Assessment of *Aphanomyces euteiches* in commercial pea fields - correlation between bioassay and DNA-analysis

Eivor Sofia Dubusc

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Eivor Sofia Dubusc

Supervisor:	**Magnus Karlsson, Swedish University of Agricultural Sciences, Department of Forest Mycology and Plant Pathology**
Assistant supervisor:	Zahra Omer, Hushållningssällskapet, HS-Konsult AB
Examiner:	Hanna Friberg, Swedish University of Agricultural Sciences, Department of Forest Mycology and Plant Pathology

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Swedish University of Agricultural Sciences
Faculty of Forest Sciences
Department of Forest Mycology and Plant Pathology

Abstract

Aphanomyces euteiches is a soil-borne pathogen that causes major yield losses in pea cultivation in Sweden. The lack of preventative measures emphasizes the importance of disease forecasting. So far, this has been done in the form bioassays, in which soil was taken from fields to be studied and peas were grown in the greenhouse on a trial basis. Molecular analysis using ddPCR is a potential alternative. In this study, it was investigated whether the amount of *A. euteiches* gene copies per gram of naturally infested soil correlates with the disease index of the peas determined in the bioassay. However, this could not be confirmed. The experiment was conducted in two parts and Spearman's correlation coefficient between the number of gene copies and the disease index was 0.11 and 0.18, indicating no statistically significant correlation. Among the main reasons for the lack of an expected correlation between the disease index and the quantity of *A. euteiches* DNA in the soil was the fact that the naturally infested soil potentially contains a range of pathogens that can cause similar symptoms and can influence each other's infectivity. In addition, it was investigated whether grinding the soil leads to improved *A. euteiches* detection. It was anticipated that soil grinding would enhance DNA extraction, improve homogeneity and aid in accessing *A. euteiches* DNA by cracking the oospores outer shell. Grinding the soil did not increase or stabilise the DNA yield, nor did it improve the detection of *A. euteiches* DNA. Among the suggested reasons for this is that the soil homogenisation during the DNA extraction process is sufficient and that the large amount of soil extracted (10 g) used for DNA extraction led to a decrease in variability between the samples.

Table of contents

List of tables

List of figures

Abbreviations

AM	Arbuscular mycorrhiza
ARR	Aphanomyces root rot
bp	Base pair
CMA	Corn meal agar
Ct	Cycle threshold
CV	Coefficient of Variation
ddPCR	Digital droplet PCR
DSI	Disease score index
ER	Emergence rate
g	gram
GH	Greenhouse soil
h	hour
MPN	Most probable number
PCR	Polymerase chain reaction
PRRC	Pea root rot complex
qPCR	Quantitative PCR
RPM	Rounds per minute
RTA	Rolled towel assay
SI	Soil indexing

Introduction

1.1 Biology and cultivation of pea

The pea genus (*Pisum*) belongs to the legume family. Legumes are the third largest family of flowering plants. (Smýkal *et al.*, 2012). Two different pea species are typically identified, green or garden pea (*P. sativum* L.*)* and field pea (*P. arvense* L.*)* (Poltoretskyi *et al.*, 2022). This study will focus on the garden pea (*P. sativum*).

1.1.1 Biology of pea

Peas are annual cool season crops. The plant has a typical legume morphology, with compound leaves, zygomorph flowers and a characteristic pod fruit that contains several small seeds. Pea plants can grow up to 2 meters tall, and their stems have a tendency to climb and twine around supports (Poltoretskyi *et al.*, 2022). The plant develops a taproot that can reach up to 1.5 m into the ground, and several highly branched lateral roots (reaching up to 1 m) in the topsoil. Further, the roots will develop nodules that harbour nitrogen-fixing bacteria called *Rhizobia*. Nitrogen fixation refers to the process of catalysing inert N_2 to biological useful NH_3. At the root nodules, a symbiotic exchange takes place between the nitrogen fixed by the bacteria and the carbohydrates synthesised by the plant (Poltoretskyi *et al.*, 2022). This leads to an accumulation of the fixed nitrogen in the plant, and eventually improves the nitrogen content in the soil.

The pea plant has a diploid chromosome set with 2n = 14 and a hermaphrodite inflorescence (Tönnberg, 2016). Reproduction usually takes place through cleistogamic self-fertilisation, in which the plant's reproductive organs are enclosed inside the unopened flower, and the pollen from the anther is transferred directly to the stigma, fertilizing the ovules and allowing the plant to produce seeds without the need for external pollination (Dordrecht, 2008). Cross-fertilisation by insects is possible, but is very rare in commercial cultivars and is estimated to be around 1%. In wild individuals, the cross-pollination rate can be significantly higher (Tönnberg,

2016). The ontogeny of pea plants is classified into four developmental phases, namely germination and emergence, vegetative growth, reproductive maturation and senescence. The pea seed consists of an embryo, two cotyledons and a seed coat. During germination of the seed, the radicle (primary root) emerges and grows along the force of gravity, exhibiting positive gravitropism, and the epicotyl develops and grows towards the soil surface (Tönnberg, 2016). Meanwhile, the cotyledons remain under the soil surface and provide the nutrients necessary for seedling development. During the development of the shoot, developmental nodes become visible, the first two nodes often remain below or at soil surface level and the third node produces the first true leaf. Vegetative growth is often rapid with approximately two nodes growing per week. Simultaneously, the radicle will develop into the taproot followed by lateral root development. During reproduction, the shoot will develop inflorescences and pollination will occur. About 24-30 days after pollination, the pod will have developed to maturity. Seeds are shed by programmed cell death of the funiculus, the attachment point between the shoot and the pod (Wu, 2018). The senescence of the entire plant is not directly introduced through the completed reproduction but through a range of environmental factors (Miryeganeh, 2021).

1.1.2 Cultivation of pea

Alongside with lentil (*L. culinaris* Medik.*),* chickpea (*Cicer arietinum* L.*),* bitter vetch (*Vicia ervilia* (L.) Willd.) and several cereal species, the pea is considered one of the eight founding crops that have been domesticated and cultivated since the origin of agriculture (Mikić *et al.*, 2014; Ambika *et al.*, 2022). The history of pea domestication began about ten thousand years ago in the Mediterranean and Middle East, with the now extinct ancestor *Pisum* sp. (Ambika *et al.*, 2022). Over time, thousands of pea varieties were developed through selection and breeding, with a lot of genotypes currently being preserved in several germplasm collections worldwide (Smýkal *et al.*, 2012). Peas are typically cultivated in regions with temperate climates, with Canada being one of the primary pea-producing countries, both historically and presently (Smýkal *et al.*, 2012). Globally, an average pea yield is approximately 1700 kilograms per hectare (Smýkal *et al.*, 2012).

Peas are one of the most popular pulse crops and are grown for various reasons. For the most part, peas are grown for human consumption. Peas are often harvested at an immature stage and then sold to the consumer either frozen or canned (Karkanis *et al.*, 2016). Peas are very nutritious due to their high mineral, fibre and protein content (23-31% of dry weight) (Karkanis *et al.*, 2016). In addition, peas contain a number of active components, such as polyphenols, vitamins and saponins. Furthermore, peas are used as feed for animals. In Europe, peas serve as a forage crop for hay and peas are fed to non-ruminants such as pigs and poultry (Karkanis

et al., 2016). Peas are also grown for their nitrogen-enriching properties of the soil. Combined cultivation with peas, either as an inter- or cover crop, or the inclusion of peas as an intermediate crop in the crop rotation is common.

The sowing time for pea depends on the growing region; in Central to Northern Europe, sowing usually takes place in spring, while in Southern Europe sowing usually takes place in mid-November (Karkanis *et al.*, 2016). Frost can damage the flowering of pea and the subsequent harvest, which is why sowing peas in autumn or early spring should be avoided in regions with winter frost. For example, a temperature of -4.5°C will cause about half of the seedlings to perish. However, pea plants are known for their ability to acclimatise to cold, thus peas are much more frost tolerant at maturity if they have been exposed to cold temperatures around freezing point at the seedling stage (Karkanis *et al.*, 2016). Optimal germination conditions are achieved at a sowing depth of 2-5 cm, a temperature between 15-20°C and low compaction of the top soil. Peas are usually sown in rows at 10-20 cm intervals with an optimum row spacing of 20-50 cm. Peas will emerge after approximately 10 to 14 days and can be harvested after about 60 days (Pavek, 2012). Nitrogen fertilisation is not common in cultivation, while the addition of phosphorus and sulphate is. The addition of phosphorus and sulphate not only plays a role as a direct nutrient for the plant, but also has an activating effect on nitrogen-fixing bacteria and thus also drives the supply of nitrogen. Peas are subjected to mechanical harvesting via a two-step process involving cutting of the plants followed by threshing to separate seeds from pods and vines (Pavek, 2012; Karkanis *et al.*, 2016).

Pea production in Sweden has a long and rich history, dating back to the Viking Age. Today, it has become an important agricultural commodity in the country due to its ability to thrive in Sweden's climate and soil conditions. With the growing demand for plant-based proteins, pea production in Sweden is set to increase in the future. This is due to the recognition of peas as a sustainable source of protein that require less water and fertilizer compared to other crops, and also have the ability to fix nitrogen in the soil. Pea production in Sweden is primarily carried out by small and medium-sized farms. The largest pea-producing regions in Sweden are Västra Götaland and Skåne, followed by Östergötland and Uppland. Companies generally contract farmers to grow peas for them, with contracts ranging from one year to several years. There are several big companies on the Swedish pea production market, including Lantmännen, a Swedish agricultural cooperative, Scandic Food, a Swedish food company that produces a range of plant-based food products, and Findus, a well-known company that has been involved in pea cultivation in Sweden since the 1940s.

From 2007 to 2016, the annual cultivation area of peas in Sweden remained stable at approximately 9,000 hectares. However, a substantial decline in pea cultivation area was observed in 2017, with only 2,500 hectares under cultivation (Karlsson, 2020). This decline can be attributed to the cessation of significant pea cultivation areas in Bjuv by the prominent producer Findus, as well as the relocation of pea cultivation operations to foreign nations such as France. Following the sharp decrease in pea cultivation in 2017, there has been a subsequent recovery with an increase in the number of pea fields being cultivated annually. As a result, in 2020, the recorded pea cultivation area had reached 4,900 hectares (Karlsson, 2020). Over the past half decade, pea production in Sweden has yielded an average of 56,000 metric tonnes per year. In the most recent harvest season of 2022, there was a significant increase in yield by 54% resulting in a total production of 86,200 metric tonnes (Ländell, 2022).

1.2 Pea root rot caused by *Aphanomyces*

1.2.1 The pea root rot complex

Pea cultivation is threatened by a range of abiotic and biotic factors. Root rot in peas is a highly prevalent disease and is a significant growth limiting factor, posing a threat to the global pea harvest and causing yield losses of up to 70% (Karppinen *et al.*, 2020). Root rot is caused by a number of soil-borne pathogens that together form a complex that infect the root (Wille *et al.*, 2021). The common causative pathogens in the pea root rot complex (PRRC) include: *Aphanomyces euteiches, Didymella pinodes, Didymella pinodella, Fusarium avenaceum, Fusarium oxysporum, Fusarium redolens, Fusarium solani, Pythium* sp., and *Rhizoctonia solani* (Wille *et al.*, 2021). Specific combinations of pathogens in the PRRC have been shown to interact synergistically and infect plant roots collectively. In this way, avirulent pathogens can break through a resistance barrier when they co-infect with a virulent pathogen (Wille *et al.*, 2021). The occurrence and progression of PRRC can differ based on factors such as the soil microbial population structure, crop rotation, meteorological conditions and other agricultural management practices, as well soil characteristics and location (Sharma *et al.*, 2022). The composition of the pathogens involved in the PRRC cannot be determined on the basis of the symptoms, as the different pathogens have overlapping symptom spectra. In general, PRRC leads to extensive seed rot, damping-off, seedling rot with subsequent seedling dieback and root rot (Wille *et al.*, 2020). Root rot prevents sufficient uptake of water and nutrients, thus leading to underdevelopment of the shoot and pod and other developmental pathological symptoms such as chlorosis

and necrosis of the leaves (Karppinen *et al.*, 2020; Sharma *et al.*, 2022). Furthermore, PRRC can negatively affect the nitrogen fixation on the rhizobia nodules, which in turn intensifies nutrient deficiency (Karppinen *et al.*, 2020).

1.2.2 The oomycetes *Aphanomyces euteiches*

The oomycetes *A. euteiche*s is a pathogen that attacks economically relevant legumes like alfalfa (*Medicago sativa*), fava beans (*Vicia faba*), beats (*Beta vulgaris*) and peas (*P. sativum*). It is part of the PRRC and is reported to cause exceptionally high economic losses in pea production (Hossain *et al.*, 2012; Karppinen *et al.*, 2020). Infection with *A. euteiches* is referred to as aphanomyces root rot (ARR) disease. The pathogen belongs to the Oomycota phylum, which is a large group of eukaryotes with about 600 to 1500 species. Although oomycetes share a number of morphological and ecological similarities with fungi, they do not belong to the group of true fungi and are more closely related to diatoms, chromophyte algae and other heterokont protists (Hossain *et al.*, 2012). The ability to infect plants has evolved at least two times within the oomycete lineage. First in an ancient lineage which diverged to Pythiales and Peronosporales (which includes the plant pathogenic genera *Phytophthora* and *Pythium*) and a second Saprolegniales lineage which includes *Aphanomyces*.

In a Europe-wide study on the genetic diversity of *A. euteiches*, three genetically distinct groups were identified. Distinct genetic differentiation was observed among strains from Italy, which displayed no genetic exchange with other groups. Similarly, strains from Finland and eastern Sweden exhibited genetic divergence compared to other groups, whereas those from other parts of Europe displayed closer genetic relatedness (Kälin *et al.*, 2022).

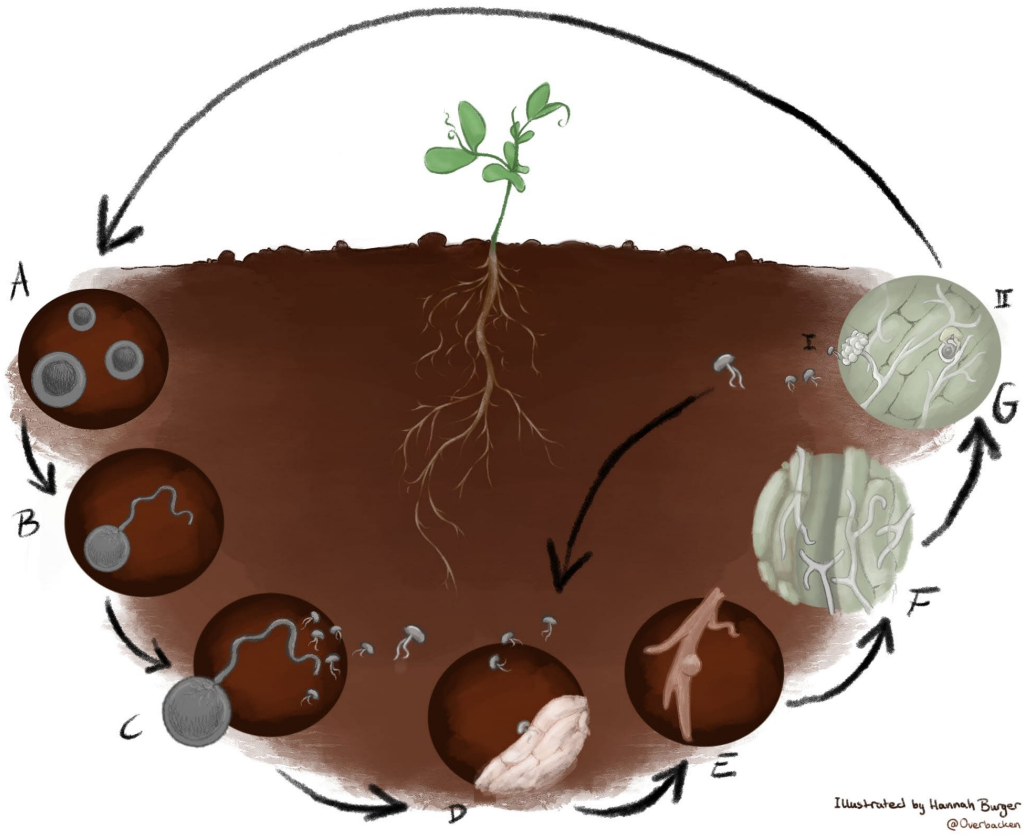


Figure 1| Life cycle of *Aphanomyces euteiches.* A) The *A. euteiches* oospore is dormant in the soil or plant residues. It retains ability to infect the host for up to 15 years. B) Infection begins when an oospore germinates in the proximity of a host plant, followed by the development of a germ tube with a long terminal zoosporangium. C) Bifoliolate, motile primary zoospores are released. D) The zoospores are guided towards their host by root exudates and initially attach to the roots. E) The zoospores encyst on the root surface. F) The pathogen advances further into the root cortex by developing hyphae. G) Reproduction of *A. euteiches*. I: sporangia develop and release zoospores that can infect new plants. II: At the end of the season, *A. euteiches* reproduces sexually by forming haploid antheridia and oogonia. The antheridia fertilizes the oogonia through a fertilisation tube, which incorporates the male nuclei into the oogonia, resulting in a diploid oospore. Illustrated by Hannah Burger @Overbacken, based on Hossain *et al.*, 2012.

The life cycle of *A. euteiches* includes both sexual and asexual stages, both occurring in the soil, allowing the pathogen to reproduce and maintain itself highly efficiently. Dispersal over long distances is facilitated through the transportation of contaminated soil, plant material or other materials (Karppinen *et al.*, 2020). An infection is initiated by the germination of an oospore in the vicinity of a host plant. Subsequently, the oospore will develop a germ tube with a long terminal zoosporangium, which can release over 300 bifoliolate, motile primary zoospores (Figure 1). Guided by host root exudates, the zoospores approach their host and initially attach to the roots. Subsequently, the zoospores will encyst on the root

surface via a cyclic uptake and release of calcium ions. Through the development of coencytic hyphae, the pathogen will advance further into the root cortex. After a few days, new sporangia will develop, which release zoospores that can infect new plants. In the end of the season, the pathogen reproduces sexually by forming haploid antheridia and spherical, about 25 to 35 µm in diameter, oogonia (Gaulin *et al.*, 2007). The antheridia fertilize the oogonia by incorporating the male nuclei into the oogonia via a fertilisation tube, creating a diploid oospore (Gaulin *et al.*, 2007) (Figure 1). The oospores of *A. euteiches* are characterised by a thick outer protective wall and dense energy reserves in form of oil deposits, enabling their survival in the soil for many years (Wu *et al.*, 2018). Survival up to 15 years have been documented so far, during which the oospore remains dormant and retains the ability to become infectious as soo potential host is present again (Hossain *et al.*, 2012).

1.2.3 Symptomology of aphanomyces root rot

An infection with *A. euteiches* can be established during any stage of plant development. Once the pathogen has penetrated the roots, it spreads rapidly from the rhizoderm to the root cortex, destroying the root structure, allowing for water to soak the roots (Wu, Chang, Conner, *et al.*, 2018) (Figure 2). The symptoms and the extent of infection can vary greatly. Initially, the roots will appear soggy and water-soaked with golden brown lesions. In severe cases, the root will rot leading the plant to collapse and wither before it can flower and produce a pod. In case of weak or late infections, the shoot may be underdeveloped in size but otherwise remain asymptomatic, with losses in pod size and yield (Wu, Chang, Conner, *et al.*, 2018) (Figure 2). Compromised plant immunity during *A. euteiches* infection may exacerbate symptoms and lead to additional infection by other pathogens (Gaulin *et al.*, 2007; Karppinen *et al.*, 2020).

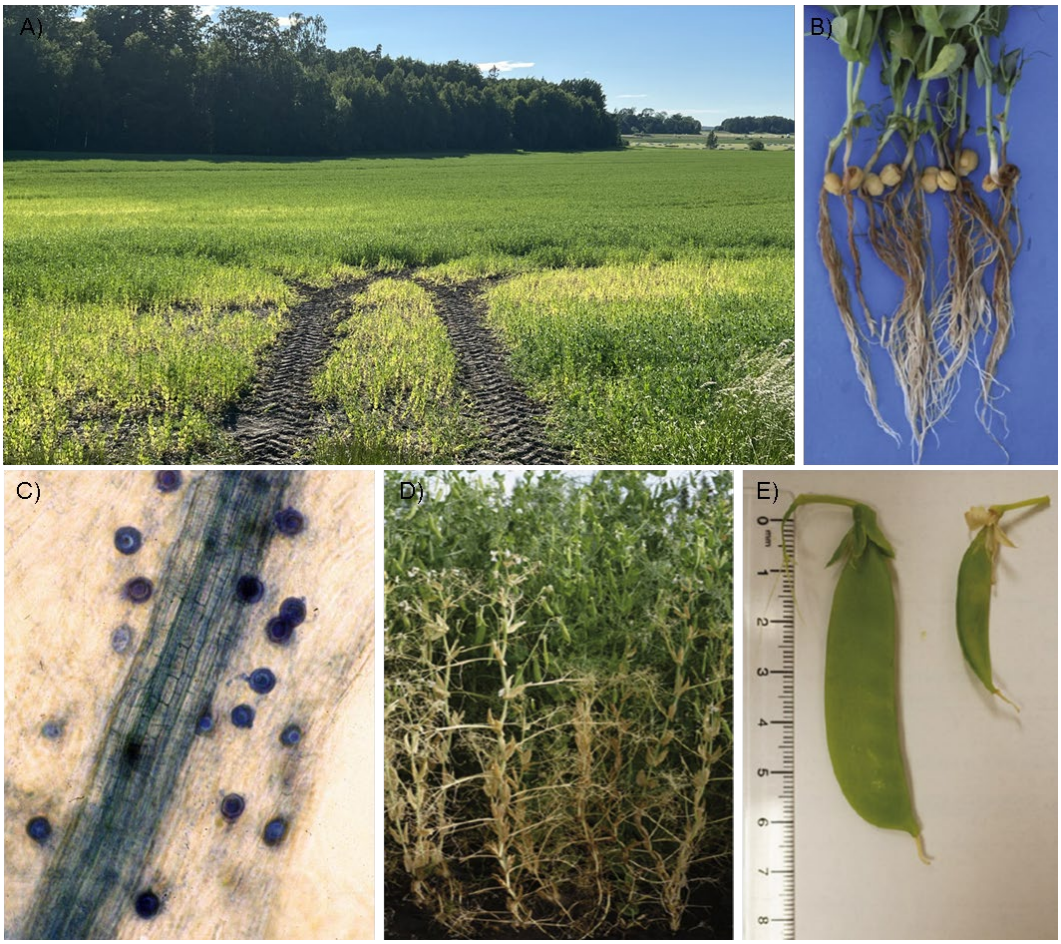


Figure 2| Root rot symptomology mediated by *A. euteiches*. The pathogen *A. euteiches* infects legumes like pea plants. A) When attacked, the pea plants are affected in clusters, which is visible in yellow shaded spots in the field. B) Infected plants have water-soaked, golden-brown roots. C) The mycelium grows inside the root towards the root cortex where it forms oospores. In the photo, the oospores are coloured blue. Both the hyphal formation and the oospores break up the root material and thus cause considerable damage to the root apparatus of the plant. D) A pea plant attacked by *A. euteiches* appears yellowed and wilted in its shoot. E) Attack by *A. euteiches* has a considerable effect on the pea crop. If the attack does not lead to total failure of pea pod development, the attack may have a negative effect on the quantity of the yield. On the left is a pea pod from an uninfected pea plant next to a pea pod from a plant infected with *A. euteiches*. Image A by Zahra Omer (HS-Konsult AB), Image B-E are adapted from Wu *et al.*, 2018.

1.2.4 Favourable conditions for disease development

The oomycete *A. euteiches* is highly tolerant to a wide range of abiotic factors. An exacerbation of disease symptoms on the host occurs when the optimal conditions of the pathogen are met. The soil-borne pathogen *A. euteiches* is well-adapted to waterlogged soil conditions, as it requires high level of moisture to complete its life cycle. Excess water in the soil can create anaerobic conditions that favour the growth of *A. euteiches* (Allmaras *et al.*, 2003; Gaulin *et al.*, 2007). Poorly drained, fine-grained soils with a high proportion of clay and loam are therefore favourable for the pathogen. Furthermore, soil compaction creates a favourable environment

for *A. euteiches* by reducing the pore spaces and in turn limiting the movement of water and air and ultimately leading to increased water retention in the soil. This condition can be enhanced by operating heavy machinery on the field (Allmaras *et al.*, 2003). Furthermore, the pathogen prefers an acidic environment with a pH level between 5.6 and 6.6 and temperatures between 15 and 20°C (Hossain *et al.*, 2012).

1.3 Aphanomyces disease management

The management of ARR includes a range of cultural practices and curative treatment. It should be emphasised, however, that none of these methods can achieve total pathogen eradication, but can mitigate disease levels. Primarily, the optimal infection conditions of the pathogen should be counteracted. This includes ensuring field drainage, avoiding cultivation on clayey and loamy soils, and preventing heavy vehicles from operating on the field as much as possible (Allmaras *et al.*, 2003). In addition, adding calcium to the soil can shift the pH to the alkaline range, which has been shown to counteract *A. euteiches* infection (Heyman, 2008).

There are also various points to consider in the context of crop rotation. Cultivation of host crops in the field leads to the build-up of *A. euteiches* inoculum in the soil, which is why a crop rotation that includes non-hosts is of great importance. However, the longevity of the oospores means that the crop rotation must be extended, sometimes up to 10 years, which puts constraints on the total production capacity (Wu, Chang, Conner, *et al.*, 2018). Some specific crops have been shown to reduce the infection potential of *A. euteiches* by integrating them into the rotation. These include, for example, the mustard plant *Sinapsis alba*. This reduction of inoculation potential is attributed to volatile aliphatic isothiocyanate components secreted from the roots that are toxic for *A. euteiches* (Hossain *et al.*, 2012, 2015).

The protection of the pea plant against *A. euteiches* by means of soil or plant treatments is not very well developed. Seed treatment with chemical fungicides can lead to a reduction in disease severity. However, it is important to emphasize that no complete mitigation of the infection can be achieved, due to the soil-borne nature of the pathogen (Wu, Chang, Hwang, *et al.*, 2018). In addition, there is much evidence to suggest that treatment of the seed with biocontrol agents can also lead to a reduction in disease. Bacterial strains of the genus *Bacillus* and *Pseudomonas* are shown to be particularly potent in greenhouse trials (Godebo *et al.*, 2022). However, field trials on the effectiveness of biocontrol agents are still pending (Godebo *et al.*, 2022).

Pea genotypes with partial resistance to *A. euteiches* have been identified and have proven to reduce ARR mediated yield loss. However, there is still a pending issue of integrating the resistance traits with other commercially desirable characteristics (Wu, *et al.*, 2018; Kälin *et al.*, 2023). This task is further complicated by the genetic linkage of some resistance traits with undesirable agronomic traits, such as shifted node length, flower and hilum coloration. Furthermore, the resistance to *A. euteiches* is highly polygenic, requiring efficient pyramiding of resistance genes to achieve high levels of resistance (Wu, *et al.*, 2018). However, development of both genetic and biogenic markers is ongoing and could help and accelerate the breeding of resistant lines in the future (Marzougui *et al.*, 2022; Wu *et al.*, 2022).

1.4 *Aphanomyces euteiches* disease forecasting

Accurate incidence forecasting is an essential component for the protection of all agricultural crops against pests, including soil-borne pathogens. As described, the majority of the control measures employed against *A. euteiches* refer to preventative measures that are undertaken prior to the cultivation process. In the event of a prevailing infection, the scope for action is restricted, and consequential yield reductions are inevitable. Therefore, developing accurate and effective forecasting methods for *A. euteiches* can facilitate prompt and strategic responses to potential infections, thereby averting significant losses in agricultural production.

1.4.1 Soil bioassays

Within the framework of a bioassay, soil samples from the field in question are taken and used in the greenhouse to cultivate the crop on a trial basis. The advantage of this method is that it covers a range of factors that contribute to disease development. These include a range of abiotic factors of the soil like grain and pore size, chemical properties of the soil, and amongst these, specifically, the presence of energy sources for the pathogen. Disease relevant biotic factors of the soil are for instance the humus content in the soil, the microbial composition of possible antagonists, and the propagule infectivity and propagule density. Propagules refer to disease-causing pathogenic structures. In the case of *A. euteiches* these include oospores, zoospores and hyphae (Malvick, 1994). The number of viable pathogen propagules in a given soil sample that are capable of infecting and causing disease in the host plant are referred to as inoculum potential. Soil testing for *A. euteiches* inoculum potential prior to pea cultivation by means of a bioassay is currently common for large-scale producers in particular.

A bioassay can be designed in different ways. Most probable number (MPN) is a term used in the rating of root rot on fresh peas, determined by a statistical algorithm that analyses serial dilutions of clean and infested soils to estimate likely oospore values (Malvick, 1994). Furthermore, the rolled towel assay (RTA) is another bioassay design, in which fresh pea seedlings are placed in wet paper towels together with a sample of soil of interest. The inoculum potential can then be calculated by determining the proportion of plants that show symptoms of root rot, based on established criteria (Malvick, 1994). The advantages of the method are the simplicity of the test set-up, the comparatively smaller quantity of soil samples required and the easier visual access to the root system. The limiting factor in the RTA is the difficulty in quantifying propagule quantities. Soil indexing (SI) is a method that has been used for over 50 years to measure inoculum potential in pea fields. During SI, susceptible plants are grown in field soil samples and disease severity on plant roots is visually scored. The SI method, however only provides general knowledge about disease tendencies in the field and provides no possibility to quantify the inoculum potential (Malvick, 1994).

All of the aforementioned bioassay methods face the common issue of unspecificity in determining the causative pathogen. These tests assess the symptomatology of all pathogens associated with PRRC, and since the symptoms of these pathogens overlap significantly, it becomes difficult to identify the causative pathogen. Even if an infestion of the soil with *A. euteiches* seems probable in the case of PRRC symptoms in pea cultivation, it cannot be confirmed without further investigation. If targeted measures are to be introduced to combat disease development in pea, then such further investigations would be necessary. If the presence of *A. euteiches* in the plants is to be investigated, phenotyping can be attempted by isolating the oomycete. To achieve this, newly harvested plant material can be promptly plated onto specialized media containing selective agents (such as metalaxyl, vancomycin, benomyl, rifampicin, and amphotericin B) for targeted isolation of *A. euteiches*. For phenotypic characterization, the mycelium can then be transferred to corn meal agar to observe sexual structures and to potato dextrose agar for asexual structures (Yokosawa et al. 1995). On the media, *A. euteiches* exhibits a white appearance with dense hyphal growth and a random arrangement. However, in the agricultural context, if only the overall suitability of pea cultivation at the given site is to be investigated, the performance of a bioassay without subsequent investigations may be sufficient.

1.4.2 DNA-based *A. euteiches* disease forecasting

Molecular methods that measure the amount of inoculum using DNA-based methods are other possibilities for disease incidence prediction. The prediction of

various soil-borne pathogens, such as *Plasmodiophora brassicae,* causing clubroot disease in crops of the brassicacae family, or *Pythium sulcatum,* causing cavity spot in turnip crops, using DNA methods is already commonly used. However, large-scale field testing for *A. euteiches* inoculum using DNA-based methods is not yet common and scientific efforts to implement this method are ongoing. Molecular detection usually involves first extracting DNA from the soil and then quantifying pathogen-specific DNA using species-specific primers.

The extraction of DNA from soil is associated with several challenges. Experiments comparing different DNA extraction methods with the aim of achieving standardisation across soil diagnostic laboratories revealed that the extraction methods had a significant influence on the recovered amount of DNA (Thomson *et al.*, 2012). The methods had an impact not only on the amount of DNA extracted but also on the ability to extract DNA from different microbial origins. For example, the amount of DNA of fungal origin extracted was particularly different between the different extraction methods. It can be concluded that the extraction method has a great influence on the ability to extract DNA from different types of soil and different types of microbes (Thomson *et al.*, 2012). It has been proven that the soil type also has a significant effect on the success of DNA extraction. DNA extraction from clayey and loamy soils proved to be more difficult than extraction from sandy soils. The presumed reason for this is the absorption of nucleic acids by clay particles (Almquist, 2016). In the case of *A. euteiches*, another challenge is the thick cell wall that has to be broken in order to obtain the DNA contained therein without the DNA being degraded by high mechanical agitation or chemical irritation. In addition, soil contains a number of substances that can interfere with DNA extraction. These include tannins and humic acids (Wydro, 2022). These can also interfere with subsequent quantitative PCR (qPCR). One way to avoid this is to dilute the DNA eluate and thus reduce the concentration of possible interfering substances. However, this also carries the risk of diluting the target DNA below the detection limit.

Another major concern regarding detection of *A. euteiches* lies in the representativeness of the sampling process. The soil infestation with *A. euteiches* tends to appear in clusters as disease foci, and thus, sampling strategies may lead to false negative or overestimated levels of predicted pathogen inoculation potential (Gangneux *et al.*, 2014). Thus, in order to predict the disease incidence of a site, several samples must be taken from the field in a systematic way. These can then be pooled to obtain an average value for the field, or tested separately (Sauvage *et al.*, 2007; Gangneux *et al.*, 2014). The latter allows for a resolution of the within-field variance and can detect possible outbreak hotspots. The more samples taken, the more accurate the resolution. In the extraction of DNA fordetection of *A.*

euteiches, extraction of the topsoil, i.e. the top 20 cm of soil, proved to be of particular interest, as it harbours the highest amount of inoculum (Gangneux *et al.*, 2014).

The design of two pairs of *A. euteiches*-specific primers is already established. The first primer pair for quantification of *A. euteiches* from soil was developed by Vandemark et al. in 2002. The sequence target is a 76 bp long region of the internal transcribed spacer (ITS) of ribosomal DNA. The ribosomal DNA gene cluster, including the ITS, is present in multiple copies in the genome of oomycetes. In a study on the copy number of ITS1, an established *A. euteiches* marker, a median copy number of 97 per haploid genome was reported by Gangeux et al. (2014), whereas Gibert et al. (2021) reported a significantly higher average of 120.5 copies per haploid genome. Another primer pair was designed in 2008 by Heyman, which amplifies a 96 bp long region of ITS1. When developing PCR primers for detection and diagnostics, it is important to ensure both species-specificity and ability to detect all isolates and genetic subgroups of the pathogen. There are three genetically distinct groups in Europe, the Central European, the Italian and a Northern European group present in Finland and Sweden (Kälin *et al.*, 2022). The species-specificity of the two primer pairs is considered assured. However, the detection capability of the primers across the genetic subgroups of *A. euteiches* has never been investigated.

Real-time PCR, or qPCR, was introduced in 1993 and is the most widespread method to quantify DNA (Vandemark and Barker, 2003; Hou *et al.*, 2023). Here, the DNA is amplified as in a normal PCR, but with continuous, real-time measurements of DNA amplification by means of fluorophores (Figure 3). The amplification of the fluorescence can be detected and by comparisons with a standard curve with known DNA concentrations, the amount of target DNA in the sample can be estimated (Vandemark and Barker, 2003). With droplet microfluidics technology, a new DNA quantification method called digital droplet PCR (ddPCR), was introduced in 2011 (Hou *et al.*, 2023). In this technique, the entire PCR reaction is divided into microreaction units in which the PCR takes place (Figure 3). Here, too, fluorophores are used to assess DNA amounts, but the number of fluorescent microunits is measured in the binary system subsequent to PCR. Each individual reaction unit is analysed for the presence of the target DNA by detecting presence or absence of fluorescence in the microreaction unit. In contrast to qPCR, no standard curve has to be generated for quantification; the DNA can be quantified directly (Figure 3). In addition, the ddPCR has several advantages compared with qPCR. For example, ddPCR should be significantly more accurate, more sensitive and more robust in the face of interference and distortion factors (Hou *et al.*, 2023). However, while qPCR only requires a qPCR-compatible cycler, which is widely

available, ddPCR requires a specialised instrument for droplet generation, a PCR cycler and a specialised device for reading the positive or negative droplets. The acquisition of such devices is associated with considerably higher investments. If a technique is too expensive or labour intensive, it may not be commercially feasible in certain situations.

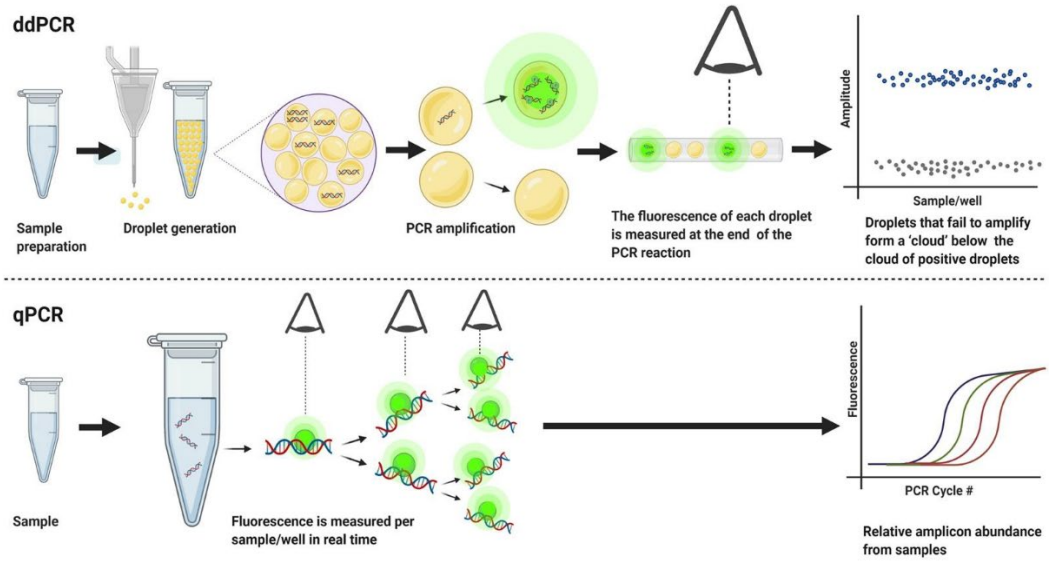


Figure 3| Droplet digital PCR (ddPCR) compared to quantitative PCR (qPCR). In ddPCR, the partitioning of samples into thousands of droplets allows for individual amplification within each droplet. The fluorescence emitted by each droplet is measured after PCR reaction and thereby the droplets are classified as either positive or negative based on their fluorescence amplitude levels on the y-axis. This is in contrast to qPCR, which provides a single measure of fluorescence per sample in real time, at the end of each PCR cycle. Figure adapted from Kokkoris *et al.*, 2021.

In the literature, several sources have already linked ARR disease incidence with *A. euteiches* DNA quantity using qPCR. However, in all sources, the soil was artificially infested with *A. euteiches* (Sauvage *et al.*, 2007; Heyman, 2008; Gangneux *et al.*, 2014). Both Sauvage *et al*., Heyman and Gangneux *et al.* extracted DNA from 0.5 g soil, with Sauvage *et al*. and Gagneux *et al*. reporting a detection limit of about 10 oospores per gram of soil and Heyman reporting a detection limit of about 1 oospore per gram of soil. All references indicate a clear correlation between the ARR disease incidence when artificially infested, otherwise disease-free soil. Sauvage *et al*. (2007) reported that inoculation of non-sterile soil significantly increased the detection limit and Gagneux *et al*. (2014) found both increased and decreased detection limit depending on the source soil and its properties. Heyman (2008) tested his detection system, established on artificially infested soil, on naturally infested soil. The result was that he could not detect correlation between the disease severity in the bioassay and the amount of *A. euteiches* DNA measured via qPCR. The target DNA was often close to or below the detection limit, despite high disease incidence. He also observed high variation

between replicates of the same homogenised soil samples. Heyman suggested using DNA from a larger amount of soil or concentrating oospores to reduce the detection limit in order to predict ARR by qPCR even in naturally infested fields.

A link between inoculum density in naturally infested pea fields and ARR severity was first reported by Gibert *et al.* (2021). In this study, 50 equally distributed soil samples per one hectare field were collected and investigated for disease severity of pea grown in the greenhouse and *A. euteiches* target DNA quantity measured via ddPCR. Various variables regarding the optimal DNA extraction, the ddPCR and the development of a forecast model were investigated. The extraction of DNA from 2 grams of soil (compared to 1 and 5 grams) was found to be the best compromise between accuracy and the cost factor in this study. In addition, homogenisation by grinding the soil lowered the coefficient of variation (CV) of the results, as did digesting the DNA before PCR with EcoRI. Gibert *et al.* (2021) proposed that digesting the DNA with EcoRI into smaller fragments, leads to a more even distribution of the DNA in the reaction units of the ddPCR and thus enabling a clearer classification of the droplets as positive or negative for target DNA amplification. For modelling the ARR disease incidence based on DNA quantity, Gibert *et al.* (2021) propose a linear mixed model with the soil type as random effect.

1.5 ARR forecasting in Sweden

When deciding where to grow peas, companies look at a variety of factors, including soil quality, climate, proximity to processing facilities, and the availability of irrigation. The disease prognosis of soil-borne diseases of a particular crop field also plays an important role. In Sweden, bioassays are the only means used for predicting ARR disease. However, there are limited possibilities for conducting such studies. Previously, Lantmännen Seed (formerly SW Seed) conducted large-scale bioassays to predict disease severity (Clarkson *et al.*, 2015). Unfortunately, these services were discontinued in 2010. Presently, Findus R&D offers a predictive test based on a bioassay in Sweden. This test includes interpretations of the results and recommendations. Findus excludes fields with a disease index above a certain threshold for pea production contracts within the company (Clarkson *et al.*, 2015). In Sweden, a large-scale project called BioSoM was conducted between 2009 and 2015 to address the need for anticipating diseases caused by soil-borne pathogens. This project was carried out by the Swedish University of Agricultural Sciences (SLU), among others, with the goal of establishing DNA-based prediction methods. The project resulted in successful

commercialization of DNA-based *Plasmodiphora brassicae* prediction (Jonsson, 2013b). The development of a method for predicting *A. euteiches* was also a part of the project. However, the establishment of a working protocol failed due to its unfunctionality in naturally infested fields and ability to detect low amounts of oospores (Jonsson, 2013a). The use of DNA-based methods to predict *A. euteiches* infestion of the soil is therefore not common in Sweden.

1.6 Research question of the thesis

In this study, the method for ARR prediction using DNA-based methods will be further developed and tested with novel protocols. The study will use soil from naturally infested commercial pea fields from southern and central Sweden.

1.6.1 Does mechanical grinding lead to improvement of molecular *A. euteiches* detection?

One of the two research questions in this thesis was whether grinding the soil could enhance the quantification of DNA using ddPCR. The fundamental assumption of this study was that grinding the soil would break the *A. euteiches* oospores, resulting in improved *A. euteiches* detection. To establish this, the first objective was to determine whether grinding the soil yielded a higher quantity of extracted DNA. Subsequently, the study investigated whether homogenizing the soil samples led to more stable results. Finally, the investigation focused on the ability of grinding the soil to improve the detection of *A. euteiches* DNA. It was believed that the internal mechanical grinding process could effectively rupture the oospores, allowing for the collection of *A. euteiches* DNA. In this regard, the study assessed whether the quantity of *A. euteiches* target DNA increased proportionally to the off-target DNA, with ddPCR being employed as the measuring tool.

Table 1| Test variables for determining whether grinding the soil leads to improved *A. euteiches* detection.

	Intended target	Research question	Variables investigated
A	Extraction of total DNA in soil	Does the amount of DNA extracted from soil increase when the soil is ground?	Comparison of DNA quantity extracted from 10 g of soil of differently ground samples
B	The amount of DNA extracted is stable across replicates	Does grinding lead to a reduction of the coefficient of variance of the extracted DNA across replicates?	Comparison of coefficient of variation between grinding methods

C	Detection of *A. euteiches*	Does grinding lead to an increase in detection of *A. euteiches*?	Comparison of *A. euteiches* gene copies detected in 100 ng DNA of differently ground samples

1.6.2 Can DNA-based detection of *A. euteiches* predict ARR disease severity in naturally infested pea fields?

The study was investigating whether molecular DNA-based *A. euteiches* detection can predict the symptom severity of ARR. For this purpose, soil samples will be taken from several fields across Sweden and pea plants will be grown in the greenhouse and evaluated according to their ARR symptomatology. Furthermore, the DNA will be extracted from the soil samples, concentrated and then the amount of *A. euteiches* DNA quantified. Both qPCR and ddPCR were available for this purpose, but the more suitable method was to be used for quantification within the framework of the project. To investigate the relationship between the disease index and the number of *A. euteiches* gene copies, the two variables were plotted against each other and examined for correlation. The hypothesis is that the amount of *A. euteiches* DNA is related to ARR symptom severity and will thus statistically significantly correlate. A positive significant correlation would indicate the possibility of predicting ARR incidence in the pea field using DNA-based detection methods prior to pea cultivation in the context of commercial pea cultivation.

2. Materials and methods

2.1 Bioassay

In order to investigate the effect of the different soil samples on ARR development, shoot dryweight and emergence, pea plants were grown in the different soil samples in a greenhouse test.

2.1.1 Soil samples

A total of 24 soil samples from 16 different locations were tested (Figure 4, Table 2). As negative control soil, the plants were also grown in greenhouse soil "U-Jord" (Hasselfors garden) and in soil in which infestation with *A. euteiches* was considered unlikely ("0") due to the absence of disease indicators and cultivating history with non-hosts. All soil samples were air dried at room temperature (approx. 22°C) for 3-4 weeks, then mechanically crushed with a hammer and sieved to a pore size of 3,4 mm.

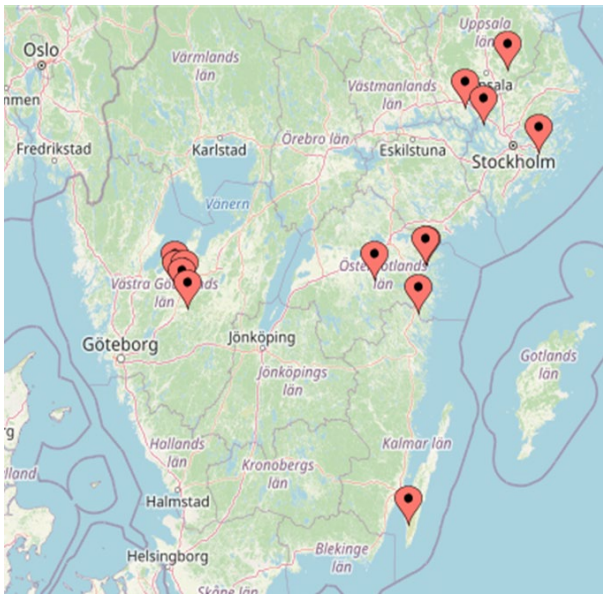


Figure 4| Map of soil sampling locations. For bioassays with soil from commercial pea fields in sweden, soil samples were collected from different locations. The map is shown from south to central sweden, red markers indicate the sampling locations.

Table 2| Soil samples used in the study, including their origin and the date of sampling.

Sample no.	County	Last cultivation of Pea	Date of Sampling	ARR indication
1	Stockholm	2022	18.10.2022	Yellowed areas of withering plants in the field
2	Stockholm	2022	18.10.2022	Yellowed areas of withering plants in the field
3	Stockholm	2022	18.10.2022	Yellowed areas of withering plants in the field
4	Stockholm	2022	18.10.2022	Yellowed areas of withering plants in the field
5	Stockholm	2022	18.10.2022	Yellowed areas of withering plants in the field
6	Stockholm	2022	18.10.2022	Yellowed areas of withering plants in the field

7	Stockholm	2022	18.10.2022	Yellowed areas of withering plants in the field
8	Stockholm	2022	18.10.2022	Yellowed areas of withering plants in the field
9	Stockholm	2022	18.10.2022	Yellowed areas of withering plants in the field
10	Stockholm	2022	18.10.2022	Yellowed areas of withering plants in the field
11	Stockholm	2017	03.11.2022	n.a.
12	Kalmar	2022	13.10.2022	n.a.
13	Kalmar	2022	13.10.2022	n.a.
14	Uppsala	2013	02.11.2022	n.a.
15	Uppsala	2009	02.11.2022	n.a.
16	Östergötland	2020	14.11.2022	n.a.
17	Östergötland	2020	14,11,2022	n.a.
18	Östergötland	2020	14.11.2022	n.a.
19	Östergötland	2020	14.11.2022	n.a.
20	Västra Götaland	n.a.	30.11.2022	n.a.
21	Västra Götaland	n.a.	30.11.2022	n.a.
22	Västra Götaland	n.a.	30.11.2022	n.a.

23	Västra Götaland	n.a.	30.11.2022	n.a.
24	Västra Götaland	n.a.	30.11.2022	n.a.
0	Uppland	n.a.	2015	n.a.

All these soil samples were used for correlation analysis to investigate the relationship between the number of *A. euteiches* gene copies and the disease index. A selection of these soil samples (soil samples 1, 2, 3, 7, 10, 11, 12, 13, 14, 15) were also used to study the effect of soil grinding on the molecular detection of *A. euteiches*.

2.1.2 Seeds

Pea plants of the susceptible variety 'Linnea' were used in the bioassay. The seeds were surface sterilised before sowing. During sterilisation, the seeds were soaked in 70% EtOH for 1 minute, then soaked in 1% NaOCl for 5 minutes and washed with sterile water. Finally, the seeds were dried at room temperature.

2.1.3 Sowing and plant growth

The trial was conducted in two parts. Part 1 was carried out from 05.12.2022 - 20.12.2023 and included samples 1-15. Bioassay part 2 was carried out from 13.01.2023 - 03.01.2023 and included samples 16-24. For sowing, 9x9x9 cm plastic flower pots were fitted with a 1003 grade filter paper, size 90 mm (Ahlstrom Munksjö) at the bottom and then filled with 340 g of soil. For each soil sample, 5 biological replicates were prepared and 5 seeds were sown per pot. The sowing was carried out differently between the two bioassays. In bioassay part 1, the full amount of soil was placed in the pot, and holes about 3 cm deep were punched for sowing using a dibber into which the seeds were sown and then covered with soil. In bioassay part 2, only part of the soil was filled into the pot and the seeds were placed on top of the soil so that a soil cover of about 3 cm could be filled over the top. After sowing, the pots were placed on 12x12 cm square petri dishes and watered with 50 ml of tap water each. The plants were arranged in the greenhouse in a randomised complete block design and grown at ca. 19.5°C with ca. 52% humidity, at long day conditions (16 hours light) and daily irrigation to the water uptake capacity limit of the corresponding soils (watering until water leaks through pot into petri dish).

2.1.4 Completion and evaluation of the bioassay

On the last day of the bioassay, the plants were first examined for the emergence rate (ER). This included all germinated plants that were visible above the soil surface. The plants were then removed from the soil and assessed into disease classes according to their symptoms (Figure 5). Different levels of ARR disease severity, as measured by the disease severity index (DSI) scale, where averaged across biological replicates.

Figure 5| Aphanomyces root rot disease severity index. Disease severity index (DSI) was assessed by the proportion of browning of the root system, 0: healthy, 25: slight root discoloration, 50: mild to moderate discoloration along with discoloration of the stem, 75: moderate browning of root and signs of wilt in shoot, 100: severe root browning leading to root degradation and wilted shoot. Photo: E. S. Dubusc.

For the recording of the dry weight of the shoots, the shoots were dried at 60°C for 16 hours (Fermarks). For qualitative analysis of *A. euteiches* infection of the roots, all roots of a soil sample were pooled and frozen at -20°C.

2.2 Qualitative analysis of the root infection with *A. euteiches*

2.2.1 DNA extraction from roots

To detect *A. euteiches* infection of the roots, the roots were first crushed in liquid nitrogen using a mortar. DNA was extracted from 100 mg of root material using FastDNA™ SPIN Kit (MP Biomedicals). The manufacturer's instructions were followed. Homogenisation was performed at 5500 rpm with two cycles of 35 seconds, including a 30 seconds intermediate paus, using precellys 24 tissue homogeniser (Bertin technologies). The DNA was then purified using illustra MicroSpin columns (Cytiva), following the manufacturer's instructions.

2.2.2 PCR and gel electrophoresis

The PCR was set up according to table 3. The *A. euteiches* microsatellite marker Ae37 (Mieuzet *et al.*, 2016; Kälin *et al.*, 2022), which has a size of 138 bp, was amplified.

Table 3| Components of Dreamtaq PCR reactions

Component	Concentration	Quantity for one reaction [µl]
DreamTaq Buffer	10 x	2.5
dDNTPs	10 mM	2.5
Forward primer	20 mM	1.5
Forward primer	20 mM	1.5
Dreamtaq	2 U	0.3
H_2O		16.7
DNA template		1

The PCR was run in a SimpliAmp™ Thermal Cycler (Applied Biosystems™), with a denutrition step at 96°C for 5 minutes followed by 30 cycles of 1 minute at 95°C, 1 minute at 58°C and 1.5 minutes at 72°C (Mieuzet *et al.*, 2016). The PCR product was separated by electrophoresis in 2% agarose gel. To prepare the gel, agarose (Saveen Werner) was dissolved by heating sodium borate buffer (19,1 gram $Na_2B_4O_7$ adjusted to the volume of 1 l with H_2O) and 2 µl of DNA staining agent Nancy-520 (Sigma Aldrich) was added per 100 ml of gel. 10 µl of the PCR product was loaded into the gel and 3 µl of GENERULER (ThermoFisher Scientific) was loaded for size calibration. The PCR products were run at a voltage of 160 volts for 45 minutes, powered by PowerPac™ Basic Power Supply (Bio-Rad). The PCR product was visualised in the GelDoc 2000 (Bio-Rad).

2.2.3 Assessment of the quantification primers for *A. euteiches* isolate of different genetic groups

The experiment involved testing the compatibility of *A. euteiches* isolates from various genetic groups with amplification primers. The testing process was carried out using PCR and gel electrophoresis, which were performed according to the guidelines specified in section 2.2.2. The DNA extracts were provided from Kälin et al. (2022). The isolates used in this study are indicated in table 4.

Table 4 | *A. euteiches* isolates used in the study

Genetic Group	Isolate ID
Finnish and Swedish	FI 43
	FI 52
	FI 11
	SE 64
Broader European	NO 1
	SE 51
	FR RB 84
	SE 58
Italian	IT 32
	IT 33
	IT 34
	IT 35

2.3 Analysis of the inoculum quantity in soil

2.3.1 Soil grinding

As part of the investigation into the effect of soil grinding on *A. euteiches* detection, subsets of the soil samples (approx. 100 g) were freeze-dried at -97°C in a ScanVac coolSafe (Labogene) for 18 hours. Subsequently, the samples were ground at Intertek Scanbi Diagnostics, Alnarp, Sweden, specifications provided by provider are indicated in table 5.

Table 5| Soil grinding specifications

Grinding method	Device	Device setting
A	Grindomix GM 200 (Retsch)	2 times reverse, 2000 rpm, 15 sec; 2 times reverse, 4000 rpm, 15 sec
B	1. Grindomix GM 200 (Retsch)	2 times reverse, 2000 rpm, 15 sec; 2 times reverse, 4000 rpm, 15 sec
	2. Geno/Grinder® (SPEX SamplePrep)	1620 rpm for 2 min with steel balls

2.3.2 DNA extraction

DNA extraction was performed using the DNeasy PowerMax Soil Kit (Qiagen) from 10 g soil. The manufacturer's instructions were followed except for the following modifications: solution C1 was heated in a heat block at 60°C for 5 minutes prior to each extraction, and in step 4 the samples were shaken for homogenisation using the mixer mill 400 (Retsch) at 30 Hertz for 2 x 60 seconds and then incubated for 10 minutes in a 65°C water bath. The amount of DNA extracted was then quantified using NanoDrop™ OneC (Thermofisher Scientific).

2.3.3 Sample concentration

After DNA extraction from the soil, a concentration of the DNA was conducted. This was carried out in two steps. In the first step, 0.2 ml 5 M NaCl (Invitrogen) and 3.5 ml 2-propanol were added to the DNA. The DNA was then filtered using NucleoBond™ Finalizer (Macherey-Nagel) according to the manufacturer's instructions and the DNA was finally eluted in 250 µl 1 x TE solution (IDT). The DNA concentration was then determined using NanoDrop™ OneC (Thermofisher Scientific). In the second concentration step, 8 µl 5 M Nacl (Invitrogen) and 416 µl 100 % EtOH cooled down to -20°C were added to the samples. The samples were then centrifuged at 14 000 x g for 30 minutes and the supernatant was discarded. The pellet was washed with 70% EtOH cooled to -20 °C, centrifuged at 14 000 x g for 10 minutes and the supernatant was discarded. The DNA was dried in a vacuum desiccator (Nalgene) for 45 minutes and then resuspended with 60 µl of 55°C MQ water. The DNA concentration was determined again using NanoDrop™ OneC (Thermofisher Scientific).

2.3.4 Quantification of DNA using ddPCR

For the quantitative analysis of the amount of *A. euteiches* inoculum for the respective soil samples, ddPCR was carried out. For this, the reaction units were prepared in a 96 well plate according to table 6. After assembly, all samples were pipetted up and down three times to ensure mixing of master mix and eluted DNA. The well plate was then sealed with piercable Heat Seal Foil (Bio-Rad) in the PX1™ PCR Plate Sealer (BioRad) and the plate was centrifuged at 1500 x g for 1 minute. Oil droplets were generated using the droplet generator (Bio-wheel) according to the manufacturer's instructions and PCR was then performed in the 96 well plate cycler ProFlex (Thermo Scientific) with 10 min at 95°C, 46 cycles of 30 sec at 94°C and 1 min at 65°C and then finally 98°C for 10 min.

Table 6| Components of ddPCR reactions

Component	Concentration	Quantity for one reaction unit [µl]
ddPCR Multiplex Supermix	4 x	5.5
Forward Primer	20 µM	1.1
Reverse Primer	20 µM	1.1
Probe	10 mM	0.55
H_2O		3.75
DNA template		10

Quantification was then performed on the QX200 Droplet Reader (Bio-Rad) and the threshold for positive or negative droplet reads was set Manually.

$$Gene\ copies * \ g\ soil^{-1} = \frac{\frac{Copies}{\mu l} * total\ volume\ of\ PCR\ unit}{g\ Soil}$$

2.3.5 Quantification of DNA using qPCR

For quantification by qPCR, the components were combined according to table 7 and subsequently amplified in the CFX Connect Real-Time 96 wellplate cycler (BioRad). Samples were incubated at 95 °C for 10 minutes, followed by 45 cycles of 15 seconds at 95 °C and 1 minute at 65 °C.

Table 7| qPCR components per reaction unit

Component	Concentration	Quantitity for one reaction unit [µl]
TaqMan™ Universal PCR Master Mix (ThermoFisher Scientific)	2 x	12.5
Forward Primer	20 µM	0.75
Reverse Primer	20 µM	0.75
Probe	10µM	0.5
ddH_2O		5.5
DNA template		5

2.4 Statistical analysis

Statistical evaluation of the experimental results was determined using the programming language R (version 4.2.2). The applied packets are listed in table 8.

Table 8| R Packages used in the study

Package	Version
dplyr	1.0.8
emmeans	1.8.4-1
ggpubr	0.5.0
lsmeans	2.30-0
multcomp	1.4-19
mvtnorm	1.1-3
readxl	1.4.0
TH.data	1.1-1

All correlation analyses were determined by means of Spearman analysis.
Significant effects of the soils on emergence, disease index and dry weight were determined using ANOVA and post hoc Tukey's test, and presented with standard error.

3. Results

3.1 The effect of soil homogenisation on the ability to detect *A. euteiches* DNA using ddPCR

To find out whether grinding the soil before DNA extraction leads to improved detection. The average DNA yield in ng/µl of the respective samples are depicted in table 9. For soil samples 2, 3, 7, 12 and 14, i.e. half of the soil samples tested, there was no significant difference in the amount of DNA extracted by grinding, neither by method A nor by method B (Table 9).

For sample 1, a significant increase in the amount of DNA extracted was noted after the sample was ground using method A. The sieved soil sample had a DNA concentration of 16.5 ng/µl while a DNA concentration of 29.3 ng/µl was obtained from the ground soil sample. When soil sample 1 was grinded using method B, a statistically higher amount of DNA of 23.3 ng/µl was obtained compared with methods S and A (Table 9). For soil samples 10 and 11, the amount of extracted DNA was significantly increased by grinding, but for both soils there was no difference in the amount of extracted DNA between the different grinding methods. For soil sample 13, grinding using method A resulted in a non significant increase in DNA concentration, while method B resulted in an insignificant decrease in the amount of DNA extracted. The amount of DNA extracted from soil treated with method B was significantly lower for soil 13 than for method A. Finally, for soil sample 15, there was a significant decrease in the amount of DNA extracted by grinding according to method B compared to the sieved soil.

In this study, the coefficient of variation (CV) was analyzed to assess the variability of the amount of DNA extracted from differently ground soil samples. The CV was divided into three classes (1, 2, and 3) and applied to the differently treated soils of one soil sample. Class 1, which had the lowest coefficient of variation, was attributed to the most consistent and reliable extraction method, while class 3, which had the highest coefficient of variation, was considered the least reliable.

Table 9| Effect of grinding soil methods (S, A and B) on DNA concentration.

Soil ID	DNA from method S (ng/µl)[1]		DNA from method A (ng/µl)[1]		DNA from method B (ngµ/l)[1]	
1	16,5	A	29,3	B	23,3	C
2	12,4	A	5,1	A	4,6	A
3	7,6	A	4,8	A	9,0	A
7	20,0	A	29,8	A	22,6	A
10	17,5	A	33,2	B	31,5	B
11	14,7	A	37,3	B	40,0	B
12	10,8	A	17,5	A	12,7	A
13	21,3	AB	29,7	A	19,9	B
14	21,8	A	28,5	A	21,8	A
15	31,0	A	19,3	AB	16,7	B

[1]Different letters indicate significant differences between methods with $P \leq 0.05$ based on ANOVA and Tukey post-hoc test.

Table 10| Effect of grinding soil on coefficient of variation[1] of DNA quantity extracted.

Soil ID	Method S	Method A	Method B
1	3	2	1
2	1	2	3
3	1	3	2
7	2	1	3
10	2	1	3
11	1	2	3
12	3	1	2
13	2	3	1
14	3	1	2
15	2	1	3
Total	20	17	23

[1]The coefficient of variation of the DNA concentrations was determined. The treatment method with the lowest variance within the soil sample was labeled as 1, and the highest 3, respectively. The total values per treatment method are indicated.

Soil samples were analyzed for the quantification of *A. euteiches* using ddPCR. The samples were collected in different conditions, where the soil was either only sieved (S), ground according to method A, or ground according to method B. The results showed that there were no significant differences in *A. euteiches* gene copies per 100 ng DNA in soil samples 1, 2, 7, 10, 11, 13 and 15 (Table 11). However, in soil samples 3, 12 and 14, the results were different. In soil samples 3 and 12, method A was found to result in significantly higher numbers of gene copies than both S

and B. On the other hand, in soil sample 14, method B generated only 0.7 gene copies per 100 ng DNA, which was significantly lower than S, where it was found to be 162.9 gene copies per 100 ng DNA (Table 11). Although the difference between method B and A was not significant, method B was significantly lower than S. These results were statistically analyzed using Anova and post hoc Tukey test, providing significant evidence for the impact of different soil preparation methods on the quantification of *A. euteiches* in soil samples.

Table 11| Effect of grinding soil on *A. euteiches* detection[1].

Soil ID	Method S[1]		Method A[1]		Method B[1]	
1	42	A	211	A	272	A
2	9	A	49	A	15	A
3	106	A	254	B	172	A
7	2054	A	38	A	167	A
10	17	A	31	A	26	A
11	0	A	7	A	1	A
12	0	A	1	B	0	A
13	86	A	12	A	14	A
14	162	B	47	AB	0	A
15	0	A	2	A	0	A

[1]*Aphanomyces euteiches* gene copies were determined by ddPCR and normalized by the DNA concentrations, and expressed as gene copies * 100 ng DNA^{-1}.

[2]Different letters indicate significant differences between methods with $P \leq 0.05$ based on ANOVA and Tukey post-hoc test.

3.2 Bioassay results

In order to evaluate the correlation between *A. euteiches* DNA quantity and ARR disease index, pea plants were grown in a bioassay in the greenhouse. After 3 weeks, the plants were harvested, disease symptoms assessed, and a disease index was calculated (Equation 1).

The bioassay of the 24 soil samples was carried out in two separate experiments, due to the different availability of the soil samples. In order to compare the two bioassays, two control samples, greenhouse soil "GH" and soil sample ID "0", were included in both experiments. The plants from the control soil "0" had a significantly ($P \leq 0.05$) higher disease index in bioassay 1 compared with bioassay

2. In the greenhouse soil, there were no significant difference in disease indices between bioassay 1 and 2. The variables DSI, shoot dry weight and emergence were tested for significant differences between the two bioassays. There was a significantly ($P \leq 0.05$) higher emergence rate in bioassay 2 for both controls (Figure 6). However, shoot weight per plant was not significantly different between the two bio-tests for either control soil (Figure 6).

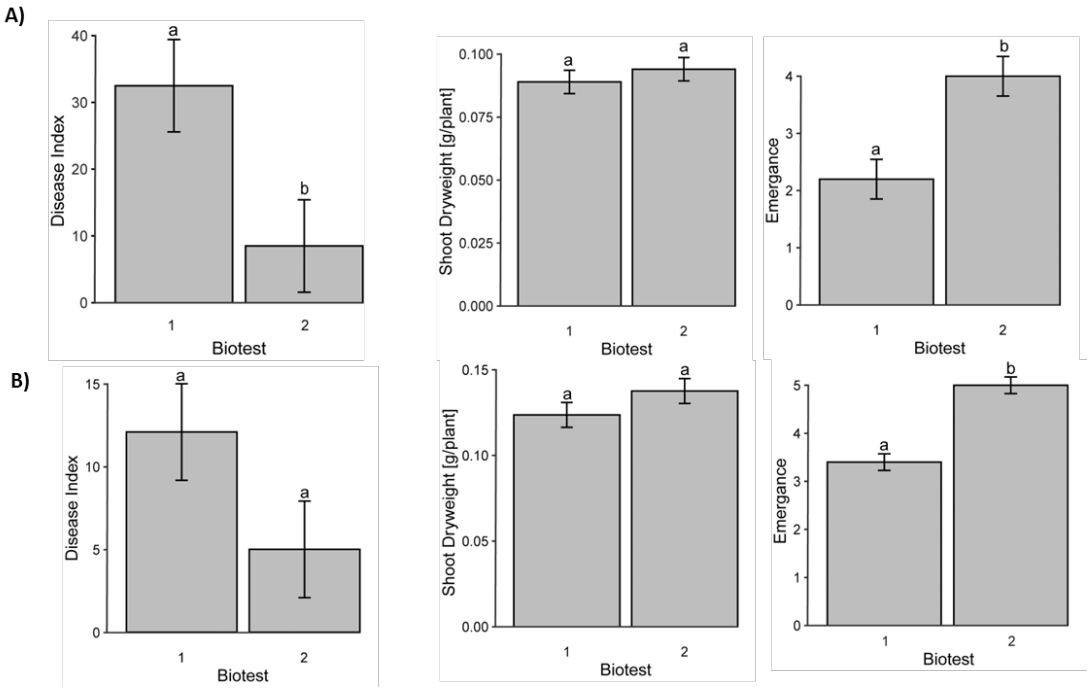


Figure 6| Comparison of the control soils of the two bioassays. The soil types 0 (A) and greenhouse soil (B) were analyzed between the bioassays carried out at different times for the mean disease index (left), shoot biomass (middle) and emergance (right). It was determined whether the values obtained were significantly different within the bioassays. The whiskers on the bar represent the mean error, and different letters indicate significant differences as measured with ANOVA and post hoc tukey test. ($P \leq 0.05$).

3.2.1 Bioassay 1

The bioassay number one was performed in December 2022. Fifteen different soil samples were tested. None of the plants were completely symptom-free. Even the controls had an average disease index of 20 for the greenhouse soil and 30 for the soil with ID 0. Of the 10 samples from the same pea field, the plants in soil samples 1, 3, 4, 5, 6, 8 and 10 had particularly high disease indices with an index value of 90 or higher, with significantly ($P \leq 0.05$) higher disease values than both control soils (Figure 7). Plants from soils 2, 7 and 9 had disease indices around 40, which was significantly higher than both control soils. Soils 11-13 and 15 had disease

index values around 60, all significantly ($P \leq 0.05$) higher than the plants in the greenhouse soil but not from soil 0. Plants from soil 14 had the lowest disease index at around 20, similar to that of the greenhouse soil and significantly lower to the plants from samples 1, 3, 4, 5, 6, 7, 8, 10, 11, 12, 1, and 15 (Figure 7).

In contrast to the disease indices, the soil had less influence on the dry weight of the shoots. Plants growing in the control soil tended to have higher shoot dry weight, with the greatest dry weight being observed in plants grown in greenhouse soil with a shot dryweight of approximately 12 mg. Its dry weight was found to be significantly ($P \leq 0.05$) higher than the dry weight of the plants from soil 5 and 6 (Figure 7). The remaining plants had a shoot dry weight between 6 and 7 mg per plant and showed neither statistical differences nor noticeable tendencies (Figure 7).

At the end of the experiment, the emergence rate was determined. All shoots that grew out of the soil were counted. The germination rate was therefore not recorded. The emergence was between 2 and 3 pea shoots out of 5 sown per pot in most soils. Emergence was particularly high in soil number 4, 13 and the greenhouse soil with an average emergence of over 3 plants per pot, making the emergence in theses soils significantly higher than in the others ($P \leq 0.05$). Emergence was particularly low in soils 14, 7 and 9, where emergence was about one plant per five sown (Figure 7).

Subsequently, the variables collected were examined for possible interactions by means of correlation analyses. The disease index correlated negatively with shoot dry weight with an R of -0.64 and a *P*-value of 6.2e-10 (Figure 8). A statistical correlation was also found between emergence and shoot dry weight. This was positive with an R of 0.44 and a *P*-value of 2.5e-05. No significant correlation was found between emergence and disease index (Figure 8).

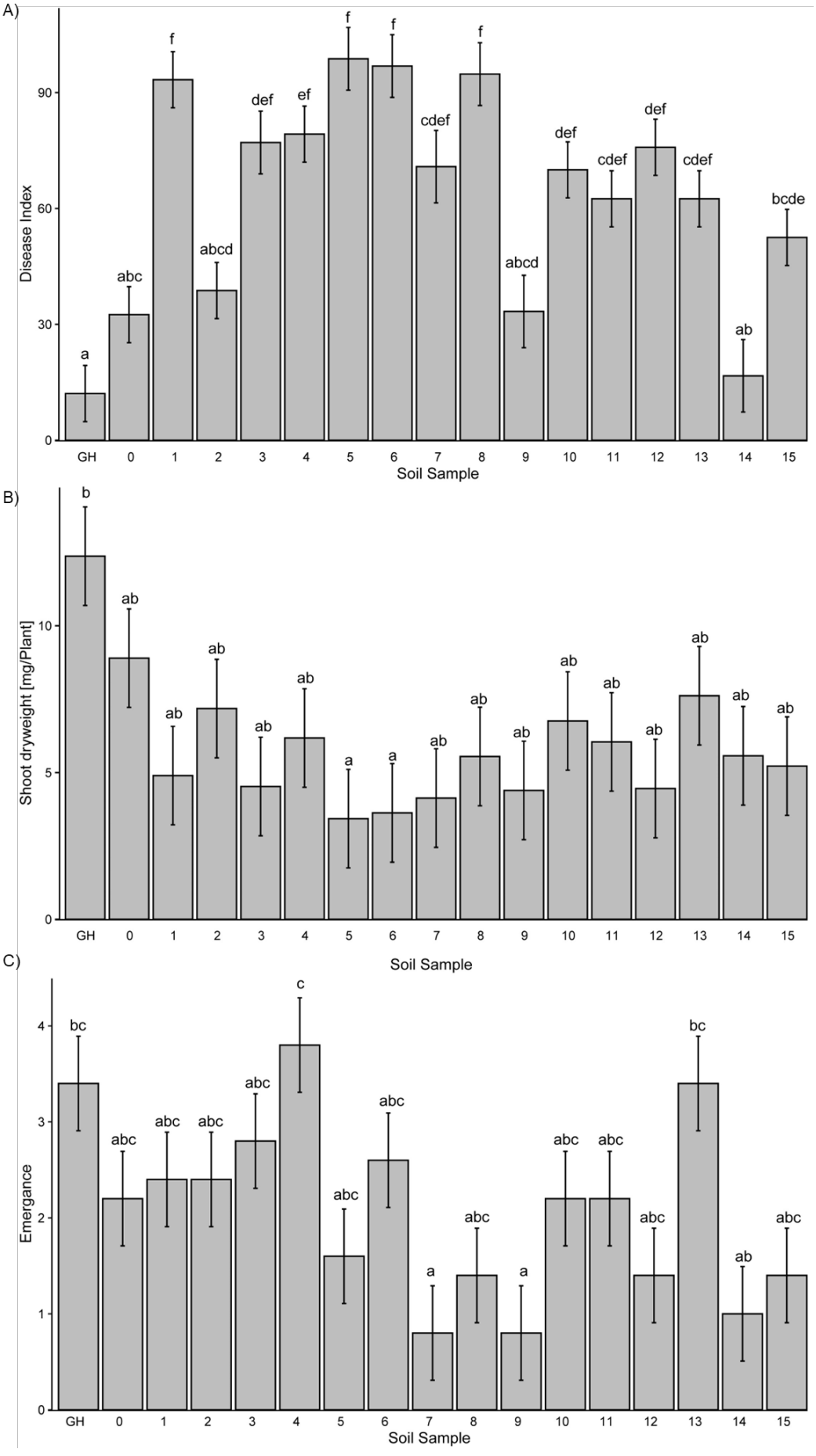

A)
Disease Index
0
30
60
90
a
abc
f
abcd
def
ef
f
f
cdef
f
abcd
def
cdef
def
cdef
ab
bcde
GH 0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
Soil Sample
B)
Shoot dryweight [mg/Plant]
0
5
10
b
ab
ab
ab
ab
ab
a
a
ab
ab
ab
ab
ab
ab
ab
ab
ab
GH 0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
Soil Sample
C)
Emergance
0
1
2
3
4
bc
abc
abc
abc
abc
c
abc
abc
a
abc
a
abc
abc
abc
bc
ab
abc
GH 0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
Soil Sample

Figure 7| Effect of soil on disease index, emergence and shoot dry weight in bioassay 1. Average disease index (A), shoot dryweight (B) and emergence (C) of pea plants after three weeks of cultivation in the greenhouse in different soil samples. The whiskers on the bar represent the mean error, and different letters indicate significant differences as measured with ANOVA and post hoc tukey test. ($P \leq 0.05$).

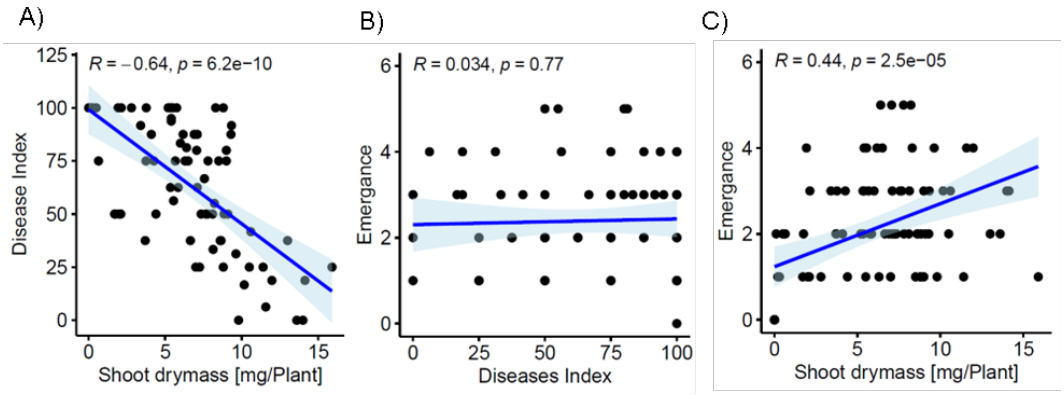
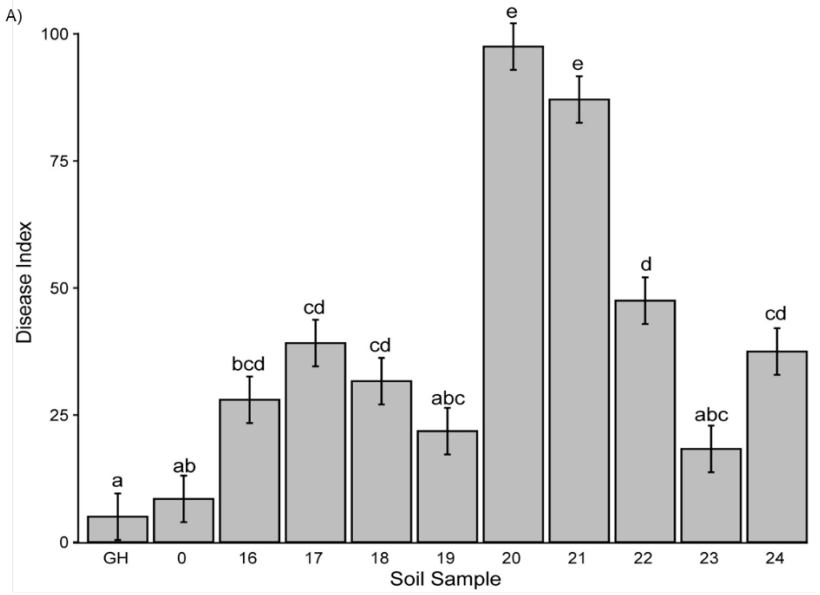
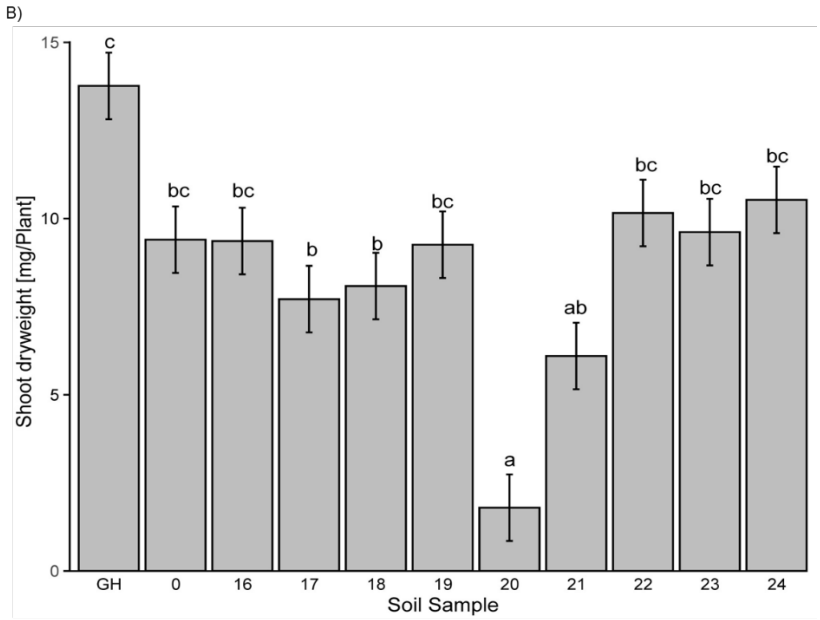


Figure 8| Correlation analyses between disease index, shoot dry weight and emergence in bioassay 1. Peas were grown in different types of soil and evaluated after about 3 weeks. Correlations of the collected data were determined using Spearman correlation analysis, the coefficient of determination (R) and the significance are given (*p*). A) Correlation between shoot dry weight and disease index. B) Correlation between disease index and emergance. C) Correlation between disease index and emergence.

3.2.2 Bioassay 2

As in bioassay 1, DSI of the control soils were significantly ($P \leq 0.05$) lower than the rest (Figure 9). The disease indices of the plants from soil 20 and 1 were conspicuously and statistically significantly higher. The dry weight of the shoot was also particularly low in plants from soil 20 and 21, with an average dry weight of 0.01 g and 0.06 g respectively. The plants growing in the greenhouse soil, on the other hand, had the highest dry weight, which was about 0.14 g, which was significantly higher than the shoot dryweight of plants grown in soil 17, 18, 20 and 1 (Figure 9). Seedling emergence was approximately 3 plants with the highest emergence rates for plants grown in soils 16 and 19 and siginifcantly lower in soils 18, 20, 21 and 24. In the correlation analyses, there was also a strong correlation between the disease index and the shoot dry weight with an R of -0.75 and a *P*-value of 4.1e-11. In contrast to the results of the first bioassay, there was no correlation between emergence and shoot drymass and, a slightly negative correlation between emergence and disease index with an R value of -0.39 and a *P*-value of 0.0035 (Figure 10).


A)
Disease Index
100
75
50
25
0
a
ab
bcd
cd
cd
abc
e
e
d
abc
cd
GH
0
16
17
18
19
20
21
22
23
24
Soil Sample


B)
Shoot dryweight [mg/Plant]
15
10
5
0
c
bc
bc
b
b
bc
a
ab
bc
bc
bc
GH
0
16
17
18
19
20
21
22
23
24
Soil Sample

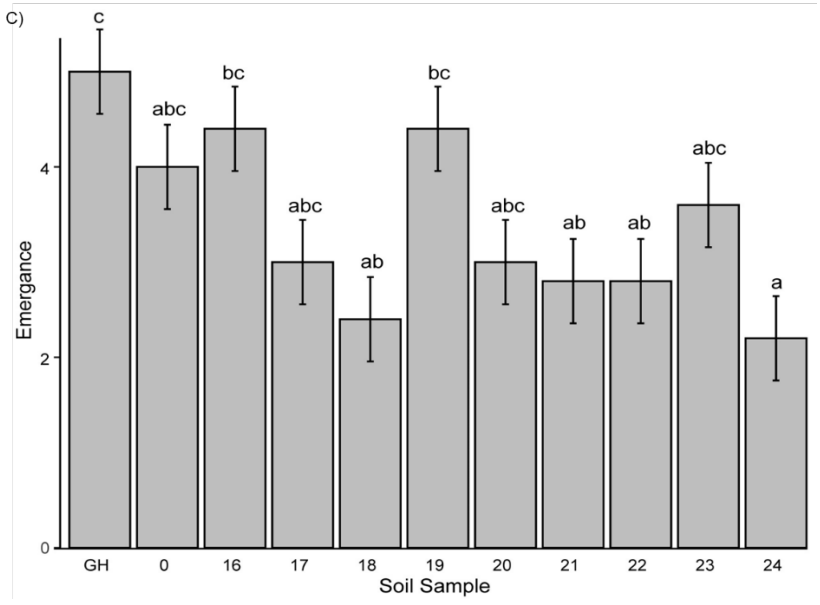

C)
Emergance
4
2
0
c
abc
bc
abc
ab
bc
abc
ab
ab
abc
a
GH
0
16
17
18
19
20
21
22
23
24
Soil Sample

Figure 9| Effect of soil on disease index emergence and shoot dry weight in bioassay 2. Average disease index (A), shoot dryweight (B) and emergence (C) of pea plants after three weeks of cultivation in the greenhouse in different soil samples. The whiskers on the bar represent the mean error, and different letters indicate significant differences as measured with ANOVA and post hoc tukey test. ($P \leq 0.05$).

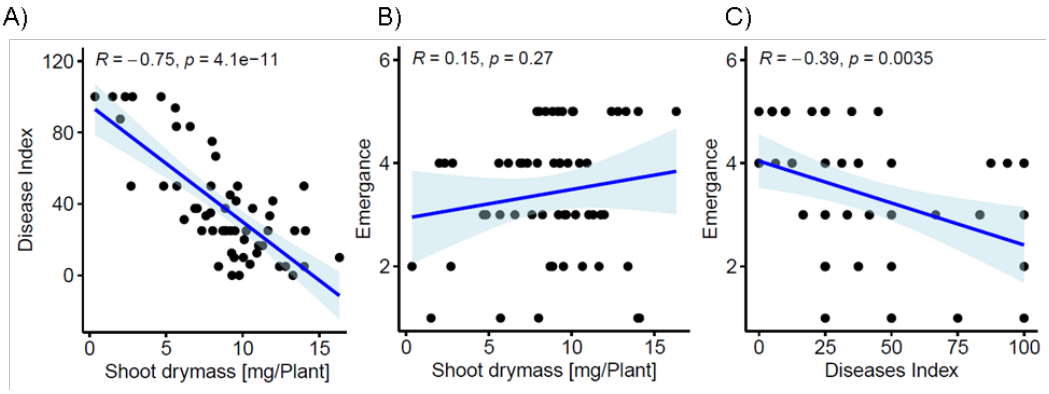


Figure 10| Correlation analyse between disease index, shoot dry weight and emergence in bioassay 2. Peas were grown in different types of soil and evaluated after about 3 weeks. Correlations of the collected data were determined using pearsman correlation analysed, the coefficient of determination (R) and the significance are given (*p*). A) Correlation between shoot dry weight and disease index. B) Correlation between shoot dry weight and emergance. C) Correlation between disease index and emergence.

3.2.3 Qualitative detection of *A. euteiches* in roots

Qualitative examination of *A. euteiches* infection of roots, showed presence of *A. euteiches* was in the roots from soil 1, 2, 3, 4, 6, 7, 8, 10 and 11 in bioassay one (Figure 11). No amplification was detected in soils 9, 12, 13, 14 and 15. In bioassay two, *A. euteiches* DNA and thus infection with it was detected in the roots from soils 16, 17, 18, 20, 21 and 22. It should be noted that the 138 bp bands of the roots of soil 18 was significantly weaker than the rest. Roots 19, 23 and 24 turned out to be negative (Figure 11). It should also be noted that a further band of about 500 bp was detected in most of the samples, which is not supposed to indicate infection with *A. euteiches.* In this context, the presumed absence of *A. euteiches* in the control soil 0 was confirmed.

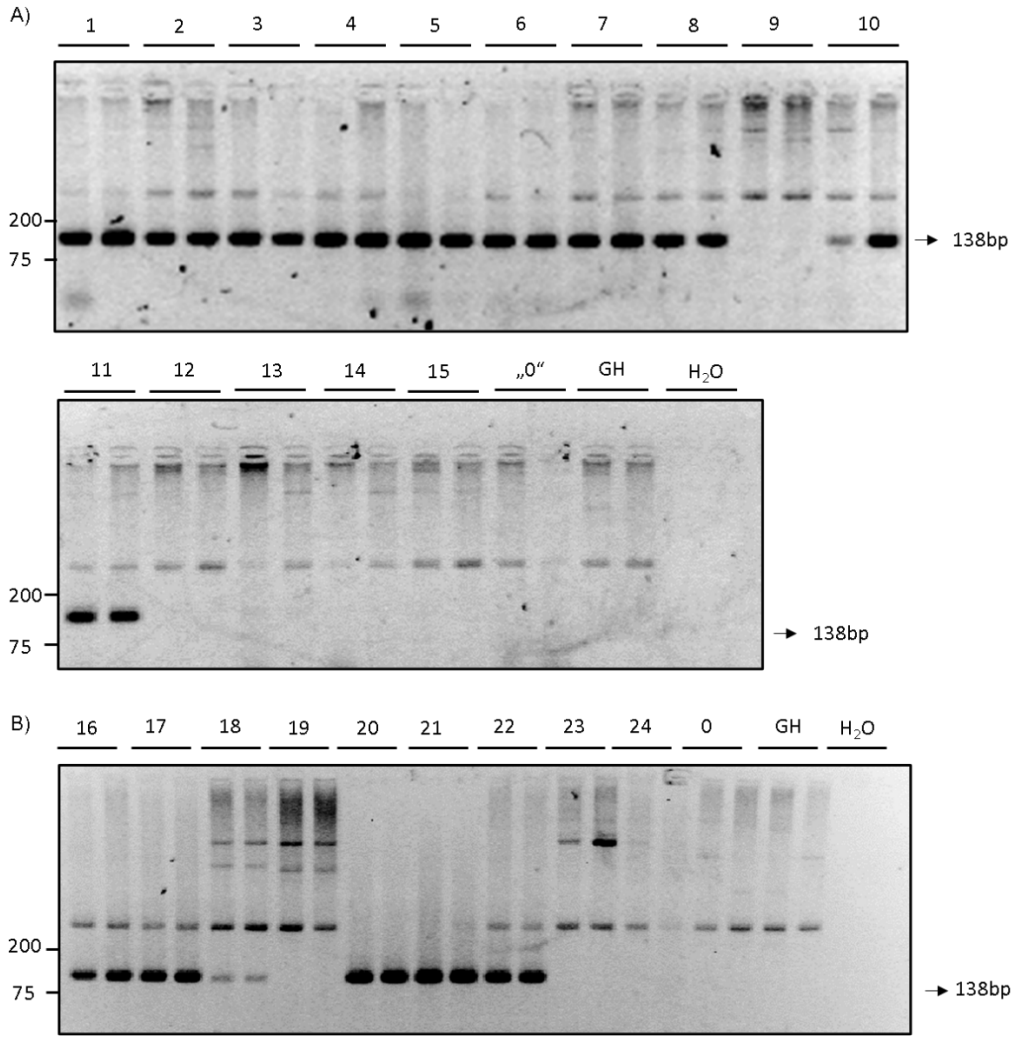


Figure 11| Qualitative assessment of *A. euteiches* infection in roots. Agarose gel electrophoresis of PCR amplified products using *A. euteiches* specific primer set. Gel A are roots samles gathered from bioassay one, gel B from bioassay two, respectively.

3.3 Correlation between DNA-based *A. euteiches* detection and root rot disease index

To investigate the relationship between the quantification of *A. euteiches* DNA in soil and the disease incidence recorded in the bioassay. For this purpose, the number of *A. euteiches* ITS1 genecopies in the soil where to be quantified. To ensure that the primers used were compatible with the *A. euteiches* genetic group in Sweden, but also in the other groups, DNA extracts were used from four isolates of each of the three genetic groups. First, the presence of the DNA was confirmed (Supplementary Figure 1) and then the DNA extracts from the isolates were amplified with the quantification primers via qPCR. The target sequence was

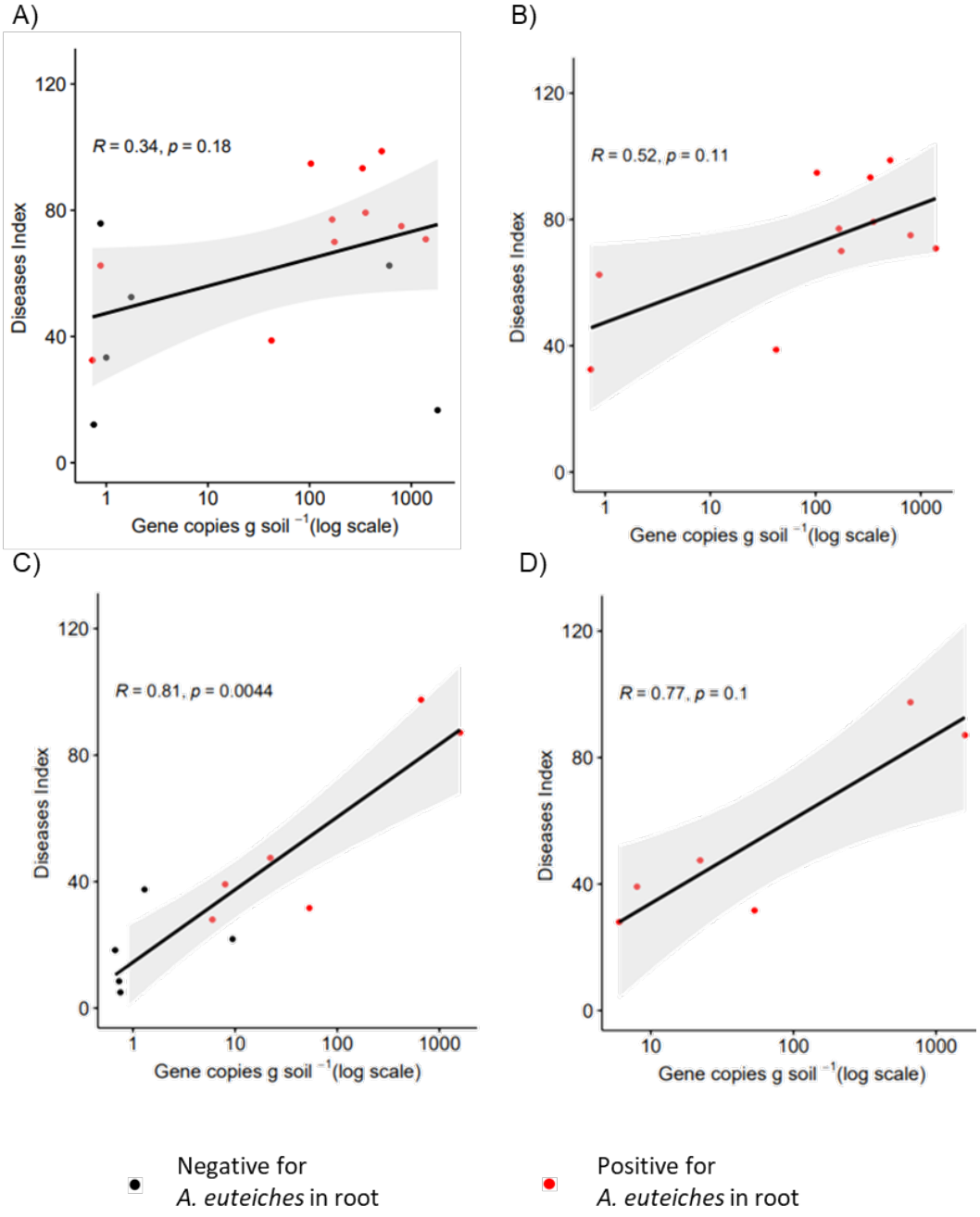


Figure 12| Correlation between disease index and *A. euteiches* gene copies per gram soil. Correlation was determined using Spearman, the coefficient of determination (R) and the significance are given (*p*). Datapoint from samples that showed up positive for *A. euteiches* infection in root are coloured red. A) Correlation between *A. euteiches* gene copies per gram soil and disease index from bioassay 1 B) Correlation between *A. euteiches* gene copies per gram soil and disease index from bioassay 1, only soil samples with positive *A. euteiches* infection of the root determined by PCR included. C) Correlation between *A. euteiches* gene copies per gram soil and disease index from bioassay 2 D) Correlation between *A. euteiches* gene copies per gram soil and disease index from bioassay 1, only soil samples with positive *A. euteiches* infection of the root determined by PCR included.

amplified in all isolates of the Finnish and European groups (Supplementary Table 1). In the Italian isolates, amplification occurred in one of the three isolates, but not in the others (Supplementary Table 1). Since soil samples from Sweden were used in this study, the applicability of the primers was considered acceptable.

Furthermore, DNA eluate was analyzed for contaminants by photospectral analysis at Nanodrop. The A260/A280 ratio for all samples was at least 1.68 as for soil sample 15 (3) or higher, thus fulfilling the requirements for an A260/A280 ratio of ≤1.6, which could indicate protein contamination (Supplementary Table 1). In addition, the A260/A230 ratio indicates whether contamination with carbohydrates, phenols or guanidine HCL may occur. The ratio should be between 2.0 and 2.2 (NanoDrop user manual). Ratios below this indicate contamination with the above mentioned substances. In 47 of the 78 (60%) DNA extracts, the A260/230 ratio is below this range. Contamination with carbohydrates, phenols or guanidine HCL may therefore be present in some of the samples.

In addition, the DNA was quantified by both qPCR and ddPCR. In the qPCR, samples 0, 1 and 7 were experimentally amplified. A spike reaction was performed per sample, which corresponds to the addition of a plasmid with the target sequence. No positive amplification was detected in any of the samples, including those to which a spike was added (Supplementary Table 2). This indicated that the reaction was inhibited. To correlate the DSI with the number of *A. euteiches* gene copies, ddPCR was used as a quantification method. To this end, disease index was plottet against the quantity of *A. euteiches* DNA per gram of soil, as measured by ddPCR, and examined the Spearman correlation for statistical significance. Note that the Spearman correlation coefficient (R) measures the strength and direction of the linear relationship between two variables, and the *P*-value indicates the probability of observing such a correlation by chance.

In bioassay one, a positive correlation was found between the disease index and the number of gene copies per gram of soil, with an R value of 0.34. However, the Spearman correlation did not reach statistical significance ($P = 0.18$) (Figure 12). In contrast, bioassay two showed a stronger positive correlation between the disease index and the number of gene copies per gram of soil, with an R value of 0.81. This correlation was found to be statistically significant with a *P*-value of 0.0044 (Figure 12).

To further investigate the results, a second correlation analysis was conducted that only included soil samples that tested positive for *A. euteiches* infection of the roots (Figure 12). In bioassay one, a weak positive correlation was found, with an R value of 0.52. No statistical significance was observed, with a *P*-value of 0.11 (Figure

12). In bioassay two, a stronger positive correlation was found, with an R value of 0.77. However, the significance was reduced to a *P*-value of 0.1, not reaching statistical significance.

4. Discussion

4.1 Grinding did not improve *A. euteiches* detection via ddPCR

The breaking of the oospores by the grinding and the resulting increased and improved detection of *A. euteiches* did not occur as anticipated. This may be due to the fact that when the oospores break open, the *A. euteiches* DNA is exposed to the environment and thus the risk of degradation is higher than when they are protected in the oospore. Direct DNA extraction of the ground earth was not feasible in this experiment, as the earth was ground externally. In other studies, a reduction in the variance of the results could be achieved by grinding the soil, which was not the case in this study (Gangneux *et al.*, 2014). In other studies, however, DNA was extracted from much smaller amounts of soil (0.5 – 2 gram). The use of 10 gram in this study suggests a higher representability of the soil in the sample and thus a decrease in the variance of the results. Finally, it also seems that the homogenisation steps in the DNA extraction protocol are sufficient to crack the oospores of *A. euteiches,* due to the fact that *A. euteiches* DNA could be amplified. In summary, improved *A. euteiches* detection by means of soil grinding could not be detected in this study, which is why all DNA samples were extracted from sieved soil in the subsequent experiments.

4.2 Bioassay 1 and bioassay 2 could not be combined to one experiment

Due to the different availability of the soil samples, the bioassays were divided into two parts that were held at different times. To allow a combination of the two parts in one experimental analysis, the bioassays were compared. The peas from soil sample 0 and GH were compared here, as they were used in both experiments. The significantly higher emergence rate in both soil 0 and GH in the second bioassay can be explained by the difference in seeding. In bioassay 1, the pot was filled up, watered and a hole was punched with a soil auger in which the pea was sown. Then

the soil above the pea was compressed by hand. The soil was already watered and moist at this point and the clay content of the soil samples made it difficult to cover the pea in a controlled manner. Pea seeds were carried down in the pot to lower soil layers. Very low peas may germinate, but with a high incidence of disease they might never reach the soil surface and be recorded in the emergence index. In the second bio-test, the pot was first filled to 80% with soil, then seeds were sown and the last 20% of the soil was then added. Finally, the soil was irrigated. This prevented the soil from being pushed around and the pea seeds from being carried away. In the future, it would therefore make sense to sow according to method B.

An alternative explanation could be that greenhouse pests that may have been prevalent at the time of the first bioassay and thus may contribute to the symptom exacerbation. During the handling of the first bioassay, an abundance of small flies was noted, which were clustered around the pea plants in the experiment. For example fungus gnats (*Bradysia* spp.*)* are one of the most common greenhouse pests (Cloyd, 2015). This fly species lays its eggs in the soil and the larvae that hatch from them cause damage to the roots. They prefer moist and warm soil (Cloyd, 2015). Since the water retention in soil 0 was significantly higher than that in soil GH, it is possible that the insect caused more damage to plants in soil 0 than to plants in soil GH. The presence of a common greenhouse pest that may have been present at the time of the bioassay could therefore explain the change in disease indices between bioassays.

In general, it can be summarised that it is not clear whether bioassay 1 and bioassay 2 can be considered as one expriment. If the conditions remain the same, it would be expected that there would be no significant differences between the measured variables. However, this is not the case. Furthermore, the plants in bioassay one were sown differently, which probably had an effect on emergence. In the statistical analyses, bioassay 1 and bioassay 2 were therefore considered as separate experiments.

4.3 The disease index did not correlate with *A. euteiches* gene copies per gram of soil

It was hypothesized that there would be a statistically significant correlation between the *A. euteiches* gene copies and the disease severity index. However, this was not achieved in this study. In the following, possible reasons why such a correlation was not measured in this study will therefore be explored.

4.3.1 DNA concentration of *A. euteiches* was not equal to *A. euteiches* disease severity

The relationship between the amount of *A. euteiches* DNA and disease severity, on the other hand, has been described as linear in previous publications (Sauvage *et al.*, 2007; Gangneux *et al.*, 2014). In both studies, soil was artificially infected with *A. euteiches* oospores suspensions. Both Sauvage et al. (2007) and Gagneux et al. (2014) grew *A. euteiches* mycelium on CMA. For oospore production, parts of the agar plates with mycelium were transferred to oat meal broth and incubated for 3 weeks to initiate oospore production. After the three weeks, the oospores were immediately (within 1-3 days) used for inoculation of the soil. The ability of the oospores to germinate at the time of soil inoculation was thus ensured in both studies. There is no such assurance of germination ability of *A. euteiches* oospores when using naturally infested soil. It is therefore possible that non-infectious *A. euteiches* structures are present in the soil which are detected by DNA quantification, but are insignificant for disease development. This is also supported by the fact that soil samples 13 and 14 both showed no infection of the roots with *A. euteiches* in the qualitative analysis. However, high gene copy numbers were measured for both of these samples using ddPCR. For soil sample 13, 608 gene copies * g soil^{-1} and for soil sample 14, 1816 copies * g^{-1} soil were measured. Assuming that these results are not due to errors in method or execution, this would mean that the presence of *A. euteiches* DNA in the soil does not necessarily lead to infection of the root. An explanation of non-infectivity due to resistance on the part of the plant is not plausible. The pea variety Linnea is susceptible to *A. euteiches* and due to the low genetic variance of pea cultivars, genetic resistance to the pathogen is very unlikely.

4.3.2 PRRC and not ARR is assessed in the bioassay

Another reason for the absence of a clear correlation between *A. euteiches* gene copies per g of soil and symptom severity is the fact that all pathogens and their symptoms contained in the soil are scored in the bioassays. The symptoms of the pathogens involved in the PRRC are largely overlapping (Marzougui *et al.*, 2022). Thus, the symptoms of the other pathogens involved in the PRRC are included in the disease assessment. The bioassay is therefore a measure of PRRC and not ARR. This would also explain the pattern of correlation analysis, in which the disease index moves independently of the quantities of *A. euteiches* gene copies per gram of soil. This illustrates the need for a holistic approach to PRRC (Wohor *et al.*, 2022). Molecular detection via DNA quantification should ideally also include several components of PRRC. For example, multiplexing technology could be used to detect several pathogens simultaneously (Gangneux *et al.*, 2014; Wille *et al.*, 2021).

4.3.3 Spearman correlation does not take into account the effect of soil properties

A relationship between *A. euteiches* symptomology and DNA quantity in the natural infested soil was found by Gibert et al. (2021). In this study, soil samples were taken from fields where *A. euteiches* was already known to be present (Gangneux et al., 2014). A total of four fields from the French region Hauts-de-France were tested, all four of which had at least one cropping season with susceptible plants in their recent cropping history (period of 6 years before sampling). In three of the four fields, *P. sativum* was cultivated directly prior to soil sampling (Gangneux et al., 2014). The amount of *A. euteiches* DNA was analysed by ddPCR and the correlation between the disease index and *A. euteiches* DNA quantity was determined by a linear mixed model. A linear mixed model is a statistical tool used to analyze data that contains both fixed and random effects (Nielsen, 2007). In their analysis, Gibert et al. (2021) set the different physico-chemical and biological soil characteristics of the soils as random effects, while the disease index and the number of gene copies were considered fixed effects. Considering that the model can account for the different effects of physico-chemical and biological soil characteristics, and the fact that the physico-chemical and biological soil characteristics of soil have a significant effect on the infectivity of *A. euteiches*, may explain why Gibert et al. were successful in showing the relationship between DI and AG. The fact that Gibert et al. (2021) took all soil samples from four fields, all from the same geographical region, whereas in this study soil samples were taken from 15 different fields from different growing regions, highlights the need to include the the effect of the soil characteristics in the statistical model, which where not accounted for in this study using only spearman correlation as statistical model. The spearman correlation applied in this study could not account for such random effects of the earth samples, which could have biased the result and therefore no correlation could be measured.

4.3.4 Composition of the rhizosphere microbiota can influence ARR and PRRC

Another explanation for the fact that Gibert et al. (2021) measured a correlation, but this study did not, could be due to the different microbial compositions of the soil. There are several publications on the microbial composition of the fungi involved in the PRRC. For example, in an analysis of PRRC-affected plant roots with soil from a field in the Swiss canton of Bern, the pathogen *Fusarium oxysporum* was detected most frequently and determined to be the most devastating pathogen in PRRC (Wille *et al.*, 2020). In a similar experiment with peas grown in

soil samples from Hesse in Germany, *Peyronellaea pinodella* was detected most frequently (Bacanovic-Sisic *et al.*, 2018). While *F. oxysporum* was also frequently detected in the German experiment, *P. pinodella* was not detected in the Swiss experiment. While such a study is not available with regard to oomycete compositions such as *A. euteiches*, it stands to reason that fluctuations of microbial composition in Sweden compared to France could be likely. Furthermore, plants have a great influence on the microbial composition of the soil (Chaudhari *et al.*, 2020). For example, the PRRC history and cropping history with *A. euteiches* hosts of the fields tested by Gibert et al. could have led to a build-up of *A. euteiches*. The presence of PRRC-diseased pea roots significantly changed the microbial composition of the soil in a study of Hossain et al. (2020). The rhizosphere of diseased roots was associated with a significant increase in bacterial, fungal and oomycete α-diversity (Hossain *et al.*, 2021). In addition, a shift in microbiome composition was observed. Healthy samples were associated with a higher relative abundance of *Rhizobium*, *Olpidium* and *Mortierella*, and lower abundance of *Fusarium* and *Pythium* (Hossain *et al.*, 2021). In this study, not all soil samples were taken from fields with host cropping history. In addition, more than 10 different pathogen species are involved in the development of PRRC, which influence and interact with each other in largely unknown ways (Wille *et al.*, 2021). Different combinations of pathogens with each other and with other microbes can act both synergistically and antagonistically on each other, thereby both relieving and exacerbating PRRC symptoms (Lamichhane and Venturi, 2015). For example, synergistic effects were noted in the coinoculation of *A. euteiches* with both *Pythium ultimum* and *Fusarium* spp. in greenhouse experiments (Pfender and Hagedorn, 1982; Willsey *et al.*, 2018). In both cases, there was an increase in susceptibility of both pathogens involved and thus increased disease incidence.

4.3.5 The method needs to be optimised further

Another way to explain the absence of a relationship between disease incidence and pathogen DNA quantity is that there is a possible constraint in the quantification method. The functionality of the primer pairs used to amplify the ITS1 genomic region of *A. euteiches* was tested on the three different genetic groups. Compitability was confirmed on both the Northern European and European groups. However, in three of the four Italian isolates tested, no amplification was detected, indicating that there is a mutation in this genetic group that prevents the primers from binding. Caution is therefore advised when using these primers for *A. euteiches* detection in Italy. However, applicability in Sweden, as in this experiment, is ensured.

The presence of inhibitors in the reaction can interfere with the amplification and affect the results. Inhibitors are substances that can interfere with the amplification of DNA in the ddPCR reaction. The lack of amplification of the spike shows that the qPCR was inhibited. The spike contained a plasmid with the target DNA and was therefore combined with the test sample in an extra well as a positive control in addition to the sub-samples. The amplification of the plasmid worked well in the wells without experimental DNA, i.e. in the standard series and the positive control. The presence of inhibitors in the experimental DNA preparations is therefore obvious. The same DNA extract preparations were also used in ddPCR, where amplification of the target DNA was possible, i.e. the reaction was not inhibited. However, a spike was not used in the ddPCR. Therefore, possible partial inhibitions that lead to deviating results but not to total inhibition of the reaction cannot be traced. In future experiments, the addition of a spike would also be useful when performing ddPCR. Nevertheless, the increased robustness of the ddPCR method compared to qPCR is exemplified by this.

PCR inhibitors often carried during DNA extraction from soil include various proteins (and proteases), calcium and metal ions, debris, fulmic and humic acids, poly-/phenols, polysaccharides, clay particles and salts (Schrader *et al.*, 2012). Inhibitors that can often be introduced during extraction are organic liquids such as ethanol and different salts (NanoDrop User manual, no date; Schrader *et al.*, 2012).

In the photospectral analysis using the Nanodrop, protein contamination was assessed based on the A260/A280 ratio, which met the specified requirements (Lucena-aguilar et al., 2016). However, the A260/A230 ratio, indicative of contamination with carbohydrates, phenols, or guanidine HCL, did not meet the recommended range (2.0 to 2.2) in 60% of DNA extracts, suggesting potential presence of these contaminants in some samples (NanoDrop User manual). These contaminants risk acting as inhibitors during qPCR and might skew the results.

PCR inhibitors can be removed by DNA purification, for which there is a wide range of methods. Heyman (2008) purified his DNA extracts from 350 mg soil using two different methods. First, the Wizard® DNA Clean-Up System (Promega) was used, which filters the eluate through a silicate membrane. This method is able to remove enzymes (including restriction enzymes, phosphatases, kinases, DNA polymerases and nucleeases), salts and nucleotides (Wizard ® DNA Clean-Up System User Manual). Subsequently the illustra MicroSpin 300 Columns (Cytiva), where utilized which use gel filtration containing different pore sizes to purify the DNA. Hereby further desalting, primer removal and buffer exchange was enabled (illustra MicroSpin Columns Product Booklet). Finally the DNA was diluted 1:5.

In this experiment, NucleoBond Finalizers (Macherey Nagel) were used to concentrate the DNA eluates. The DNA is hereby filtered by a polypropylene filter containing a silica membrane, allowing for desalting (NucleoBond® Finalize User manual). Subsequently, however, the samples were further concentrated by a method involving the addition of salt and ethanol. The absence of salt and ethanol in the final eluate is therefore not given. Additional filtration with, for example, the illustra MicroSpin 300 Columns (Cytiva) would be advantageous. Subsequent dilution of the sample could also be beneficial. This would allow the dilution of any inhibitors that might remain.

It should also be noted that too high DNA quantities during qPCR can also lead to a complications of the reaction. Excessive amounts of DNA in the reaction vessel can cause the DNA to become densely packed in the limited space, resulting in the possibility of false priming and hindered diffusion of Taq polymerase molecules. Further, too high DNA quantities can also result in the saturation of the PCR reaction, where the amplification of the target sequence reaches a plateau phase due to the depletion of PCR reagents, such as dNTPs or Taq polymerase. This can lead to reduced amplification efficiency (Ma, Bell and Loker, 2021). Depending on the composition of the qPCR components and the reaction volume, DNA quantities from 0.1 ng to 1000 ng can be applied in qPCR (Meyer, 2010; Life Technologies Corporation, 2012). For high DNA quantities of 1000 ng per reaction unit, larger reaction volumes of 50 µl are recommended (Ma, Bell and Loker, 2021). In the qPCR carried out in this project, the DNA quantities per reaction unit ranged between 1600 and 6550 ng per 25 µl reaction unit, which is significantly higher that the recommended amounts. Dilution of the DNA template would therefore make sense from two points of view: on the one hand, dilution of residual inhibitors and, on the other hand, avoidance of blockage of the qPCR due to excessively high DNA quantities.

Furthermore, during ddPCR, if the concentration of DNA is too high, it can lead to an overloading of the droplets, which can affect the accuracy and precision of the ddPCR measurement. In addition, high concentrations of DNA can lead to an increased likelihood of partitioning errors, where more than one template molecule is present in a droplet, leading to false positives (Bio-Rad). According to the manufacturer guidelines of the digital droplet PCR systems (Bio-Rad), no more than 1000 ng of DNA should be loaded per PCR unit. This was exceeded many times over in this study. The average amount of DNA loaded in this study was about 8000 ng, which is eight times higher than the upper limit set by the manufacturer. In addition, the manufacturer recommends digesting the DNA into smaller fragments before performing the test for quantities of 60 ng DNA per reaction unit or more. This is also very important for the correct partition into the droplets.

Gagneux *et al.* (2021) investigated the effect of DNA digestion in the detection of *A. euteiches* with ddPCR and were able to achieve a more conclusive result, whereby the difference between positive and negative droplets became clearer subsequent to DNA digestion. Such a DNA digest was not carried out in the context of this study. In future, therefore, the template DNA should be purified, diluted and digested prior to the performance of ddPCR.

In summary, in future experiments with both qPCR and ddPCR, the DNA should be purified and diluted before quantification. In addition, when performing ddPCR, the DNA should be digested into smaller fragments. For both qPCR and ddPCR, the manufacturer's instructions should be followed more closely to ensure optimal reaction conditions.

5. Conclusions and outlook

Pea growers seek to evaluate *A. euteiches* infection risk before planting, aiming to prevent potential crop losses. This study determined whether grinding the soil samples improves the detection of *A. euteiches*. No such effect was found. Milling did not stabilise or increase DNA harvest or increase target DNA detection with ddPCR. Therefore, in the following experiments, DNA was extracted from unground soil samples. Furthermore, the relationship between ARR symptom development and quantified amount of *A. euteiches* DNA was investigated. For this purpose, peas from 26 different soil samples were grown in the greenhouse and evaluated after about 3 weeks according to their symptoms. The *A. euteiches* DNA in the soil was determined by ddPCR and examined for correlation with the disease index. No significant correlation was found. The reason for this could be the microbial composition of the soil which can influence the symptoms, the strong effect of physico-chemical and biological soil characteristics on disease development which cannot be accounted for in the statistical analysis, and obstacles in the quantification method.

The quantification of DNA by qPCR was also tested on a selection of samples, but because the reaction was inhibited, no results could be obtained. The fact that ddPCR was not (completely) inhibited underlines the increased robustness of ddPCR to interfering factors like inhibitors.

In the future, quantification by qPCR could possibly be made available by eliminating inhibitors in die DNA eluate. In addition, a DNA digest in the context of ddPCR could lead to an improvement of the method.

It is questionable whether optimising the ddPCR would also change the result of the soil grinding analysis in terms of the amount of *A. euteiches* DNA detected. It would therefore be advisable to repeat the experiments as soon as the ddPCR could be optimised.

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Popular science summary

Aphanomyces euteiches is a plant pathogen that lives in the soil. It infects the roots of economically important plants such as peas, leading to considerable crop loss. If there is an infestation in the field, there is not much you can do to protect your crop. Therefore, farmers want to know in advance if the soil they are planting in is at high risk for *A. euteiches* infection. You can either test the soil in a green house test and then see if the plants show symptoms, or use molecular methods to detect the pathogen in the soil. The latter would save a lot of time and money. Molecular prediction relies on quantifying *A. euteiches'* specific DNA in the soil. A commercial experimental protocol for this does not yet exist because previous attempts to establish it have failed. Particularly difficult is the fact that even very small amounts of *A. euteiches* in the soil are enough to cause great damage to the plant, because this means that the soil test has to be very sensitive to work. In addition, *A. euteiches* forms spores that are very robust and need to be broken open to detect the DNA in them. In this study, it was investigated whether grinding the soil improves the detection of *A. euteiches*. However, grinding did not improve the detection of *A. euteiches.* In addition, two methods were used to quantify the DNA in the soil. One is slightly older and is called qPCR, the other method is newer and is called ddPCR. Quantification with qPCR was not possible under the given conditions and the reaction appeared to be inhibited. Therefore, it should be investigated in the future whether the inhibition of the experiment can be remedied. Using ddPCR, it was possible to quantify *A. euteiche's* DNA, which is due to the fact that this method is significantly more robust against interfering factors. Finally, it was investigated whether the amount of *A. euteiches* DNA can be used to predict the extent of the disease. For this purpose, it was investigated whether these two factors correlate with each other. This was not the case. This could either be because the quantification method did not work properly or because there were other pathogens in the soil that also made the plant sick. A combination of both would also be possible.

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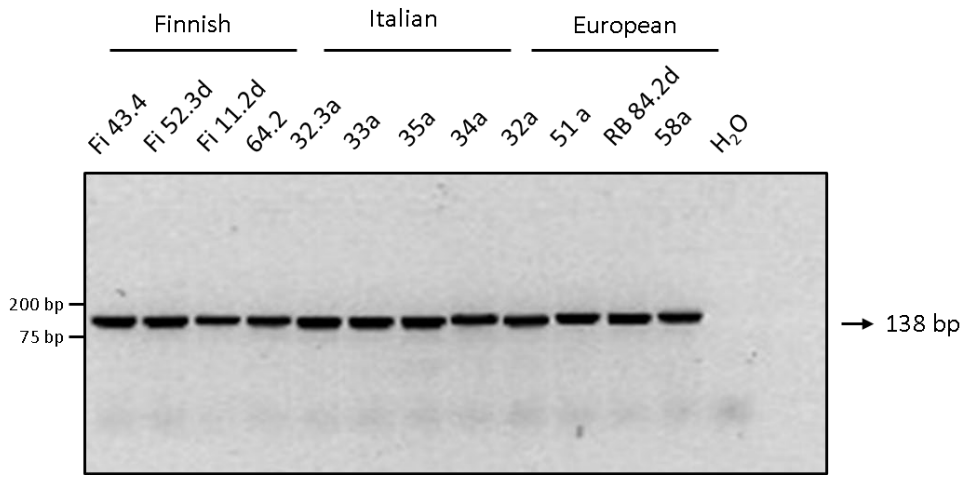
Supplementary Figures



Supplementary Figure 1| Confirmation of DNA content of DNA preparation of *A. euteiches* isolate DNA extracts. PCR amplification with primers Ae37 (Kälin *et al.*, 2022), that bind to all genetic groups, was peformed on DNA samples and subsequently visualised by gel electrophoresis. The sought-after product of 137 bp was found in all DNA preparations, indicating presence of *A. euteiches* DNA in all samples.

Supplementary Table 1| Test of PCR primer specificity towards three genetic *A. euteiches* groups[1].

Genetic group	*A. euteiches* isolate	Amplification
Finnish	FI 43	+
	FI 52	+
	FI 11	+
	SE 64	+
European	NO 1	+
	SE 51	+
	FR RB 84	+
	SE 58	+
Italian	IT 32	+
	IT 33	-
	IT 34	-
	IT 35	-

[1]PCR primers used for amplification by qPCR and ddPCR was tested on DNA from three genetic groups (Finnish, European and Italian).
[2]Successful amplification is indicated by +, failure to amplify is indicated by -.

Supplementary Table 2| Amplification of *A. euteiches* DNA by qPCR[1].

Sample	Dilution	CT
0	Undiluted	n.a.
0 + Spike	Undiluted	n.a.
0	1:5	n.a.
0 + Spike	1:5	n.a.
0	1:10	n.a.
0 + Spike	1:10	n.a.
1	Undiluted	n.a.
1 + Spike	Undiluted	n.a.
1	1:5	n.a.
1 + Spike	1:5	n.a.
1	1:10	n.a.
1 + Spike	1:10	n.a.
7	Undiluted	n.a.
7 + Spike	Undiluted	n.a.
7	1:5	n.a.
7 + Spike	1:5	n.a.
7	1:10	n.a.
7 + Spike	1:10	n.a.
Plasmid	$1:10^{-7}$	25.9
Plasmid	$1:10^{-8}$	27.
Plasmid	$1:10^{-9}$	30.3
Plasmid	$1:10^{-10}$	34.3

[1]Samples 0, 1 and 7 were amplified by qPCR undiluted, diluted 1 to 5 and diluted 1 to 10. In addition, a spike corresponding to 2 µl of a $1:10^{-6}$ dilution of a plasmid containing the amplicon was added to a replicate. A standard series with the same plasmid from dilutions 10^{-7} to 10^{-10} was also included.

Supplementary Table 3| DNA concentration and quality extracted from soil samples.

Soil ID	Grinding	Replicate	Final DNA eluate: DNA concentration [ng/µ]	Final DNA eluate: A260/A280	Final DNA eluate: A260/A230	DNA quantitity per reaction unit ddPCR
1	S	1	737.90	1.86	2.02	7379
1	S	2	524.40	1.84	1.93	5244
1	S	3	789.10	1.85	1.98	7891
1	A	1	928.20	1.85	1.89	9282
1	A	2	1383.00	1.86	1.97	13830
1	A	3	1570.30	1.86	1.99	15703
1	B	1	765.20	1.85	2.05	7652
1	B	2	640.60	1.85	2.00	6406
1	B	3	569.90	1.85	1.96	5699
2	S	1	220.70	1.79	1.73	2207
2	S	2	552.40	1.78	1.59	5524
2	S	3	509.20	1.76	1.55	5092
2	A	1	9.30	1.55	1.43	93
2	A	2	106.50	1.73	1.27	1065
2	A	3	9.60	1.72	1.24	96
2	B	1	319.80	1.83	1.75	3198
2	B	2	165.30	1.77	1.44	1653
2	B	3	7.70	1.55	1.04	77
3	S	1	143.10	1.82	1.91	1431
3	S	2	96.70	1.79	1.67	967
3	S	3	227.30	1.81	1.75	2273
3	A	1	215.20	1.79	1.60	2152
3	A	2	82.90	1.69	1.14	829
3	A	3	24.40	1.63	1.00	244
3	B	1	329.80	1.84	1.85	3298
3	B	2	237.90	1.79	1.58	2379
3	B	3	412.10	1.81	1.77	4121
4	S	1	573.30	1.82	1.79	5733
4	S	2	455.90	1.83	1.70	4559
4	S	3	522.40	1.82	1.70	5224
5	S	1	271.50	1.81	1.64	2715
5	S	2	316.50	1.80	1.62	3165
5	S	3	351.20	1.79	1.68	3512
6	S	1	1.406.80	1.88	2.10	14068
6	S	2	1.350.60	1.87	2.13	13506

6	S	3	1.920.90	1.87	2.03	19209
7	S	1	625.80	1.85	1.99	6258
7	S	2	1.303.60	1.87	2.06	13036
7	S	3	761.70	1.89	2.06	7617
7	A	1	1.491.40	1.87	2.07	14914
7	A	2	1.255.00	1.87	2.05	12550
7	A	3	1.587.90	1.87	2.05	15879
7	B	1	1.263.30	1.86	2.04	12633
7	B	2	324.60	1.82	1.99	3246
7	B	3	817.20	1.84	2.08	8172
8	S	1	570.70	2.02	4.50	5707
8	S	2	465.90	1.85	1.89	4659
8	S	3	689.30	1.86	1.90	6893
9	S	1	1.061.80	1.85	1.95	10618
9	S	2	1.323.10	1.87	2.05	13231
9	S	3	1.089.70	1.85	1.96	10897
10	S	1	1.068.20	1.87	2.02	10682
10	S	2	924.00	1.85	2.00	9240
10	S	3	937.7	1.86	1.94	9377
10	A	1	885.7	1.86	2.01	8857
10	A	2	1218.8	1.88	2.07	12188
10	A	3	1651.8	1.88	2.1	16518
10	B	1	1239	1.86	2.1	12390
10	B	2	1439.6	1.86	2.02	14396
10	B	3	2169.6	1.87	2.09	21696
11	S	1	639.7	1.87	1.99	6397
11	S	2	599.7	1.86	2.01	5997
11	S	3	771	1.87	2.06	7710
11	A	1	2524	1.89	2.16	25240
11	A	2	1273.6	1.87	2.13	12736
11	A	3	1421.4	1.86	2.13	14214
11	B	1	3761.7	1.87	2.1	37617
11	B	2	1790	1.87	2.03	17900
11	B	3	1416.2	1.88	2.06	14162
12	S	1	1195.5	1.85	1.86	11955
12	S	2	501.5	1.82	1.72	5015
12	S	3	362	1.81	1.66	3620
12	A	1	342.1	1.84	1.96	3421
12	A	2	719.9	1.86	1.9	7199
12	A	3	1011	1.87	1.98	10110
12	B	1	339.5	1.8	1.65	3395
12	B	2	507.2	1.82	1.76	5072
12	B	3	742.3	1.84	1.83	7423
13	S	1	1345.6	1.87	1.95	13456

13	S	2	1127.7	1.88	2.07	11277
13	S	3	658.8	1.85	2.07	6588
13	A	1	551.5	1.81	1.69	5515
13	A	2	441.5	1.77	1.51	4415
13	A	3	340	1.79	1.75	3400
13	B	1	873.9	1.86	1.98	8739
13	B	2	797	1.84	1.86	7970
13	B	3	1185.5	1.85	1.98	11855
14	S	1	1306.2	1.88	2.08	13062
14	S	2	1014.9	1.87	2.09	10149
14	S	3	822.6	1.86	2.04	8226
14	A	1	146.7	1.79	1.78	1467
14	A	2	256.4	1.75	1.43	2564
14	A	3	230.6	1.74	1.45	2306
14	B	1	1053.3	1.85	2.05	10533
14	B	2	679	1.85	1.95	6790
14	B	3	1033.1	1.87	1.99	10331
15	S	1	948.5	1.86	2.11	9485
15	S	2	1608.4	1.89	2.15	16084
15	S	3	1599.6	1.88	2.16	15996
15	A	1	1346.3	1.89	2.15	13463
15	A	2	492.1	1.83	1.82	4921
15	A	3	157.5	1.68	1.18	1575
15	B	1	448.6	1.82	1.82	4486
15	B	2	727.5	1.83	1.82	7275
15	B	3	1051	1.85	1.98	10510
16	S	1	384.9	1.79	1.93	3849
16	S	2	671	1.84	2.12	6710
16	S	3	319.4	1.83	1.87	3194
17	S	1	442.8	1.83	2.08	4428
17	S	2	516.3	1.82	1.98	5163
17	S	3	503.7	1.82	1.83	5037
18	S	1	627.30	1.86	2.03	6273
18	S	2	912.30	1.86	2.14	9123
18	S	3	1.228.20	1.87	2.12	12282
19	S	1	425.7	1.82	1.97	4257
19	S	2	450.2	1.82	1.94	4502
19	S	3	1962	1.88	2.12	19620
20	S	1	1028.5	1.87	2.03	10285
20	S	2	968.5	1.87	2.12	9685
20	S	3	1337.7	1.87	1.97	13377
21	S	1	624.7	1.85	1.98	6247
21	S	2	1162.3	1.87	2.07	11623
21	S	3	1024.4	1.86	2.06	10244

22	S	1	1012.1	1.85	2.09	10121
22	S	2	752.3	1.91	2.36	7523
22	S	3	1001.9	1.85	1.98	10019
23	S	1	1156.1	1.85	1.97	11561
23	S	2	735.6	1.83	1.76	7356
23	S	3	552.4	1.81	1.73	5524
24	S	1	610.8	1.71	1.32	6108
24	S	2	584.6	1.75	1.44	5846
24	S	3	633.1	1.75	1.38	6331
0	S	1	518	1.81	1.64	5180
0	S	2	339.8	1.79	1.5	3398
0	S	3	471.2	1.82	1.57	4712
KJ	S	1	762.9	1.85	1.88	7629
KJ	S	2	752.5	1.86	1.96	7525
KJ	S	3	863.5	1.85	1.94	8635
Average			814.58			8145.7

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