longest coherent lesion length, index and fresh weight, see Figure 9.

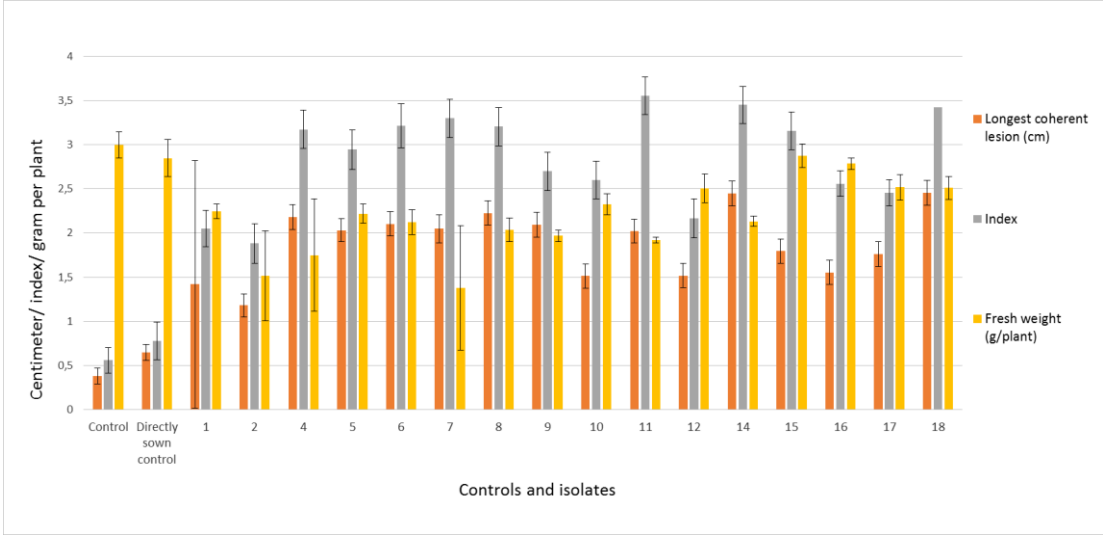


Figure 9. Results from the virulence assessment. Error bars for standard error.

Emergence at seven days after sowing differed significantly between only few of the isolates, and only correlated (positively) with the amount of leaked solutes during the inoculation. The emergence time sometimes differed between replicates within the same accession. The proportion of wilting plants out of the emerged ones at 14 days showed no significant differences, and was not treated further. For more details on correlation, please see next section.

Correlation between measured parameters

The replicates' means for longest coherent lesion length, index and plant fresh weight were all strongly correlated to each other (Pearson correlation coefficient), see Table 6. Epicotyl length had a positive correlation with longest coherent lesion length and index, and a negative correlation with fresh weight. Increase in electrical conductivity during the inoculation was positively correlated with emergence seven days after sowing.

Table 6. Pearson correlation coefficients and p-values for the correlations between measurements in the virulence assessment. Significant correlations are marked in yellow.

Measurement	Value	Epicotyl length	Longest co-herent lesion	Index	Plant freshweight	Emer-gence 7 days after sowing	Electrical conductivity in inoculum
Longest coherent lesion	corr. coeff.	0,493	-	*	*	*	*
	p-value	0,000	-	*	*	*	*
Index	corr. coeff.	0,481	0,929	-	*	*	*
	p-value	0,000	0,000	-	*	*	*
Plant fresh weight	corr. coeff.	-0,659	-0,699	-0,645	-	*	*
	p-value	0,000	0,000	0,000	-	*	*
Emergence 7 days after sowing	corr. coeff.	-0,373	-0,269	-0,170	0,185	-	*
	p-value	0,001	0,016	0,135	0,107	-	*
Electrical conductivity in inoculum	corr. coeff.	-0,194	0,143	0,145	-0,109	0,373	-
	p-value	0,106	0,233	0,227	0,372	0,001	-
Electrical conductivity increase during inoculation	corr. coeff.	0,329	0,182	0,070	-0,173	0,653	-0,415
	p-value	0,009	0,153	0,584	0,182	0,000	0,001

Resistance in pea accessions

Experiment I

The problem with sciarid fly larvae increased in this experiment compared to the virulence assessment. The flies have to be controlled in a better way in future assessments.

Inoculum production

To grow enough spore solution to inoculate all accessions at the same time first seemed impossible due to limitations with the available lab equipment. Initial problems with bacterial contamination were replaced with problems of aerating the big E-flasks. The problem with aeration occurred mainly in E-flasks with larger volumes of inoculum solution, noticed by spores growing slower than expected.

Effect of inoculum batch

Longest coherent lesion length differed significantly (Tukey's test p>0,05) between all of the three controls of 'Bolero' that had been sown with one inoculum batch each. It would be necessary in future studies to use the same inoculum in comparisons, or on routine include a larger set of "standard cultivars" for each inoculum batch.

Due to the significant differences between batches of 'Bolero' it could be clearly concluded that a just comparison between accessions demands the same spore solution.

Effect of pea accession

There were significant differences between accessions regarding longest coherent lesion and index (Tukey's test p<0,05) within spore batches, see **Error! Reference source not found.** Seeds with seed treatment were compared only against each other; this since they as a group had shown suspected phytotoxic effects from the seed treatment (poor germination, poor plant survival, stunted or deformed growth)."Longest coherent lesion length" better revealed differences between cultivars than "Index".

RESULT: EXPERIMENT I

Table 7. Result on longest coherent lesion and index from the experiment I. Each inoculum batch should be compared separately. Significance groupings are provided (Tukey's test p<0,05).

Accession no.	Longest coherent lesion (cm)	Standard deviation	Longest coherent lesion grouping	Longest coh. lesion group compared to 'Bolero'	Index	Standard deviation	Index grouping	Index group compared to 'Bolero'	No of evaluated replicates
Inoculum batch no. 1									
2	0,9	0,6	C	+	1,3	1,2	B	+	4
6	1,6	0,7	B		3,7	0,5	A		4
5	1,6	0,5	B		3,6	0,6	A		3
'Bolero'	1,7	0,7	B		3,6	0,3	A		4
4	2,0	0,6	A	-	3,7	0,4	A		4
3	2,3	0,6	A	-	4,0	0,5	A		3
Inoculum batch no. 2									
25	1,9	0,4	D	+	3,3	0,5	A B		4
22	2,1	0,5	C D		3,2	0,4	A B		2
24	2,2	0,5	C D	+	3,4	0,5	A B		4
10	2,2	0,3	C D	+	3,3	0,4	A B		4
7	2,2	0,3	C D	+	3,0	0,0	B	+	4
8	2,2	0,5	C D	+	3,2	0,3	A B		4
9	2,2	0,5	C D	+	3,0	0,6	B	+	3
12	2,4	0,6	A B C D		3,4	0,6	A B		4
13	2,4	0,5	A B C D		3,3	0,5	A B		4
11	2,5	0,5	A B C D		3,4	0,5	A B		4
23	2,5	0,5	A B C D		3,3	0,4	A B		4
26	2,5	0,6	A B C D		3,5	0,5	A B		4
19	2,6	0,6	A B C D		3,5	0,6	A B		4
18	2,8	0,6	A B C D		3,7	0,5	A B		4
17	2,9	0,7	A B C		3,6	0,6	A B		4
15	3,1	1,0	A B C		3,7	0,7	A B		3
'Bolero'	3,4	0,9	A B		3,8	0,5	A		4
20	3,4	0,9	A B		4,2	0,4	A		4
16	3,5	0,8	A B		3,6	0,5	A B		2
21	3,7	1,0	A		4,1	0,4	A		2
14	3,9	1,0	A		4,3	0,5	A		1
Inoculum batch no. 3, without seed treatment									
28	1,5	0,58	A		2,9	0,5	B	+	4
30	1,6	0,57	A		2,9	0,4	A B		4
27	1,7	0,86	A		3,0	0,4	A B		4
29	1,7	0,64	A		3,0	0,4	A B		4
32	1,8	0,60	A		3,1	0,3	A B		4
'Bolero'	1,9	0,64	A		3,1	0,2	A		4
31	1,9	0,56	A		3,2	0,4	A B		4
Inoculum batch no. 3, with seed treatment									
39	1,4	0,9	C		2,8	0,8	A		4
33	1,7	0,7	B C		3,5	0,6	A		3
37	2,0	0,8	A B C		3,2	0,4	A		4
41	2,1	0,6	A B C		3,0	0,1	A		4
36	2,2	0,7	A B C		3,1	0,3	A		4
35	2,3	0,5	A B C		3,2	0,3	A		4
38	2,6	0,6	A B C		3,3	0,5	A		4
40	2,8	0,9	A B		3,1	0,3	A		4
34	3,3	1,4	A		3,4	0,6	A		3

Experiment II

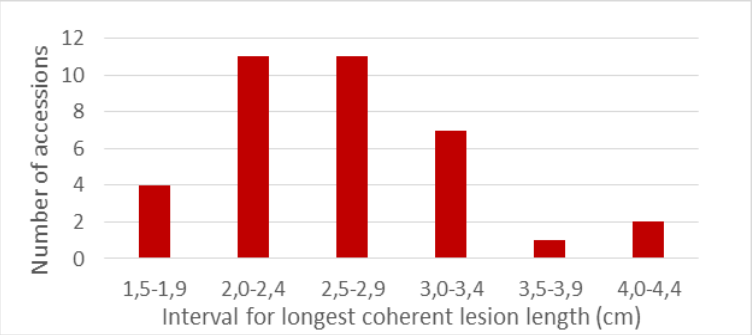
Inoculum production

The inoculum production succeeded well with the learnings of the importance of aeration, by using smaller amounts of volume in each flask and smaller flasks, with absence of bacterial contamination and with a ten times lowered spore concentration need.

Effect of pea accession

The longest coherent lesion length among the accessions without seed treatment were distributed according to **Error! Reference source not found.**

Figure 10. Distribution of coherent lesion length among the 44 accessions in the second resistance assessment.



The standard cultivar 'Bolero' obtained an average lesion length of 3,1 cm, which gave it a lesion length slightly above the median value 2,5 cm. Results in Table 9, please see next page.

Correlation between measured parameters

The correlation between the values on epicotyl lesion length, hypocotyl lesion length and longest coherent lesion length was assessed, see. The strongest correlation between measurements was between hypocotyl lesion length and longest coherent lesion length (0,981; p = 0,000), see Table 8.

Table 8. Pearson correlation coefficients and p-values for the correlations between measurements in the second resistance assessment. Significant correlations are marked in yellow.

Measurement	Value	Epicotyl lesion ength	Hypocotyl lesion length	Longest coherent lesion
Hypocotyl lesion length	corr. coeff.	0,463	*	*
	p-value	0,002	*	*
Longest coherent lesion	corr. coeff.	0,627	0,981	*
	p-value	0,000	0,000	*
Index	corr. coeff.	0,408	0,557	0,580
	p-value	0,007	0,000	0,000

RESULT: EXPERIMENT II

Table 9. Result from experiment II ordered after longest coherent lesion. Accessions with seed treatment should be compared separately (columns to the left).

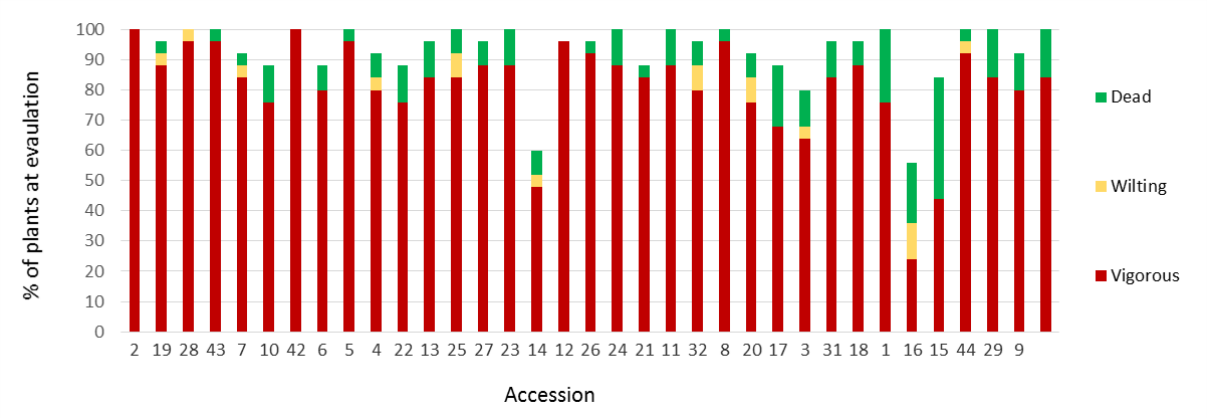
Accessions no., without seed treatment	Longest coherent lesion (cm)	Accession no., with seed treatment	Longest coherent lesion (cm)
2	1,5	39	2,6
19	1,8	37	2,8
28	1,8	34	3,4
PI652445	1,9	36	3,6
7	2,0	33	4,2
10	2,0	41	4,4
PI652444	2,0	40	5,4
6	2,0	38	6,1
5	2,1	35	7,3
4	2,1		
22	2,2		
13	2,3		
25	2,4		
27	2,4		
23	2,4		
14	2,5		
12	2,5		
26	2,5		
24	2,6		
21	2,6		
11	2,7		
32	2,7		
8	2,7		
20	2,8		
17	2,9		
3	2,9		
31	3,0		
18	3,0		
’Bolero’	3,1		
16	3,1		
15	3,1		
PI652446	3,2		
29	3,3		
9	3,8		
30	4,3		

RESULT: EXPERIMENT II

The accessions' mean coefficient of variation for the hypocotyl measurement was 31%, and for longest coherent lesion length 26%. Longest coherent lesion length would be preferred in future measurements thanks to the significantly lower coefficient of variation $p=2*10^{-5}$ (paired t-test, assumed equal variances, Excel).

The plant status results, with the final reading at plant evaluation, are accounted for in Figure 11. The distribution of non-germinated, dead, wilting and vigorous plants provided no distinct pattern of affected plants in relation to longest coherent lesion length.

Figure 11. Plant status of accessions without seed treatment 21 days after sowing, with the accessions ordered after lowest lesion length from origo. The height of the staple shows the total number of emerged plants.



Comparison between experiment I & II

Spearman's rank correlation coefficient for longest coherent lesion length between the accessions in the first and second assessment was for spore batch one -0,60 (p=0,208), for spore batch two -0,270 (p=0,236), for untreated in batch three -0,371 (p=0,468) and for treated in batch three 0,133 (p=0,732). Accession no. 2 was the best performing accession in both tests.

Marker screening

The marker screening revealed that the markers AA160 and AD53 were both present and absent in the studied lines, in different combinations, see Table 10. The marker distribution did not explain these accessions' performance in experiment 1 or 2.

Table 10. Results from the marker screening.

Code	AA160	AD53	Longest coherent lesion length (cm)
2	+	-	1,5
19	-	+	1,8
28	-	+	1,8
43	+	-	1,9
7	+	-	2,0
5	-	+	2,1
37	-	+	2,8
1	+	-	3,1
9	-	+	3,8
30	-	+	4,3
41	+	+	4,4
40	+	+	5,4
38	-	+	6,1
35	+	+	7,3
Carman (pos. control)	+	+	*
Reward (neg. Control)	-	-	*

Discussion

Conclusions

There were significant differences in fusarium root rot resistance between accessions. It would be possible to breed for higher levels of resistance within the material.

Measurement of longest coherent lesion length gave higher accuracy, more significant differences, and left less room for the evaluators judgement than using an index system. Since lesion length increase correlated well with fresh weight decrease, which confirmed its' validity as a resistance measurement, would this measurement be recommended for use in future studies.

The resistance could not be explained by the available markers, which highlighted the need for marker development.

Validity of the study in relation to other work

The accessions PI65244, PI652445 and PI65246 were reported to have high resistance against fusarium root rot (Coyne et al. 2008). The two first mentioned accessions clearly showed the expected high resistance in comparison with all the other tested accessions in this study. On the contrary, PI652446 was shown to be relatively susceptible in this study. Coyne et al. (2008) used disease severity ratings of plant foliage in a field infected with fusarium root rot. Different environmental conditions, different virulence of the fungi, and the different evaluation methods might have contributed to this discrepancy. It is well established that aggressiveness of a pathogen depends on environmental conditions (Andrivon 1993).

Epicotyl length was earlier reported to decrease with increased virulence of different isolates (Ondrej et al. 2008). The pattern in this study was the opposite. Epicotyl length would however be impractical for comparing cultivars; since epicotyl length is likely to depend on the cultivar's height rather than resistance.

Sources or error

The different disease severities in different inoculum batches, experiment I, might have been caused by several factors such as: differing spore viability, differing nutrient availability and differing availability of bioactive organic compounds.

Unattended sciarid fly larvae could have destroyed the results. The flies need to be managed carefully in future studies. The fly larvae fed on the lesions and surrounding healthy tissue, which might have contributed to slight but limited overestimations of the lesion length. The treatment interval with active *Bacillus* strains against the larvae can be shortened to have better effect, and should preferably be combined with scheduled and repeated insecticide sprays directed toward the pot substrate to target the adult flies. Even high numbers of sticky traps are inefficient for control, since the adult flies spend most of their time on the pot substrate.

Differing environmental conditions during the experimental period might have influenced the outcome, due to restrains in the precision of the greenhouse chamber's climate control.

Different physiological status of the seed accessions could be a source of error. Since the accessions used in Coyne et al. (2008) were grown, harvested and hand threshed is the

physiological status probably not contributing to the level of resistance observed in these three accessions.

Suggestions for improvement of the method

The low repeatability of the results may be improved with standardisation of the methodology:

The inoculum production should be standardised in terms of inoculum age, growth conditions, size and time for cultivation. The spores should be separated from the nutrient media and re-suspended in a standardized media to avoid effects of the nutrient solution. The E-flasks used during inoculum production should have cotton stoppers, instead of aluminium foil lids to provide better aeration with less bacterial contamination.

Ondrej et al. (2008) used a similar inoculum production technique as in this paper, and also a method were petri dish cultures were mixed with water to a slurry. Ondrej et al.did unfortunately not report on the repeatability of the results. Grünwald et al. (2003) seems to have used the original nutrient solution in the inoculum, but reported of high repeatability. Porter (2010) produced inoculum by a similar method as Grünwald et al., but centrifuged a sieved spore suspension, and re-suspended the pellet (in some undefined media) before dilution to final concentration.

The number of standard cultivars could be increased to make interpretations for both limited assessments and large scale assessments easier. In large scale assessments, were it is necessary to use more than one inoculum batch could the statistical treatment take advantage of the standard cultivars to predict the compared resistance for accessions in several batches, similarly to the work in Grünwald et al. (2003). By using a set of standard accessions with varying resistance, each accession's resistance can be quantified in relation to the standard accessions in future studies.

The spore concentration is recommended to be held at 10^5 spores/ml, or possibly lower, with this isolate. The concentration recommendation is in accordance with the recommendation in Ondrej et al. (2008). A lower the spore concentration will also ease with the possible number of tried accessions for each inoculum batch. A shorter time between inoculation and evaluation could give better resistance estimations, since the spread of infection decline both in the epicotyl and hypocotyl when reaching less susceptible tissue types, as shown by Stahl et al. (1994).

It is recommended to relate the sowing depth to the seed size by just cover the seeds with substrate when sowing.

Explanation of found tendencies

The plate study in the methodological investigations indicated that the inoculation was successful and that the evaluated lesions likely had been caused by *F. solani* f. sp *pisi.* Fungi isolated from the uninoculated plants might come from inoculum present on the seed, or inoculum brought to the plants by air.

Method II, seed soaking and addition of inoculum to the pot substrate, had a tendency to higher fresh weight than method I. An explanation model would be that addition of spore solution to the pot provided plant nutrients that promoted plant freshweight increase.

DISCUSSION

Plant status measurements (vigorous, wilting, dead) added no information of greater value to evaluate resistance against fusarium root rot. To count the proportion of surviving plants in the end of the experiment would probably be enough in future studies.

Markers and future

The molecular resistance markers did not correlate with level of resistance in this study. Coyne et al. (2015) recently developed new molecular markers for fusarium root rot resistance. It would be interesting to see if these markers correlate with the results from this study. Several of the QTLs found by Coyne et al. (2008) coincide with QTLs associated with resistance to aphanomyces root rot, which is knowledge that could be interesting when breeding peas for Swedish conditions. Integration of marker-assisted selection into existing breeding programmes could aid to obtain new cultivars with increased disease resistance against both aphanomyces and fusarium root rot.

If the markers developed by Coyne et al. wouldn't be sufficient in predicting resistance, the use of a fine tuned phenotyping measurement as lesion length would aid in the marker development. Accurate phenotyping will continue to be an important tool for marker development.

Ultimately, comparisons of resistance between in vitro and in situ studies would be required to elucidate the validity of in vitro resistance markers as predictors of field resistance. The outcome can help adjust in the methodology of marker-assisted selection.

Main conclusions

- There were significant differences in fusarium root rot resistance between accessions.
- Measurement of longest coherent lesion length gave higher accuracy, more significant differences, and leaved less room for the evaluators judgement than using an index system. It also had significantly higher robustness Lesion length increase correlated well with fresh weight decrease, which confirmed its' validity as a resistance measurement. It is recommended to use this measurement in future studies.
- The resistance could not be explained by the used markers, which highlighted the need for marker development.

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Appendix

Appendix 1. Differences between isolates in the virulence test with the proc mixed procedure. Untreated control is described as isolate 19, and the directly sown control as isolate 20.

Epicotyl length (mm)				Longest coherent lesion (mm)				Index			
Isolate	Estimate	Std Error	Grouping	Isolate	Estimate	Std Error	Grouping	Isolate	Estimate	Std Error	Grouping
8	1,44	0,069	A	18	2,49	0,141	A	11	3,56	0,21	A
1	1,36	0,066	AB	14	2,48	0,141	A	18	3,46	0,22	A
4	1,35	0,070	AB	8	2,23	0,139	AB	14	3,45	0,22	A
7	1,31	0,079	ABC	4	2,20	0,142	ABC	7	3,30	0,25	AB
9	1,30	0,069	ABC	6	2,13	0,137	ABC	6	3,23	0,22	AB
5	1,29	0,066	ABC	9	2,09	0,140	ABCD	4	3,21	0,22	AB
6	1,29	0,068	ABC	5	2,05	0,132	ABCD	8	3,21	0,22	AB
2	1,22	0,065	ABCD	7	2,04	0,159	ABCD	15	3,17	0,22	AB
14	1,21	0,070	ABCD	11	2,04	0,135	ABCD	5	2,94	0,21	ABC
10	1,14	0,068	ABCDE	15	1,78	0,140	ABCDE	9	2,71	0,22	ABC
12	1,08	0,068	BCDE	17	1,75	0,139	BCDE	10	2,59	0,22	ABC
15	1,03	0,069	BCDE	16	1,57	0,136	BCDE	16	2,57	0,21	ABC
11	1,03	0,067	BCDE	10	1,52	0,138	BCDE	17	2,46	0,22	ABC
16	1,00	0,067	CDE	12	1,51	0,137	CDE	12	2,15	0,22	BC
17	0,98	0,069	CDE	1	1,40	0,133	DE	1	2,01	0,21	C
18	0,93	0,070	DE	2	1,19	0,130	EF	2	1,90	0,21	C
20	0,87	0,045	E	20	0,65	0,090	FG	20	0,78	0,14	D
19	0,85	0,046	E	19	0,38	0,092	G	19	0,55	0,15	D