Root Rot of Pea Caused by *Aphanomyces euteiches*

Calcium-dependent Soil Suppressiveness, Molecular Detection and Population Structure.

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Root rot of pea caused by *Aphanomyces euteiches*. Calcium-dependent soil suppressiveness, molecular detection and population structure.

Abstract

Aphanomyces root rot, caused by the oomycete *Aphanomyces euteiches*, is the most important and destructive disease of pea. Since the inoculum is persistent in the soil, and no resistant cultivars or means of chemical control are available, the disease is difficult to manage. The occurrence of soil suppressiveness against the disease was previously reported, and the main aim of this study was to understand the mechanism.

By analysing chemical data for naturally infested soil samples from a large number of fields, we found that disease suppression was correlated to the Ca content of the soil. This was also true for artificially inoculated soil samples. Suppressiveness was only inconsistently affected by soil sterilisation, and was concluded to be abiotic in nature. Amendments of non-suppressive soils with both neutral and alkaline Ca salts strongly reduced disease severity. In contrast, amendment of suppressive soils with $NaHCO_3$ solutions lowered the content of soluble Ca, increased pH and made the soil less suppressive. It was concluded that high concentrations of Ca in the soil solution was a major factor causing suppressiveness.

The effect of Ca in the field was evaluated by adding a neutral Ca salt, $CaSO_4$ in a naturally infested field trial. A clear delay in the onset of disease symptoms was seen at all doses evaluated.

A quantitative molecular detection method based on quantitative PCR was developed and compared with the greenhouse biotest traditionally used to detect the pathogen in soil samples. Using the qPCR assay, it was possible to detect and quantify the pathogen at concentrations under 10 oospores g soil^{-1}.

The Swedish population of *A. euteiches* was analysed using co-dominant DNA sequence markers. It was found that the population was dominated by a globally distributed genotype, but that distinctly different genotypes also exist. These results were interpreted as evidence of a predominantly clonal mode of reproduction, but also of occasional sexual recombination.

Keywords: *Aphanomyces euteiches*, oomycetes, pea, soil suppressiveness, calcium, detection, population

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Till Dag och Nils, och minnet av morfar Torgny.

Contents

List of Publications **7**

1 Introduction **9**

2 Background **11**
2.1 Oomycetes as plant pathogens. 11
2.1.1 General 11
2.1.2 The genus *Aphanomyces* 13
2.1.3 *Aphanomyces euteiches* and the root rot disease of pea 14
2.1.4 Population biology studies of oomycetes 15
2.2 "Soil suppressiveness" phenomena. 15
2.2.1 Suppression of oomycete pathogens in the soil environment 17
2.3 Molecular detection in disease management 19

3 Present investigation **21**
3.1 Aims and outlines 21
3.2 Preliminary studies and approaches, “evolution of hypotheses” 22
3.3 Materials and methods 24
3.3.1 Examining the mechanism for soil suppressiveness (paper I) 24
3.3.2 Application of calcium sulphate (gypsum) to suppress *A. euteiches* in the field (paper II) 25
3.3.3 Development and evaluation of a quantitative molecular detection method (paper III) 26
3.3.4 Genetic analysis of the Swedish population of *A. euteiches* (paper IV) 26
3.4 Results 27
3.4.1 Mechanisms behind soil suppressiveness (paper I) 27
3.4.2 Practical use of calcium amendments to suppress *A. euteiches* in the field (paper II) 28
3.4.3 Evaluation of a molecular detection method (paper III) 28
3.4.4 Population study using co-dominant markers (paper IV) 29

4 Conclusions **31**

5 References **33**

6 Acknowledgements **37**

List of Publications

This thesis is based on the work contained in the following papers, referred to by Roman numerals in the text:

I Heyman F, Lindahl, B, Persson L, Wikström M, Stenlid J. (2007). Calcium concentrations of soil affect suppressiveness against Aphanomyces root rot of pea. *Soil Biology and Biochemistry* vol (39), 2222-2229.

II Heyman F, Wikström M, Persson L, Lindahl B, Stenlid J. Effect of calcium sulphate on Aphanomyces root rot of pea in field experiments (manuscript submitted for publication).

III Heyman F, Almquist C, Jonsson A, Lindahl B, Stenlid J. Evaluation of a quantitative PCR method for detection and quantification of *Aphanomyces euteiches* in soil samples (manuscript).

IV Heyman F, Lindahl B, Wikström M, Stenlid J. Analysis of the Swedish population of *Aphanomyces euteiches* using co-dominant markers (manuscript).

1 Introduction

This thesis consists of four studies related to the biology and management of the soil inhabiting pea root pathogen *Aphanomyces euteiches* Drechsler. Aphanomyces root rot is considered the most serious disease of pea, due to its persistence in soil and ability to rapidly destroy the crop. *A. euteiches* belongs to the oomycetes, a group which superficially resembles fungi, but are more closely related to photosynthetic brown algae. The group contains many destructive plant pathogens. To date, effective control measures against Aphanomyces root rot are lacking, since no pea varieties with strong resistance are available, and seed dressing fungicides normally used to control oomycetes are ineffective.

In the first study, the goal was to understand the mechanism behind soil suppressiveness against the disease. Suppressive soils are soils where a soilborne plant disease fails to develop despite the presence of the pathogen, a susceptible host and environmental conditions favourable for disease. We were able to show that a high amount of soluble calcium in the soil was an important cause of soil suppressiveness against Aphanomyces root rot. In the second project we studied the practical application of this important suppression mechanism, by adding a neutral calcium salt to naturally infested fields. The third study consists of the development and preliminary evaluation of a DNA-based method for quantification of the pathogen in soil samples. Finally, in the fourth study, we investigated the genetic structure of the population of *A. euteiches* in Sweden, using co-dominant DNA sequence markers.

2 Background

2.1 Oomycetes as plant pathogens.

2.1.1 General

Oomycetes contain some of the most destructive pathogens of wild and domesticated plants. Modern study of plant pathology was born out of the potato blight epidemic affecting Europe in the 1840´s, causing the Irish potato famine (Agrios, 1988). A second historic oomycete epidemic of important economic magnitude was caused by the 1878 introduction into Europe of wild grape rootstocks from North America. While successful in saving European viticulture from the devastating root disease caused by the aphid Phylloxera (which was also the result of an earlier transantlantic import), the introduction meant that a serious leaf disease was introduced, the downy mildew disease of grapevine caused by *Plasmopara viticola* (Gobbin *et al.*, 2006). Current important oomycete epidemics include the "Sudden Oak death", which causes great damage in ecosystems of the North American Pacific region, and is caused by a pathogen, *Phytophthora ramorum*, that is thought to have been introduced to the region via long-distance imports of ornamental plant material (Ivors *et al.*, 2004). Extensive damage on wild and cultured woody plants worldwide is also caused by *Phytophthora cinnamomi* which is reported to have a host range in excess of 3000 species (Hardham, 2005).

Oomycetes resemble fungi in morphology (mycelial growth) and in the pathogenic lifestyle of many species. However, it has long been known that they differ from fungi in many physiological traits. For example, their cell wall is composed mainly of cellulose as opposed to chitin in fungi. Modern DNA based phylogenetic research places the oomycetes far from true fungi

in the kingdom *Stramenopila,* which also includes brown algae and diatoms. The original ancestor of the *Stramenopila* clade is believed to have been a photosynthetic organism. The photosynthetic ability was later lost by some groups, such as the oomycetes (Baldauf *et al*., 2000).

As a group, oomycetes are mainly associated with aquatic habitats, where both saprotrophic and parasitic lifestyles exist. The vegetative stages of their lifecycle is diploid, and both heterothallic (outcrossing) and homothallic (self fertilising) modes of reproduction exist within the group. The female sexual organ is called an oogonium, which when fertilised by the male organ (antheridium) produces a unicellular oospore. Oospores are typically desiccation resistant and capable of long-term survival.

A key feature of oomycetes is the asexual spore, called the zoospore, which consists of a wall-less cell equipped with two flagella, making the spore mobile in free water. Zoospores have been shown to react to a diversity of chemical and even electrical signals in the environment (Judelson & Blanco, 2005), and in pathogenic species, this is used to locate favourable infection sites on the plant surface and accurately "dock" to the host, positioning the ventral surface of the spore against the host surface. In addition, they possess an ability to rapidly (within seconds) shed their flagella and encyst, using preformed substances stored in vesicles to form a spherical walled cyst which is more protected than the motile spore. The cyst is then capable of either germinating to form a mycelium, or releasing a new secondary zoospore (Hardham & Hyde, 1997).

Besides oospores and zoospores, some oomycete groups have developed additional reproductive organs. Sporangia are capable of germinating on their own, as well as forming and releasing zoospores. These sporangia are easily released from the mycelium and in some species function as propagules for spread of the organism by wind. As a further adaptation to life above ground, the sporangia in the genus *Peronospora* (causing downy mildew diseases) have in some species lost the ability to produce and release zoospores (Cooke *et al*., 2000). Another structure present in some oomycetes is the chlamydospore, which is an asexual resting spore with a thickened cell wall.

Despite their economic importance, oomycetes were for long neglected (Judelson & Blanco, 2005) as models for understanding pathogenic interactions in detail, and the molecular basis for their powerful, often broad-host pathogenicity was not known. In contrast to true fungi, they are diploid organisms in their vegetative state, and are thus less attractive as models for genetic studies. In recent years the molecular biology research on oomycetes has increased, with large scale genome sequencing projects being

undertaken for several species of *Phytophthora*. Examples of major results that have emerged from these studies include: 1) that *Phytophthora* spp. possess a wide range of effectors proteins that are injected into the host cell and whose main function is believed to be interference with different plant defence processes (Kamoun, 2006). These effector proteins have similarities with proteins secreted by the malaria parasite *Plasmodium falciparum,* showing that mechanisms for pathogenicity directed at plant and animal cells can be similar (Birch *et al*., 2005). 2) Despite the great phylogenetic distance between fungi and oomycetes, parts of the molecular machinery for plant infection shows unexpected similarity at the protein sequence level (Latijnhouwers *et al*., 2003) between the groups, suggesting a complex evolutionary origin. Evidence of horizontal gene transfer between fungi and oomycetes has also been demonstrated (Richards *et al*., 2006).

2.1.2 The genus *Aphanomyces*

The genus *Aphanomyces* belongs to an order of oomycetes (*Saprolegniales*) that is phylogenetically separated from other orders such as the *Peronosporales* and *Pythiales* where other important plant pathogens are found (Petersen & Rosendahl, 2000). From a pathology point of view, the genus is interesting in that it in addition to plant pathogens contains specialised pathogens of arthropods (e.g. *A. astaci*, causative agent of the crayfish plague), and vertebrates (*A. invadans*, an important fish parasite causing ulcerative syndrome) as well as saprotrophic species. In the future, comparative studies between *Aphanomyces* species with different host ranges might shed further light on key genes and traits responsible for the ability to infect radically different host taxa.

Morphologically, *Aphanomyces* species lack several of the specialised organs mentioned above. The structures called sporangia are basically just branched sections of the mycelium without capacity for aerial dissemination or independent germination, and chlamydospores are absent. Zoospores are differentiated within the mycelium. As the plant pathogenic species in the genus mainly attack and develop in roots, they are considered to be strict soil-borne pathogens. As with many oomycetes, zoospore production under controlled conditions is stimulated by flooding of the mycelial culture, a phenomenon which has been interpreted as an adaptation to the need to react and exploit temporary flooded conditions in soils, which are favourable for zoospore function, and thus infection of the host (Papavizas & Ayers, 1974).

2.1.3 *Aphanomyces euteiches* and the root rot disease of pea

A. euteiches was first described by Drechsler in 1925 (Jones & Drechsler, 1925). During that time, the pathogen caused great damage in the pea canning industry in the American Midwest, where pea was often grown in monoculture on company-owned land. Within a few years of the first report, the disease was reported from many areas around the world, indicating that it was already globally distributed at the time it was first described (Papavizas & Ayers, 1974). Almost a century later, Aphanomyces root rot is still considered the most important and destructive disease of pea.

A. euteiches grows readily on culture media, but in nature, saprophytic growth outside the host is not considered important (Papavizas & Ayers, 1974). It survives as oospores in the soil, and the entire lifecycle is completed in the host roots and surrounding soil. The oospores are 20-35 μm in diameter, have a thick protective wall and contain energy reserves in the form of a large oil globule. Upon germination, the oospores either form a mycelium, or a short mycelial strand called a germ sporangium. From a single oospore, hundreds of zoospores can be released through the germ sporangium. The motile zoospores locate and encyst on a host root within minutes, and the cysts are able to germinate and penetrate cortical cells within hours (Papavizas & Ayers, 1974). The mycelium then grows mostly longitudinally and intracellularly through the root tissue and, within a few days of infection, forms large amounts of oogonia, which are fertilised by antheridia and develop new oospores. Live mycelium in infected roots is also capable of releasing new zoospores.

The symptoms of the plant appear as water soaked lesions, which develop into a straw coloured soft-rot of the cortical cells affecting the entire root system if the infection is severe. Symptoms also appear on the epicotyl, giving the lower stem a shrunken appearance. The destruction of the root system leads to stunted growth, wilting symptoms and chlorosis of the lower leaves (Hagedorn, 1984).

A. euteiches is capable of causing disease in a range of different legumes (e.g. Faba bean, vetch, lentil, red clover, Phaseolus bean, alfalfa) and can also complete its life cycle in some plant species from other families; but pea is the crop where the pathogen causes the greatest economic damage (Papavizas & Ayers, 1974). In North America, genetically distinct pathotypes causing significant disease on other hosts such as Phaseolus bean and alfalfa have been described (Pfender & Hagedorn, 1982; Malvick & Percich 1998). Such host specialisation has not been conclusively reported from Europe, although Levenfors observed isolates preferentially infecting vetch (Levenfors *et al.*, 2003).

Pea varieties showing partial resistance have been described, but the resistance does not seem to be equally effective against all strains of the pathogen, and no fully resistant variety is able for use in commercial pea culture. In contrast, resistance breeding in alfalfa has been more successful (Malvick & Grau, 2001) and is being employed in the U.S. The molecular basis for host resistance is now being more closely examined by QTL mapping of pea (Pilet-Nayel *et al.*, 2002) and work with the model legume *Medicago truncatula* (Gaulin *et al.* 2007).

2.1.4 Population biology studies of oomycetes

The ability of crop plant pathogens to overcome resistance in plants, or gain resistance to agrochemicals, is dependent on the occurrence of genetic variation in the population. This variation is strongly influenced by the biology of the pathogen, such as the ability to spread geographically, the generation time and relative importance of sexual vs. asexual reproduction under field conditions, and the ability to self-fertilise. For many plant pathogens, the population biology is also influenced by anthropogenic factors, such as the breaking of geographical barriers by man-assisted transport, or adaptation to domesticated plants and their traits, as opposed to the original host in a natural ecosystem.

Population studies using molecular genetic markers can resolve important questions relating to these issues, and the increase in availability and ease of use of such markers, together with a range of statistical methods being developed and refined to analyse the results, has lead to greatly increased knowledge about the populations of plant pathogens. Examples of topics investigated in population studies of oomycete pathogens include the determining of the geographic origin of *Phytophthora infestans* (Gomez-Alpizar *et al.*, 2007), the occurrence of sexual mating in Swedish *P. infestans* populations (Widmark *et al.*, 2007), and the high clonality of the global population of *P. cinnamomi* (Dobrowolski *et al.*, 2003). Interspecific hybridisation events in oomycetes have been proven to occur (Ioos *et al.* 2006; Nechwatal *et al.*, 2007), and can be the source of new aggressive pathogenic phenotypes.

2.2 "Soil suppressiveness" phenomena.

In cases where disease resistant varieties of crop plants are not available, soilborne plant diseases with persistent inoculum, such as *A. euteiches*, are in practice very difficult to control. The soil, as such, is difficult to manipulate

and can only be reached by biocidal treatment at a great environmental and monetary cost. Likewise, despite the advent of systemic fungicides suitable for seed-dressing, the effect against soilborne diseases is seldom complete, and decreases with time after planting. As a consequence, the concept of naturally occurring soil suppressiveness has for a long time attracted interest by plant pathologists. Suppressive soils have been defined as "soils in which the pathogen does not establish or persist, establishes but causes little or no damage, or establishes and causes disease for a while but thereafter the disease is less important, although the pathogen may persist in the soil" (Baker & Cook, 1974).

The goal of research on soil suppressiveness is not necessarily just to identify suitable fields where the crop can be grown without disease problems, but also to identify the factors behind the suppressiveness and use this knowledge for disease management in all fields. An attractive feature of the study of soil suppressiveness is that the methodology of first identifying consistently suppressive soils and later elucidating the mechanisms, if successful, guarantees identification of mechanisms that are robust enough to have an effect in the complex soil environment. As an example of a contrasting methodology, one can screen potential biological control agents (or novel agrochemicals) for *in vitro* antagonism against the pathogen. However, this will frequently lead to disappointments when trying the candidate antagonist in the field, or even when trying it under any non-sterile conditions such as in greenhouse experiments with soil.

In several classic cases, sterilisation of suppressive soils reduced or abolished suppressiveness, and, together with other experiments, showed that suppression was of biological origin (Weller *et al.*, 2002). These observations, along with the discovery of the strong antifungal and plant growth-promoting activity of many fungi and bacteria found in the rhizosphere, has lead to a strong research interest and understanding of soil suppressiveness and rhizosphere biology that goes beyond practical agronomy aspects.

A theme common to several cases of biological suppressiveness is that certain cultural practices, such as monocultures of the same crop species over several growing seasons, lead to the accumulation of a microflora (in most cases bacterial) that is antagonistic to the pathogen. Take-all is a root disease of cereals caused by *Gaumannomyces graminis* var. *tritici*. The decline of the take-all disease, after repeated monoculture of the host following a disease outbreak, is a phenomenon that has been observed on different soils around the globe and is frequently associated with the accumulation of a specific

group of bacteria (flourescent pseudomonads) that produce metabolites that are toxic to the pathogen (Weller *et al.*, 2002). Similar effects of monoculture leading to the development of an antagonistic, disease suppressing microflora have been described for plant diseases caused by actinobacteria, fungi and nematodes. Strategies of actively using these accumulation phenomena in disease management are being investigated (Mazzola, 2007). A disease for which the mechanism behind soil suppressiveness seems to be more complex is the Fusarium wilt. Soils suppressive to this disease, which affects many host species, have long been known to exist in banana plantations of Central America as well as in melon growing districts in France. The suppression is believed to be caused by a combination of bacteria, non-pathogenic *Fusarium* strains and soil chemical properties, and the biota involved act through a range of different mechanisms, including the triggering of systemic induced resistance reactions in the host (Mazzola, 2002).

Despite the clear influence of biological factors in many cases of suppressiveness, physical and chemical soil factors are also important. In a review of the literature on this topic, Höper and Alabouvette saw few general patterns but rather many specific examples for different systems. Ca concentrations, pH, clay mineralogy and micronutrient availability were among the important factors mentioned, which can act directly on the pathogen or indirectly, by supporting a certain microflora or affecting the ability of the plant to withstand disease (Höper & Alabouvette, 1996).

2.2.1 Suppression of oomycete pathogens in the soil environment

Biological suppression of soil diseases caused by oomycetes has been demonstrated or implied in many studies. Many of the examples involve systems where large amounts of organic materials have been added, such as in the composition of horticultural potting mixtures, mulching of tree crops or incorporation of green manure. A variety of biological processes leading to disease suppression have been shown in these cases, such as antibiosis, enzymatic activity and competition from the community of microorganisms in the amended soil (Downer *et al.*, 2001; McKellar & Nelson 2003; Wiggins & Kinkel, 2005). Suppressiveness against diseases caused by *Pythium spp.* coupled with increased disease severity after biocidal treatments has also been shown for other, less manipulated soils (Knudsen *et al.*, 2002; Adioboa *et al.*, 2007).

Chemical suppression, involving calcium, has been reported in several cases of suppression against oomycetes in both soil and soil-less systems

(Lewis 1973; Broadbent & Baker, 1974; Kao & Ko 1986; Engelhard, 1989; vonBroembsen & Deacon, 1997; Messenger *et al.*, 2000).

For Aphanomyces root rot specifically, seed inoculations with bacteria (Parke *et al.*, 1991; Wakelin *et al.*, 2002) and crop amendments with paper mill residues (Cespedes Leon *et al.*, 2006) are examples of cases where biological disease suppression has been shown to occur. Of interest is also the work done by Rosendahl (1985) describing interactions between arbuscular mycorrhiza and Aphanomyces root rot, where it was shown that pea roots pre-colonised by AM were less severely affected by rot. These studies have recently been complemented by proteomic work on the reaction of the host, using *Medicago truncatula* as a model (Colditz *et al.*, 2005). It is clear that AM colonisation can affect the induced resistance status of the host, and that this is a mechanism for protection against Aphanomyces root rot. Some aspects of this phenomenon have also been demonstrated in the field (Bödker *et al.*, 2002).

Before the present study, two earlier studies (Oyarzun *et al.*, 1998; Persson, 1998) have been published that approach the topic of suppressiveness against Aphanomyces root rot by studying disease responses in a large number of samples from different fields and analysing correlations between soil properties and disease suppression. Oyarzun *et al.* focused on pea root rot as a disease complex, which in addition to *A. euteiches* includes other pathogens, such as *Thielaviopsis basicola* and *Fusarium solani* f.sp. *pisi*. They studied the disease severity on pea caused by these pathogens in bioassays, using a collection of Dutch field soils in carefully controlled microcosm systems. Artificial inoculation of soils with *A. euteiches* zoospores at doses between 50 and 5000 g soil^{-1} was included in one of the studies. In-depth multivariate analysis of a large number of chemical and biological parameters did not uncover any chemical factors related to suppressiveness against *A. euteiches*, but suppressiveness was positively correlated to the presence of *Gliocladium* and *Acremonium* in the rhizosphere, as well as to certain cropping practices. The experiment as a whole revealed little variation in suppressiveness against this disease, with strong disease severity observed in all doses and samples. Soil sterilisation reduced suppressiveness to all pathogens in all soils tested.

In contrast, the work of Persson *et al.* (1999) revealed a large and reproducible variation in disease response among soils from southern Sweden, when inoculating soils with dried oospores in greenhouse experiments. Fields scoring high suppressiveness in pot experiments showed slower disease build-up compared to fields classified as conducive, when cultivated with prolonged monocultures of pea. Suppressiveness was also

shown to be correlated to a range of abiotic characteristics of the soils, such as high pH, high Ca levels and high vermiculite/smectite content of the clay minerals (Persson & Olsson, 2000). Sterilisation experiments yielded somewhat variable results depending on the method used, but sometimes suppressiveness was reduced. Biotic factors were postulated to be involved in suppressiveness, based on this observation and the fact that the soil temperature at sampling influenced the disease response (Persson, 1998). The present work was initiated as a continuation project based on Persson's work.

2.3 Molecular detection in disease management

The devastating effects on yield of root rot outbreaks and the lack of effective control measures have made it useful for growers to obtain information about the degree of infestation, in order to be able to avoid high-risk fields. Traditionally, in Sweden and other areas where peas are grown, this is done by growing peas in the greenhouse in representative soil samples, and quantifying the root symptoms after visual examination. This assessment of root rot potential provide an index representative of the soil; in Sweden a scale of 0-100 is used, where the number roughly corresponds to the mean percentage of the root system that is rotted. In contract-growing of fresh green peas for the food processing industry, a threshold value is used when assigning contracts. Although the relevance of the root rot index for the field situation is well established (Olofsson, 1967), false negatives can occur.

The ability of the PCR technique (Polymerase Chain Reaction) to detect single copies of specific DNA fragments in a mixed DNA sample, has among a huge range of other applications made it an attractive option for the detection of plant pathogenic organisms. Quantitative PCR (qPCR) is a development of the original PCR technique that allows accurate quantification of the target template, through quantification of the amplicon in each cycle based on fluorescence of dyes included in the reaction. The original concentration is then inferred from the rate of increase in fluorescence, as the amount of PCR product grows exponentially.

There are now many qPCR based methods for detecting plant pathogens using DNA extracted directly from soil (Okubara *et al.*, 2005). Compared to other substrates, soils are problematic, since many substances in soil can inhibit the PCR reaction. One way of overcoming the inhibition is to dilute the DNA template. However, when detecting organisms present in small numbers, the dilution lowers the detection limit. Other possible problems

associated with DNA extraction from soil are low extractability due to incomplete disruption of resistant propagules and shearing of DNA. The need for effective and reproducible extraction procedures has led to a number of commercial kits being developed for the purpose. These are now commonly used in analysis of total DNA in soil samples.

3 Present investigation

3.1 Aims and outlines

The original aim of this thesis work was to investigate the influence of biological factors on soil suppressiveness to Aphanomyces root rot. However, some of the preliminary results indicated that non-biological factors were so important in determining differences in suppressiveness among soils, that the experimental system used was probably not precise enough to be able to discern biological factors. The objective to investigate biological factors also seemed somewhat premature, as long as the mode of action of important abiotic factors remained unknown. In the revised plan, soil suppressiveness mechanisms became the focus of one study, while the overall scope of the thesis was expanded to include practical application of suppression knowledge through calcium applications in field trials, development of a quantitative molecular detection method for *A. euteiches* in soil, and a study of the population biology of the pathogen in Sweden.

Based on this revised plan, the following aims were set up:

- to investigate the importance of potential chemical factors behind soil suppressiveness against Aphanomyces root rot, using a reductionistic approach (paper **I**).
- to test whether calcium amendments in the form of moderate doses of gypsum can suppress *A. euteiches* in naturally infested fields (paper **II**).
- to develop and evaluate a qPCR-based method for detection and quantification of *A. euteiches*, which can be used for

complementing or replacing biotests of the disease potential of soils (paper **III**).

- To investigate whether *A. euteiches* populations in Sweden are differentiated based on their geographical origin (paper **IV**).

3.2 Preliminary studies and approaches, "evolution of hypotheses"

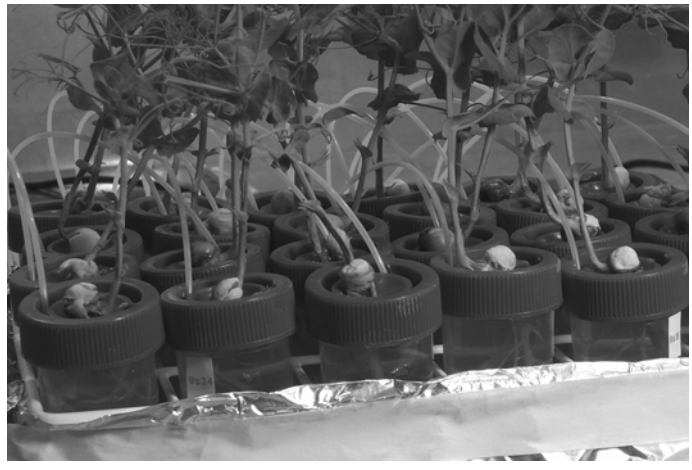
Originally, the project aimed at studying the involvement of soil biota in disease suppression. This aim was based on the preliminary observations by Persson that soil irradiation and autoclaving could decrease suppression, as well as other interesting phenomena, such as variation in disease suppression due to time of sampling (Persson 1998). Several possible approaches were considered. One was to isolate bacteria from suppressive soils and screen these in a biotest for antagonism against *A. euteiches*. This approach was dismissed, as it was based on a chain of non-confirmable assumptions about the mechanisms behind suppression. Although a large-scale screening of soil bacteria might have been fruitful, as such studies almost invariably lead to the discovery of bioactive bacterial isolates, it seemed weak as a main approach to address the research question, which was to identify the main causes of soil suppressiveness.

It later also turned out that it was difficult to reproduce the results from some of the soils in Persson's study, as well as practically working with them, since the fields are situated in the southern part of Sweden. A new collection of soils from another district was tested and displayed good and reproducible differences in disease suppression. Of special value was the occurence of a very "reliable" conducive soil (soil #4 in paper **I**) which was free of natural infestation.

The labour and inconvenience involved in handling large amounts of soil from different fields and managing pot experiments in growth chambers, as well as the fact that large scale soil testing coupled with soil analysis had already been done in previous studies, called for homing in on two model soils (the most suppressive #9 and the most conducive soil #4 from the new collection). Experimental systems were refined to a reproducible scaled down biotest, which would facilitate studies in a smaller format accessible to different kinds of manipulation or microscopic observations.

One of the several experimental systems tried involved growing pea plants hydroponically in microcosms where the individual pea roots were

submerged in aerated soil-water suspensions in 50 ml Falcon tubes. When such systems were employed, there was a clear delay in the onset of visible symptoms in suspensions prepared from soil #9, compared to when the conducive soil #4 was used. The effect was reproducible between experiments and worked both with oospore infected soil and with inoculum supplied as zoospores during the experiment.



This seemed to be the scaled down experimental system we were looking for. Unfortunately, the use of this system led to a number of artefacts and wrong conclusions. One was the observation that ferrous iron supplied as FeEDTA stimulated the disease in nutrient solutions or when applied to soil suspensions.This interesting observation was not possible to reproduce when the growth chamber for the experiment was changed. The reason was that the EDTA complexed traces of copper ions in the water used to water the microcosms, and thus gave the pathogen an advantage, since copper is toxic to oomycetes.

Another artefact was uncovered when we tried to refine the system further by replacing the microcosms with mycelial cultures grown in soil water extracts. We could see that mycelial growth was slower in soil extract prepared from soil #9 compared to the conducive model soil #4. However, when returning to the collection of soils and repeating the experiment on a larger scale, we could see no general correlation between mycelial growth and disease suppression data. The only soil that suppressed mycelial growth in this system was the one we had chosen as a model suppressive soil.

The collection of soil water extracts used for the experiment mentioned above, were the ones used for the calcium analysis mentioned as "water soluble calcium" in paper **I**.

In hindsight, it seems that the lesson to be learned (or re-learned) is that it is very easy for researchers in general to get "married" to the hypotheses or the experimental system we are working with. The hydroponic system should have been tested on a few more soils to confirm the correlation between results in pots and in test tubes. Nevertheless, analysis of the correlation between the chemical composition of the water extracts prepared for the mycelial growth experiment and the disease suppression data from pot experiments, in combination with earlier observations on the influence of Ca on *Aphanomyces* physiology (Lewis 1973), led to the hypothesis that Ca was involved in disease suppression. Furthermore, we found that the "suppressive factor" did not inhibit mycelial growth but zoospore production.

3.3 Materials and methods

3.3.1 Examining the mechanism behind soil suppressiveness (paper I)

The basic assessment of the degree of disease suppressiveness in soil samples was similar to the one used by Persson (1999). Soil samples from the topsoil were collected from agricultural fields, and each sample was mixed, sieved and dried. They were then artificially inoculated with the pathogen using a dry oospore-talcum mixture with known oospore concentration, prepared from mycelial cultures in the laboratory. Peas were then grown in pots with this inoculated soil, and after four weeks, the roots of the pea plants were washed, and the disease symptoms were quantified by visual examination and grading, resulting in a disease severity index. Non-inoculated pots of each soil were included in all experiments. Chemical analysis data from the soils were used, in order to discern potential chemical factors behind soil suppressiveness.

A similar bioassay, but without the inoculation step, is used in commercial pea production to determine the infestation level of fields, and thus the suitability of fields for pea planting. In study **I**, data from chemical analysis of 1549 such soil samples, collected and tested by Findus AB over a period of five years, were analysed to elucidate relationships with the disease response.

Effect of soil manipulations on suppressiveness were also examined in inoculated pot experiments. Manipulations included a: soil sterilisation prior to inoculation, b: admixture of calcium salts into the soil prior to

inoculation, c: watering of soils with $NaHCO_3$ in order to bind Ca ions as $CaCO_3$, which has a low degree of bioavailability due to low solubility.

Finally, the response of the pathogen to calcium *in vitro* was examined by allowing mycelial cultures of the pathogen to grow in calcium solutions of varying strength. Zoospore concentration in the medium was measured after 7 days, and the effect on mycelial growth was assessed by drying and weighing of the mycelia.

3.3.2 Application of calcium sulphate (gypsum) to suppress *A. euteiches* in the field (paper II)

The conclusion (paper **I)** that Ca is an important factor behind naturally occurring soil suppressiveness made us want to try to apply it in the field. Liming experiments have been done earlier in Sweden with the goal of controlling Aphanomyces root rot, but the effect was thought to depend on the elevation of soil pH, so alkaline salts like $Ca(OH)_2$ and $CaCO_3$ were used (Persson, 1998). The stronger disease suppressive effect of $CaSO_4$ compared to $CaCO_3$ in the laboratory, in combination with anecdotal reports in the literature of strong effects of low doses (Schroeder, 1953), made it interesting to apply a neutral Ca salt in a naturally infested fields.

The field trial was carried out in two commercial pea fields in southern Sweden where farmers grew vining pea on a contract basis with the food processing company Findus AB. The fields were assessed for root rot potential as described above and both had been found to contain some degree of infestation (index 25 and 66 on a 0-100 scale). Four treatments (0, 1500, 3000 and 6000 kg gypsum/ha) were included in four replicates (randomized block design), and the plot size for each observation was 39 m^2. Gypsum was applied by hand to the plots at the day of sowing and mixed into the soil by harrowing before planting of the peas.

Disease development during the growing season was assessed by digging up 10 randomly selected plants from each plot at four time points (37, 42, 49 and 56 days after sowing). The root systems were washed and root rot symptoms quantified by visual grading of each plant. The fields were harvested after 77 or 73 days, and the yield of each plot was measured. Five months after the field trial, we returned to the field and collected soil samples for use in the evaluation of the molecular detection method (paper **III**). These samples were also subjected to a pot bioassay in the greenhouse, where we obtained a disease index value for each sample under experimental conditions equivalent to the ones used in the suppressiveness assays.

3.3.3 Development and evaluation of a quantitative molecular detection method for *A. euteiches* (paper III)

In the first step, sequences of the ITS region of the ribosomal genes of closely related *Aphanomyces* species were aligned, and suitable regions for designing species-specific PCR primers were identified. The primers were then evaluated experimentally for specificity, using genomic DNA templates of different species. A fluorescent probe was then designed to match with the target region amplified by the chosen primers. A template standard was prepared by cloning a 524 base pair fragment containing the amplified region. This standard was used to prepare the standard curves necessary for quantitative PCR, and for spiking soil DNA samples to estimate PCR inhibition.

Total DNA was extracted from 0.3 or 0.5 g soil samples by the use of a commercial extraction kit. Initial trials showed that the templates obtained strongly inhibited the PCR reaction, and it became necessary to routinely further purify the DNA extracts using two commercial purification kits, as well as to try to assess the degree of remaining PCR inhibition. The inhibition was quantified by comparing the qPCR signal of spiked soil DNA extracts with spiked water controls.

The method was evaluated using a range of soil samples, most of which were also subjected to the bioassay method. These included soil samples of a single soil, which were artificially inoculated at different doses, as well as naturally infested soil samples from different fields. In addition, the field trial samples described in paper **II** were intensely sampled, with the aim to evaluate the hypothesis that calcium amendments would lead to detectable reductions in the amount of inoculum remaining after harvest of the pea crop. The cloning work and most of the qPCR runs in the project were done at Eurofins, Lidköping (formerly Analycen AB) by the second author of paper **III**.

3.3.4 Genetic analysis of Swedish populations of *A. euteiches* (paper IV)

A collection of 82 isolates of *A. euteiches* from different regions was established. In addition to old isolates from foreign countries previously in the collection of Findus AB, we made new isolations from diseased pea plants collected from two fields in southern Sweden. Isolations were also made from pea plants used in commercial root rot potential tests at Svalöf Weibull AB. This facility receives soil samples from fields in many different parts of Sweden. Thus, we could add isolates from western and northern parts of the country.

To analyse the population genetics of diploid organisms such as *A. euteiches*, more information is obtained if co-dominant molecular markers are used, allowing distinction between homozygous or heterozygous presence of each allele. Sequences containing such markers were published by Akamatsu *et al.* (2007). Some of the variable regions in these sequences could be classified as microsatellites, while other contained other kinds of polymorphisms. We designed PCR primers to amplify variable regions and amplified each fragment for all isolates. The PCR products were then sequenced at Macrogen Inc (Seoul, Korea). Allele frequencies in the different populations were calculated, and haplotype trees based on the genetic distances between the different alleles were constructed for each locus.

3.4 Results

3.4.1 Mechanisms behind soil suppressiveness (paper I)

The hypothesis that was tested experimentally in the study reported in paper **I** was that suppression was non-biological and that calcium ions, as such, were a major factor behind soil suppressiveness, in contrast to being merely one of several variables correlated to suppressiveness, as had been observed in the study by Persson and Olsson (2000). This hypothesis fits well with oomycete biology and previous studies done on soil amendments as a method of controlling oomycete root diseases (Engelhard 1989, Messenger *et al.*, 2000).

We supported this hypothesis with several lines of evidence. First, we could show that sterilisation did not abolish differences among soils in suppressiveness, even if autoclaved soils in some cases showed increased disease severity. We concluded that inconsistent and, sometimes, marginal increases in disease severity after autoclaving of a suppressive soil do not support a mainly biological origin of suppressiveness. In fact, it seems likely that such effects can just as well be the results of changes in soil chemistry and nutrient availability due to the autoclaving process itself. Interestingly, a similar effect of autoclaving was noted by Sauvage *et al.* (2007). Sterilisation experiments in studies of soil suppressiveness are important, but the results should be interpreted with caution and have little explanatory power, if conducive control soils are not included in the experiments.

Secondly, we showed that there was a negative correlation between Ca content and disease severity both in naturally infested and in artificially inoculated soils from a different district than the one previously studied. The

correlation was strongest when calcium was extracted with weaker extractants such as water, more closely reflecting the ion concentrations in the soil solution.

Thirdly, we were able to increase suppressiveness in a conducive soil by adding two different calcium salts, gypsum and calcium carbonate. Gypsum was more soluble and resulted in stronger disease suppression. In addition to this, we were able to increase disease severity in suppressive soils by adding $NaHCO_3$, which raised pH dramatically, but lowered the amount of extractable calcium.

Finally, we could confirm that mycelial cultures of *A. euteiches* were strongly impaired in their ability to release zoospores, when exposed to low concentrations of Ca. Mycelial growth was not affected. This allowed us to postulate that zoospore release from germinating oospores might be an important mechanism of Ca-mediated soil suppressiveness.

3.4.2 Practical use of calcium amendments to suppress *A. euteiches* in the field (paper II)

Of the two fields included in the experiment, a root rot outbreak occurred only in one (the field with the highest root rot potential). There was a significant effect of the gypsum applications on the field disease severity, even at the lowest dose (1500 kg/ha). However, as the season progressed, it became clear that disease suppression was not complete at any of the doses. In the pot bioassays of the field samples collected five months after harvest of the pea crop, disease suppression was high in samples from the plots receiving the highest doses (Paper **II**). This shows that high Ca levels still were present, leading to a strong disease suppressive effect as measured in the bioassay.

Inoculum levels in these samples can be assumed to have been higher than the one prevalent during the previous growing season in the field, since a root rot outbreak had occurred, leading to a batch of fresh oospores being produced in the roots of the infected plants.

3.4.3 Evaluation of a molecular detection method for *A. euteiches* (paper III)

The primer-probe system designed to detect *A. euteiches* proved to be selective with respect to closely related *Aphanomyces* species with similar sequences and was able to detect the target molecules in amounts as low as a few copies in qPCR reactions. It also worked well in detecting and quantifying the pathogen in soil samples inoculated with dry oospores and subjected to a pot experiment. In these experiments, the pathogen was

detected at levels corresponding to 0 in disease index and ~1 oospore/g soil. Correlation between separate extractions from the same soil sample was good, indicating that variation due to the extraction and purifying procedures was acceptable. PCR inhibition was low in the soil used.

For naturally infested soil samples, the results were different. The detected levels were generally very low, despite the soils having a high root rot potential. Several samples yielded no PCR signal. There was also a high degree of variation between repeated extractions from single soil samples, and also some variation between technical duplicates of the PCR reaction. Consequently, there was no correlation between detected amounts and disease index. DNA extracts from some soils showed PCR inhibition despite the additional purifying procedures performed. However, the variation among technical replicates did not seem to be caused by the inhibition, but rather by the low amounts present, as qPCR is less accurate at very low concentrations of the target molecule. By comparing PCR-signals from naturally infested soils with signals obtained from soils artificially inoculated with washed and counted oospores, we conclude that the qPCR assay is sensitive enough to detect single oospores. However, it seems as oospore densities lower than one per sample (0.5 g of soil) still may cause significant disease in the field.

The reason for the difference in results between soil inoculated with dry (non-washed) oospores and naturally infested soils was concluded to be a higher ratio between detected pathogen template and the disease severity in the inoculated samples. This could possibly have been caused by free DNA in the artificial inoculum preparations.

We concluded that naturally infested soils with high root rot potential generally contain oospore amounts too low to quantify by the method used. As a consequence, for the method to be useful, it is necessary to change the sampling strategy. This could be done by enriching the oospores or possibly by extracting DNA from larger soil samples.

3.4.4 Population study using co-dominant markers (paper IV)

The main finding from the analysis of the sequence data from the 82 *A. euteiches* isolates showed that a globally distributed genotype (present in France, North America and Sweden) dominated the Swedish population. An American bean infecting isolate was the genetically most distant from this genotype. One genotype (named genotype 2) present in a field in northern Sweden differed in many alleles from the dominant genotype. The population as a whole had a strong heterozygosity deficit for all alleles, a sign of inbreeding, which in homothallic organisms such as *A. euteiches* can be

caused by self fertilisation. However, there was also evidence of sexual recombination and widespread geographic distribution of the rarer alleles. As an example, isolates from western Sweden and a Norwegian isolate shared an allele with the genotype 2. There were also signs of heterozygosity, in the form of double peaks being formed in the sequence chromatograms for certain isolates, while other homozygous isolates had single peaks at the same site.

Allele frequency analysis in different populations indicated that the composition of the northern Swedish population was different from the southern one. However, for such an analysis to be informative, the datasets should be clone corrected, counting identical individuals as one individual. The low degree of total variation in Sweden seen with the markers used, did not permit such an analysis.

4 Conclusions

Based on the results of the studies presented, the following conclusions may be drawn:

1. Naturally occurring soil suppressiveness against Aphanomyces root rot seems to be abiotic in nature and largely depend on the soluble Ca content of the soil.

- Weak extraction methods are probably the most suitable for analysing the Ca content in soil samples with regards to suppressive effects on Aphanomyces root rot.

2. Amendments of soil with gypsum in moderate doses can lead to a significant decrease in disease symptoms in the field under Swedish conditions.

- Soils with a total absence of symptoms in a pot bioassay may still lead to disease symptoms in the field.

3. A qPCR assay directed towards ribosomal genes can detect single oospores of *A. euteiches* by using DNA directly extracted from soil as a template.

- Molecular detection methods of plant pathogens should be validated on naturally infested soils.

- Despite the availability of kits designed for the purpose of extracting and purifying DNA from soil samples, PCR inhibition remains an important issue when using soil DNA templates for detection and quantification of organisms present in low numbers.

- *Aphanomyces* inoculum in naturally infested soils is capable of causing severe disease symptoms at spore concentrations below 10 spores/g soil.

Thus, enrichment procedures or larger sample sizes are necessary for molecular quantifications to be meaningful.

4. The ability to quantify the pathogen, coupled with the knowledge of the importance of Ca for disease suppression, will help further attempts to predict the risk of disease, and possibly in searching for other factors causing naturally occurring disease suppression. For example, one could imagine a risk model where the threshold value of inoculum quantity over which pea planting is discouraged will be dependent on the Ca status of the soil.

5. The Swedish population of *Aphanomyces euteiches* is dominated by one globally distributed genotype. However, other genotypes exist. There is evidence both of clonality and occasional sexual recombination, but the population is far from a Hardy-Weinberg equilibrium.

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