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Aphanomyces root rot in pea

Genomic insights into pathogen diversity and disease resistance

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Cover: Pea seedlings showing various grades of typical aphanomyces root rot symptoms (photo by Cajsa Lithell).

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Abstract

The cultivation of vining pea (*Pisum sativum*) faces significant challenges due to root rot, primarily caused by *Aphanomyces euteiches*, a soil-borne oomycete pathogen. Pea cultivars resistant to aphanomyces root rot (ARR) are currently lacking and common mitigation methods include crop rotation and avoidance of highly infested fields. The thesis work integrates population genetic, transcriptomic, and comparative genomic analyses to enhance our understanding of aphanomyces root rot in pea, providing valuable knowledge for future breeding and disease management efforts.

Genetic diversity analyses of *A. euteiches* in Europe revealed three distinct groups on a north to south gradient: a north-eastern (NE), central European (CE) and southern (S) group. Shared multilocus genotypes between geographically distant regions indicate genetic movement between countries. The S group differed in virulence and oospore size compared to NE and CE and displayed signs of genetic isolation, all of them indications to view group S as a separate *Aphanomyces* species.

The use of partial resistance in the pea genotype PI180693 was assessed in crosses with the susceptible, commercial cultivar Linnea in controlled and field conditions. The new breeding lines displayed enhanced disease resistance to ARR compared to the susceptible parent. The results further highlighted the difficulties of predicting breeding line performance in the field based on trials in controlled conditions and of breaking the linkage between ARR resistance and unfavourable breeding traits in classical breeding. Characterization of the transcriptomic immune response in Linnea and PI180693 to *A. euteiches* infection showed to be time- and genotype-dependent, involving differential regulation of transcription factors and genes associated with hormone signalling. Cross-referencing with genes located in resistance quantitative trait loci led to the identification of 39 candidate disease resistance genes, including a putative immune receptor with NLR structure that was polymorphic between the pea genotypes.

Keywords: aphanomyces root rot, genetic diversity, oomycete, pea breeding, quantitative trait loci, resistance genes, speciation, transcriptomics, virulence

Aphanomyces ärtrotröta: genomiska insikter om patogenens diversitet och sjukdomsresistens

Sammanfattning

Odlingen av ärt (*Pisum sativum*) står inför betydande utmaningar på grund av rotröta, främst orsakad av patogenen *Aphanomyces euteiches*, en marklevande algsvamp. Det saknas kommersiella ärtsorter som är resistenta mot rotröta, och metoder för att begränsa sjukdomen inkluderar en varierad växtföljd och att undvika odling av ärt på mycket angripna fält. Avhandlingsarbetet integrerar populationsgenetiska, transkriptomiska och jämförande genomiska analyser för att förbättra vår förståelse av aphanomyces rotröta i ärter, och ger värdefull kunskap att basera framtida insatser inom förädlings- och sjukdomsbegränsning på.

Genetiska analyser av *A. euteiches* i Europa avslöjade tre distinkta grupper på en nord-sydlig gradient: den nordöstra (NE), centraleuropeiska (CE) och södra (S) gruppen. Gemensama multilocus-genotyper mellan geografiskt avlägsna regioner indikerar förflyttning av *A. euteiches* mellan länder. S-gruppen skiljde sig åt i virulens och storlek på oosporer jämfört med NE och CE, och visade tecken på genetisk isolering, sammantaget tyder resultaten på att grupp S kan betraktas som en separat *Aphanomyces* art.

Användningen av partiell resistens mot ärtrotröta från genotyp PI180693 utvärderades i korsningar med den mottagliga, kommersiella sorten Linnea under kontrollerade förhållanden och i fält. De nya förädlingslinjerna visade förbättrad sjukdomsresistens mot ärtrotröta jämfört med den mottagliga föräldern. Dessutom underströk resultaten svårigheterna med att förutsäga förädlingslinjernas prestanda i fält baserat på försök under kontrollerade förhållanden och att bryta kopplingen mellan sjukdomsresistens och ogynnsamma egenskaper i klassisk förädling. Det transkriptomiska immunsvaret hos Linnea och PI180693 mot infektion med *A. euteiches* visade sig vara tids- och genotypberoende, och involverade differentierad reglering av transkriptionsfaktorer och gener associerade med hormonell signalering. En jämförelse med gener som är fysiskt lokaliserade i genomiska regioner som segregerar med sjukdomsresistens ledde till identifiering av 39

kandidatgener för sjukdomsresistens, inklusive en förmodad immunreceptor med NLR-struktur som var polymorf mellan ärtgenotyperna.

Nyckelord: ärtförädling, aphanomyces rotröta, oomycet, genetisk mångfald, virulens, artbildning, transkriptomik, loci för kvantitativa egenskaper, resistensgener

Aphanomyces-Wurzelfäule der Erbse: Genomische Erkenntnisse über Pathogenvielfalt und Krankheitsresistenz

Zusammenfassung

Der Anbau der Gartenerbse (*Pisum sativum*) ist stark eingeschränkt durch das Auftreten von Wurzelfäule, die hauptsächlich durch den bodenbürtigen Krankheitserrerger *Aphanomyces euteiches* verursacht wird. Kommerzielle Erbsensorten mit vollständiger Resistenz gegen Wurzelfäule gibt es derzeit nicht, und zu den üblichen Methoden zur Schadensbegrenzung im Erbsenanbau gehören Wechsel in der Fruchtfolge und die Vermeidung von stark befallenen Feldern. Diese Dissertationsarbeit beinhaltet populationsgenetische, transkriptomische und vergleichende genomische Analysen, die zum Verständnis der Aphanomyces-Wurzelfäule bei Erbsen und zukünftigen Resistenzzuchtprogrammen beitragen.

Analysen zur genetischen Diversität von *A. euteiches* in Europa beschrieben drei verschiedene genetische Gruppen: eine mitteleuropäische, eine nordöstliche und eine genetisch sehr unterschiedliche Gruppe im Süden. Gemeinsame Multilocus-Genotypen zwischen geografisch entfernten Regionen deuten auf Gentransfer zwischen europäischen Ländern hin. Die Gruppe der *A. euteiches*-Isolate aus dem Süden unterschied sich in Bezug auf Virulenz und Oosporengröße im Vergleich zu den Gruppen in Zentral- und Nordosteuropa. Zudem zeigte sie Anzeichen genetischer Isolation innerhalb der Gruppe auf - zusammengenommen Indikatoren dafür, die Gruppe im Süden als differenzierte *Aphanomyces*-Spezies zu betrachten.

Das Potenzial der partiellen Resistenz des Erbsen-Genotyps PI180693 wurde in Kreuzungen mit dem anfälligen, kommerziellen Kultivar Linnea in Gewächshaus- und Feldversuchen geprüft. Die neuen Introgressionslinien zeigten eine erhöhte Resistenz gegen Wurzelfäule im Vergleich zur anfälligen Parentalgeneration. Die Ergebnisse verdeutlichten zudem die Schwierigkeiten bei der Vorhersage der Leistung von Zuchtlinien im Feld auf der Grundlage von Versuchen unter kontrollierten Bedingungen, und die Kopplung von *A. euteiches*-Resistenzgenen und ungünstigen Zuchtmerkmalen. Die transkriptomische Immunreaktion auf *A. euteiches*-Befall in Linnea und PI180693 zeigte eine zeit- und genotypabhängige

Regulierung von Transkriptionsfaktoren und Genen, die mit der Regulierung von Hormonen verbunden sind. Ein Vergleich mit Genen, die in sogenannten ”quantitative trait loci” (QTL) für *A. euteiches*-Resistenz liegen, führte zur Identifizierung von 39 potenziellen Resistenzgenen für zukünftige markergestützte Selektion. Darunter befand sich unter anderem ein mutmaßlicher Immunrezeptor mit ”nucleotide-binding leucine-rich repeat” (NLR)-Struktur, der Polymorphismen zwischen den Erbsengenotypen aufwies.

Stichworte: Aphanomyces-Wurzelfäule, Erbsenzucht, genetische Diversität, quantitative trait loci, Resistenzgene, Transkriptomische Immunreaktion, Virulenz

Dedication

To my parents

Contents

List of publications

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

I. **Kälin, C.**, Berlin, A., Kolodinska Brantestam, A., Dubey, M., Arvidsson, A.-K., Riesinger, P., Elfstrand, M., Karlsson, M. (2022). Genetic diversity of the pea root pathogen *Aphanomyces euteiches* in Europe. Plant Pathology, 71, 1570-1578. 10.1111/ppa.13598

II. **Kälin, C.**, Kolodinska Brantestam, A., Arvidsson, A.-K., Dubey, M., Elfstrand, M., Karlsson, M. (2023). Evaluation of pea genotype PI180693 partial resistance towards aphanomyces root rot in commercial pea breeding. Frontiers in Plant Science, 14. 10.3389/fpls.2023.1114408

III. **Kälin, C.**, Piombo, E., Bourras, S., Kolodinska Brantestam, A., Dubey, M., Elfstrand, M., Karlsson, M. (2024). Transcriptomic analysis identifies candidate genes for Aphanomyces root rot disease resistance in pea. BMC Plant Biology, 24, 144, 10.1186/s12870-024-04817-y

IV. **Kälin, C.***, Piombo, E.*, Dubey M., Dubusc, S., Karlsson, M. Comparative genomics provides insight into the evolution of European *Aphanomyces euteiches* strains. (**shared first authorship, manuscript*)

Papers I - III are reproduced with the permission of the publishers.

The contribution of Carol Kälin to the papers included in this thesis was as follows:

I. Performed the lab experiments, performed main parts of data analysis and writing.

II. Contributed to conceptualization and performed greenhouse and growth chamber experiments; analyzed the data and wrote the manuscript.

III. Designed and performed experiment, analyzed the data and was the leading author of the manuscript.

IV. Contributed to conceptualization and planning of experiments, contributed to data analysis and manuscript writing.

List of tables

List of figures

Abbreviations

aa	Amino acid
ABA	Abscisic acid
ABC	ATP-binding cassette
ARR	Aphanomyces root rot
ATP	Adenosine 5’-triphosphate
bp	Base pair
BSR-seq	Bulked segregant RNA-seq
BUSCO	Benchmarking universal single-copy orthologs
CAFE	Computational analysis of gene family evolution
CAZyme	Carbohydrate-active enzymes
CE1	Carbohydrate esterases 1
DEGs	Differentially expressed genes
DI	Disease index
EST	Expressed sequence tag
ET	Ethylene
ETI	Effector-triggered immunity
ETS	Effector-triggered susceptibility
FLS2	FLAGELLIN SENSING 2

GCPSR	Genealogical concordance phylogenetic species recognition
GSTs	Glutathione S-transferases
GWAS	Genome-wide association studies
hpi	Hours post inoculation
JA	Jasmonic acid
LRR	Leucine-rich repeat
MAMP	Microbe-associated molecular pattern
MAS	Marker assisted selction
MFS	Major facilitator superfamily
MLG	Multilocus genotypes
MSN	Minimum spanning networks
MTI	MAMP-triggered immunity
MYB	Myeloblastosis
NIL	Near isogenic line
NJ	Neighbour-joining
NLR	Nucleotide-binding domain leucine-rich repeat
PAMP	Pathogen-associated molecular patterns
PCA	Principal component analysis
PCoA	Principal coordinate analysis
PCR	Polymerase chain reaction
PRR	Pattern-recognition receptor
PRRC	Pea root rot complex
Psd2	*P. sativum* defensin 2
PTI	PAMP-triggered immunity
QTL	Quantitative trait loci
RAPD	Randomly amplified polymorphic DNA

RIL	Recombinant inbred lines
RLK	Receptor-like kinase
ROS	Reactive oxygen species
RUN	Resistance to *Uncinula necator*
SNP	Single nucleotide polymorphisms
SP	Signal peptide
SSR	Simple sequent repeat
TM	Transmembrane domain

1. Introduction

The worldwide demand for pea and pea-based products, is increasing as the legume offers a great source of protein in food and feed. Pea cultivation, however, faces several biotic and abiotic stresses that can result in severe yield reductions. Among the biotic threats, aphanomyces root rot (ARR), caused by the oomycete *Aphanomyces euteiches* is the most devastating disease in commercial pea production. Mitigation methods are limited and resistance sources in the pea host are scarce. This thesis work aims at a better understanding of the *A. euteiches* – pea pathosystem. More specifically, the genetic diversity of *A. euteiches* and its virulence phenotype, as well as the pea immune response to *A. euteiches* infection and single genes associated with disease resistance are under investigation. The identification of candidate disease resistance genes and their use in marker-assisted selection breeding aims at the deployment of resistant pea cultivars in the future.

The thesis builds on previous knowledge on known resistance loci in the pea genome and the use of pea genotypes that have proven to be partially resistant to ARR. In this thesis, I demonstrate that we can observe genetic diversity of *A. euteiches* in Europe on a north-to-south gradient with signs of genetic isolation in strains from Italy. In pea, I evaluated new breeding lines for their potential use in future breeding programs and identified a set of candidate genes for ARR disease resistance.

1.1 *Pisum sativum*

Pea (*Pisum sativum* L.) belongs to the family of the Fabaceae and is known not only as part of the human diet and fodder but also for being the first genetic model species, used by the catholic monk Gregor Mendel in his studies on central concepts of genetics and inheritance (Mendel 1865). The exact origin of pea as we know it today is still debated. One hypothesis describes its domestication from the wild pea *Pisum elatius* around 10'000 years ago and subsequent spread from the Middle East to temperate areas worldwide (Jing *et al.* 2010).

The legume has a beneficial nutritious profile, is rich in protein, starch and fibre. Pea seeds contain up to 32% protein and offer a great alternative to animal-based proteins and fats (Burstin *et al.* 2007; Shanthakumar *et al.* 2022). These properties make pea not only an important crop in food but also feed production. It is often grown in crop rotation as a 'natural fertilizer' that has shown to increase yield of subsequent crops, due to its capacity for nodule symbioses with nitrogen-fixing bacteria.

1.1.1 Pea cultivation in Sweden

Historically, pea has been cultivated in Sweden since Neolithic times and is considered one of the most important protein crops in Nordic countries (Hjelmqvist 1979). In the 19th century, pea cultivation accounted for more than 3% of Swedish farmland (Leino & Nygårds 2008). Pea cultivation in Sweden is mostly based in southern parts of the country due to a warmer climate and longer days. As green pea is a summer crop, the growing season starts already in mid-March and ends with the final harvest in autumn.

In the last 20 years, the production of green peas in Sweden has been mostly stable with between 40-45 kt annual production quantity (FAOSTAT 2024). In the year 2017, production drastically declined to roughly 12 kt on only 2'500 ha harvested. Since then, both the production area and quantity have been increasing steadily and reached 20'340 kt on nearly 6'400 ha harvested area in 2022 (**Figure 1**). There is an ongoing political discussion about increasing the self-sufficiency on legume protein in Sweden and it is therefore likely that pea production will continue to increase in the coming years.

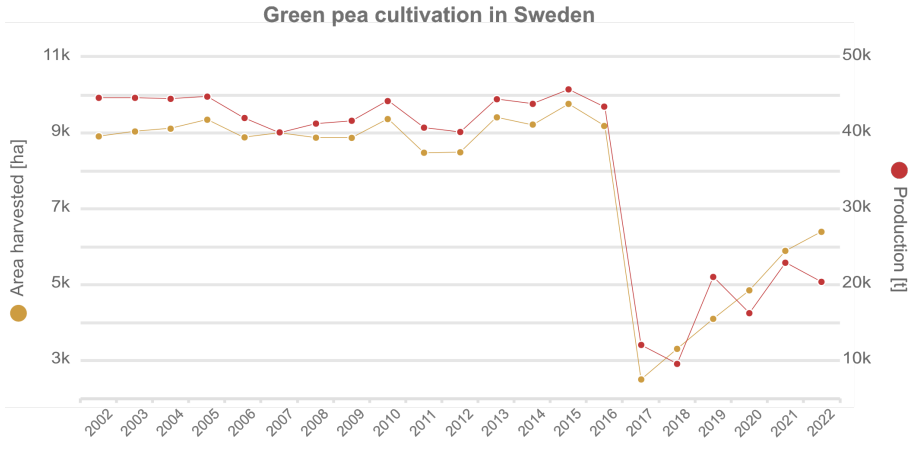

Figure 1 Harvested area [ha] and production quantity [t] of green pea in Sweden over the last 20 years (Source: FAOSTAT, 20.01.2024).

1.2 *Aphanomyces euteiches*

1.2.1 Oomycetes and the genus *Aphanomyces*

Oomycetes, commonly known as water molds, are filamentous eukaryotic microorganisms that were long believed to be most closely related with fungi. Even though they share similar filamentous growth and morphological traits, they are phylogenetically distinct and in fact more closely related to brown algae and diatoms in the eukaryotic kingdom of Stramenopiles (Gaulin *et al.* 2007; Gleason *et al.* 2018).

One of the main differences to fungi is the composition of the oomycete cell wall. In oomycetes, cell walls contain cellulose, more specifically beta-1,3- and beta-1,6-glucans and little to no chitin (Mélida *et al.* 2013). In *A. euteiches* however, non-crystalline chitin and glucan-linked chitosaccharides account for 10% of total cell wall components (Klinter *et al.* 2019).

Oomycetes have evolved either pathogenic or saprobic lifestyles and occur in terrestrial and aquatic habitats. The genus *Aphanomyces* occurs in both habitats and comprises species that can infect plants and animals. *Aphanomyces invadans* and *Aphanomyces astaci*, for example, can infect fish species and crustaceans respectively. Among phytopathogenic species

of *Aphanomyces*, *Aphanomyces cochlioides* is the causal agent of root rot in sugar beet and spinach, *Aphanomyces cladogamus* infects spinach and *Aphanomyces euteiches* is the most destructive soil-borne pathogen of pea and other legumes (Gaulin *et al.* 2007).

1.2.2 *Aphanomyces euteiches* life cycle

Aphanomyces euteiches Drechs. was first described in Wisconsin in 1925 as a damaging pathogen to pea roots (Jones 1925). It has in fact a rather broad host range, including several legume species such as common bean, vetch and alfalfa.

As *A. euteiches* is a hemibiotroph, it completes a biotrophic and necrotrophic growth phase on its host. The infection cycle starts with oospore germination, formation of sporangia and the production of asexual zoospores (**Figure 2**). The bi-flagellate zoospores have chemotaxis behaviour and actively swim towards their host guided by chemical gradients of legume isoflavones, such as prunetin, as well as arabinogalactan proteins (Sekizaki & Yokosawa 1988; Cannesan *et al.* 2012). After arrival at the host root surface, the zoospores encyst and germinate by formation of a germ tube. Host tissue is penetrated via hyphae derived from the germ tube and the pathogen continues to colonize the root tissue during the first six days of infection, the biotrophic phase (Hughes & Grau 2013). Within the plant stele and vascular tissue, hyphae differentiate into antheridia and oogonia in the necrotrophic phase. The consequent degradation of host tissue by pathogen-derived enzymes leads to root browning and wilting, the typical symptoms of ARR (Djébali *et al.* 2009; Kiselev *et al.* 2022). As the oogonium and antheridium are both produced by the same individual and are compatible, *A. euteiches* can be classified as homothallic (self-fertile) species (Hughes & Grau 2013). The infection cycle comes to an end with the production of the double cell- walled, sexual oospores within dead plant tissue. As the plant decomposes, the oospores accumulate in the soil where they are highly resilient against abiotic stress and remain viable for long periods of time (Hughes & Grau 2013). Due to the soilborne nature of *A. euteiches*, its dispersal capacity is rather limited but it is assumed to spread by transportation of infested soil and infected plant residue over longer distances (Grünwald & Hoheisel 2006; Wu *et al.* 2018).

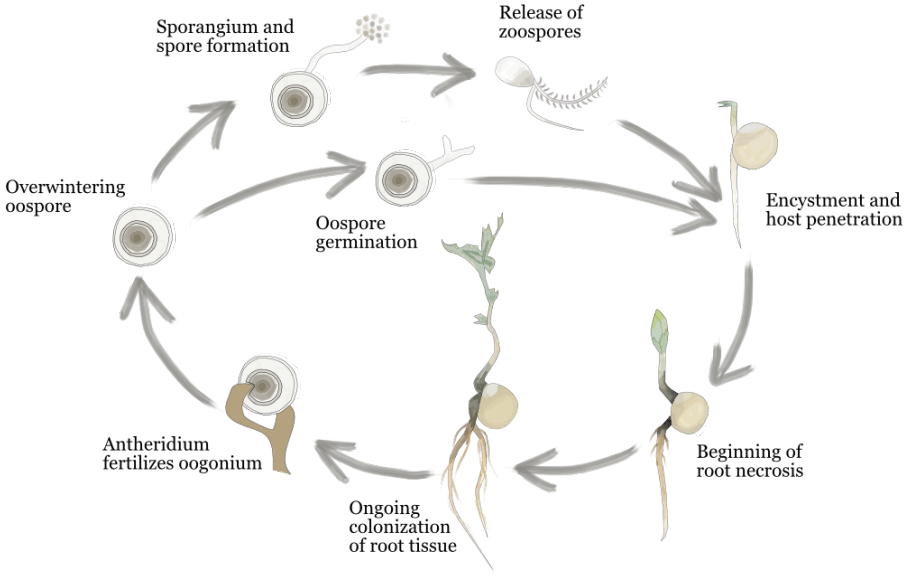

Figure 2. Illustration of the *Aphanomyces euteiches* life cycle.

1.2.3 *A. euteiches* genetic and pathogenic diversity

Genetically differentiated *A. euteiches* groups were reported in the United States and France (Malvick & Percich 1998; Grünwald & Hoheisel 2006; Quillévéré-Hamard et al. 2018). In the US, high genetic diversity within fields but a lack of genotypic differentiation among populations based on geographic origins was reported using random amplified polymorphic DNA (RAPD) and amplified fragment length polymorphism (AFLP) markers (Malvick & Percich 1998; Malvick *et al.* 2008b). Screening *A. euteiches* strains using simple sequent repeat (SSR) and sequence related amplified polymorphism (SRAP) markers showed no genetic structure within strains from France but grouping into three different populations within strains from the United States (Mieuzet et al. 2016; Le May et al. 2018). In a subsequent study of French *A. euteiches* strains using co-dominant SSR markers, a lack of genetic structure was confirmed with the exception of strains from the Bourgogne region, suggesting occasional outcrossing in the predominantly

homothallic (self-fertile) reproduction mode of the pathogen (Quillévéré-Hamard et al. 2018).

The separation of *A. euteiches* strains into races was defined based on their pathogenicity on alfalfa (*Medicago sativa*) cultivars (Hudelson & Grau 1998). Currently, many available alfalfa cultivars carry resistance to the initially described race 1. The later identified race 2 is more virulent and can overcome race 1 resistance (Grau et al. 1991). Both races are reported to occur throughout the United States but so far, no association between genotypic diversity and disease severity or race phenotype has been found (Malvick & Grau 2001; Malvick *et al.* 2008a).

In *A. euteiches* isolates from France, Wicker *et al.* (2001) described four pathotypes (I-IV) according their host range and aggressiveness on pea, vetch, alfalfa and broad bean. In extended screenings of *A. euteiches* strains from Europe, Canada, the United States and New Zealand, eleven virulence types using the six pea genotypes Baccara, Capella, MN313, 902131, 552 and PI180693 were identified (Wicker & Rouxel 2001; Wicker *et al.* 2003). In the latter study, highest aggressiveness was reported for virulence type I, including isolates originating from France.

The different nomenclature in defining pathogenic diversity in *A. euteiches* (pathotypes and virulence types on pea and races on alfalfa), complicate a global comparison of pathogen occurrence and aggressiveness.

1.2.4 Pea root rot complex

Among the biotic stresses impacting pea cultivation, the pea root rot complex (PRRC) consists of co-occuring soil-borne fungi and oomycetes and can account for 30-57% yield reduction depending on the field situation (Sharma *et al.* 2022). Pathogens belonging to the PRRC cause seed, root and foot rot, damping-off, wilting and chlorosis in the pea host (Sharma *et al.* 2022). The PRRC includes several fungal and oomycete species, among them *Fusarium* spp., *Didymella* spp, *Rhizoctonia solani*, as well as *Pythium* spp., *A. euteiches* and *Phytophthora pisi* (Baćanović-Šišić *et al.* 2018; Chatterton *et al.* 2019; Wille *et al.* 2021).

Among the pathogens of the PRRC, *A. euteiches* is considered the most devastating causal agent of root rot in pea cultivation (Gaulin *et al.* 2007). Typical symptoms of ARR in pea include water-soaked brown lesions, a reduction in root volume and function, as well as stunted seedlings (Giles *et*

al. 2022) (**Figure 3**). The oomycete *P. pisi*, was first described in southern Sweden in 2013 as an emerging root rot pathogen of pea and faba bean (Heyman *et al.* 2013). Both oomycetes are reported to be able to co-infect a single pea plant, but the disease development of coinfection is not fully understood. Their oospores can be differentiated microscopically whereas disease symptoms are rather similar, with the exception that *P. pisi* infection is rarely observed on the epicotyl (Heyman *et al.* 2013).

Figure 3. Pea seedling of the susceptible cultivar Linnea infected with a highly virulent *A. euteiches* strain displaying typical ARR symptoms: brown root discoloration, water-soaked lesions and reduced root volume. Photo by Cajsa Lithell.

1.2.5 ARR mitigation measures

Soil testing and crop rotation

So far, crop rotation and diversification with non-host crops has been among the most efficient mitigation measures against root rot in pea. Growing non-host crops during a field season, allows for a break in *A. euteiches* oospore inoculum buildup in the soil. Crop rotation however, faces limitations with the thick-walled morphology of *A. euteiches* oospores that can remain in the

soil for long periods of time together with the pathogen's broad host range (Pfender & Hagedorn 1983; Grünwald & Hoheisel 2006). Soil testing prior to a pea growing season determines the inoculum of root rot-causing pathogens present in the soil and highly infested fields can be avoided. The inoculum potential of root rot pathogens in the soil can be determined by growing susceptible pea genotypes in the sampled soil and assessing disease symptoms visually and microscopically. The development of molecular methods for disease risk prediction includes quantitative and real-time PCR assays with species-specific primers for detecting *A. euteiches* DNA in the soil (Sauvage *et al.* 2007; Gangneux *et al.* 2014). Once available for industrial diagnostics, such PCR-based predictions could pose a much faster and more cost-effective way of assessing *A. euteiches* inoculum in the field than classical soil testing. Field and greenhouse trials have shown the potential of biofumigation in reducing disease severity on pea caused by ARR (Chan & Close 1987; Muehlchen *et al.* 1990; Hossain *et al.* 2015). Alternatively, Brassicaceae species can be used as cover crops. They release glucosinolates that produce volatile compounds upon hydrolysation, which are toxic to various soilborne pathogens (Kirkegaard *et al.* 2000; Potter *et al.* 2000).

Manipulating soil properties

Humid, compact soils with a high clay content are favourable environments for *A. euteiches* inoculum build-up and spread (Allmaras *et al.* 2003). On the other hand, reduced ARR disease occurrence is observed in soils with low clay contents and high percentage of light-textured sandy components (Persson & Olsson 2000). The avoidance of soil flooding and subsequent zoospore production and spread can also be maintained by proper drainage using subsurface drainage tiles and tillage methods (Giles *et al.* 2022). There is a risk of spreading the disease with contaminated equipment, emphasising the need for proper cleaning of tools and equipment in agricultural practices. Additionally, high calcium contents in the soil can have an inhibiting effect of *A. euteiches* zoospore production, suggesting beneficial effects of soil liming as ARR management strategy (Heyman *et al.* 2007).

Chemical control

Some commercially available fungicides have been shown to inhibit *A. euteiches* and improve pea seedling emergence when applied in seed coating. Fosetyl-Al (Bayer CropScience), a specific fungicide against oomycetes,

showed to have suppressing effect on *Peronospora pisi*, *Pythium* spp. and *A. euteiches* in greenhouse trials (Oyarzun *et al.* 1990). Pyraclostrobin (Stamina; BASF) is used in legume seed coating against root rot caused by *A. euteiches*, *Rhizoctonia solani* and *Phytophthora medicaginis* by preventing fungal respiration (Venancio *et al.* 2003). Ethaboxam (INTEGO Solo, Valent Canada Inc.) seed coating suppresses early-season ARR in pea caused by *Phytophthora* spp., *Pythium* spp. and *A. euteiches* (Wu et al. 2018). Treatment of naturally infested soils with herbicides can also reduce the severity of ARR in pea (Grau & Reiling 1977; Jacobsen & Hopen 1981). However, the use of chemical control in ARR disease management is not only costly but also strictly limited in big parts of Europe, as negative environmental effects of fungicide accumulation in soil and groundwater have become a rising concern. In Sweden, there is currently no fungal control agent registered for use against ARR. Furthermore, large-scale application of fungi- or herbicides can also add selective pressure on pathogen populations and enhance selection for resistant *A. euteiches* strains.

Biological control

The use of biological control of ARR using microbial antagonists is considered an environmentally friendly disease management strategy and commercial application is studied intensively. Certain soil bacteria act as antagonists and can inhibit *A. euteiches* mycelial growth and zoospore germination when applied in seed coating (Wakelin *et al.* 1998; Wakelin *et al.* 2002; Oubaha *et al.* 2019; Godebo *et al.* 2020). Wildtype strains of *Pseudomonas* spp. have been showed to inhibit *A. euteiches* growth on plates, suggesting their possible use in bacteria-mediated biocontrol against ARR (Bowers & Parke 1993; Lai *et al.* 2022). In Sweden, the biocontrol agent Cedress (Lantmännen, BioAgri AB) is used in the suppression of ascomycete fungi but has shown no effect on *A. euteiches*. Seed coating with the mycoparasite *Clonostachys rosea* has shown to significantly reduce root rot severity caused by other members of the PRRC but has not been effective against *A. euteiches* (Xue 2003).

Resistant pea cultivars

Genetic resistance is considered the most preferable mitigation method for ARR in pea but currently no pea cultivar with complete resistance is available. However, several genotypes with partial, polygenic resistance have been identified and are used in pea breeding programs. Cultivars with

partial resistance to ARR have higher germination and emergence rates as well as improved seedling health, eventually leading to higher yield compared to susceptible cultivars as seen in various legume hosts (Vincelli *et al.* 2000; Conner *et al.* 2013). The application of resistant cultivars can further decrease the use of chemical control harmful for the environment and strategic deployment of resistant cultivars in pea cultivation should reduce the selective pressure on *A. euteiches*. Most likely, a combination of above-mentioned mitigation methods is the most effective and durable control of ARR in pea.

1.2.6 Pea resistance breeding to ARR

Sources of ARR resistance in pea are scarce but a number of genotypes have shown to carry partial resistance and are used in resistance breeding programs. The old landrace PI180693, for example, originated in Germany and has been identified as a source of partial resistance to *A. euteiches* (Lockwood 1960). Even though being partially resistant to ARR, PI180693 carries unfavourable breeding traits such as long internode length, a normal leaf type, round seeds and a starchy flavour. In contrary, commercial pea cultivars, such as the Swedish cultivar Linnea, carry desirable breeding traits alongside with high susceptibility to ARR (Kälin *et al.* 2023).

Classical disease resistance breeding focuses on controlled crosses between parental pea lines with desirable breeding traits. Selection of the new generations of breeding lines require controlled infections and disease resistance screenings to select the most resistant lines for backcrossing to the parental line carrying the desired traits for commercial breeding. However, classical breeding is a rather slow and costly process including large breeding populations, growth facilities and field trials. The use of marker genes in marker-assisted selection (MAS) breeding could speed up the process by enabling early selection at the seedling stage. Compared to classical breeding, MAS minimises the risk of undesirable effects caused by linked genes but requires the markers to be tightly linked to the resistance genes (Ghafoor & McPhee 2012). Transgenic breeding methods and the production of genetically modified cultivars have been limited due to safety concerns for health and environment (Turnbull *et al.* 2021). In the future however, precise genome editing techniques like CRISPR/Cas9 are likely to play an important role in keeping up with the demand for increased pea production.

1.2.7 Polygenic resistance to ARR – what we know so far

With the publication of a reference genome for pea in 2019 and the rapid development of next-generation sequencing, valuable tools and genomic resources have been made accessible for accelerating crop improvement (Kreplak *et al.* 2019). There is currently no pea cultivar with full resistance to ARR but old landraces or wild lines can offer sources of resistance. Resistance in pea is therefore characterized by slower lesion growth, reduced lesion size, as well as lower levels of oospores on roots and zoospore production than in susceptible pea genotypes (Kraft & Boge 1996). Pea resistance to ARR is polygenic, i.e. multiple genes in so-called quantitative trait loci (QTL) are controlling a resistant phenotype.

In the last 20 years, substantial progress has been made on understanding the genetics underlying root rot resistance in pea. Pilet-Nayel *et al.* (2002) genotyped recombinant inbred lines (RILs) with a collection of molecular and morphological markers and identified several major and minor QTL using linkage mapping. Major QTL were defined as being consistent over the years in their genomic location and resistance to ARR whereas minor QTL were specific to one environment and one resistance criterion. The same mapping population was then screened for specificity and consistency of resistance QTL using controlled *A. euteiches* infections in greenhouse and field trials (Pilet-Nayel *et al.* 2005). Hamon *et al.* (2011) used mapping populations derived from crosses of partially resistant pea genotypes to identify consistent QTL associated with partial resistance to *A. euteiches* and a meta-analysis of the diversity of resistance QTL across a broad collection of germplasm lead to the identification of consistent QTL combinations for durable resistance to ARR (Hamon *et al.* 2013). In 2015, (Lavaud *et al.*) validated previously identified QTL in different pea genetic backgrounds by introgression in near-isogenic lines (NILs). The resistance QTL in these NILs were further associated with different steps of the *A. euteiches* life cycle. More recently, genome-wide association studies (GWAS) were used to validate and refine confidence intervals of reported QTL and comparative mapping identified common loci controlling root system architecture and ARR resistance (Desgroux *et al.* 2016; Desgroux *et al.* 2018). Wu *et al.* (2021) used single nucleotide polymorphism (SNP) array and simple sequence repeat (SSR) markers to map QTL associated with partial resistance to ARR and published a list of genes segregating with major-, moderate- and minor-effect QTL. The list was expanded with novel genes

associated with partial resistance to ARR that were found by bulked segregant RNA-seq (BSR-Seq) analysis and the combined list of genes served as a basis for one of the thesis projects (Wu *et al.* 2022).

However, QTL segregating with partial resistance to ARR are often involved in diverse functions and linked with unfavourable breeding traits, such as a tall growth phenotype or coloured flowers. High-resolution analyses are required to break the linkage between loci controlling ARR resistance and unfavourable breeding traits (Desgroux *et al.* 2016).

1.2.8 Plant-pathogen interactions and the pea immune response to *A. euteiches* infection

Plants are in continuous interactions with their environment, including beneficial or pathogenic microorganisms. In order to defend against biotic stresses, plants have developed several protection strategies. Initial barriers include physical modifications in plant architecture that prevent pathogens from entering host tissue, or changes in host metabolism such as the release of enzymes and secondary metabolites. However, if pathogens overcome these initial barriers, they face a two-layered system of plant immunity that was first proposed as the 'zigzag' model in 2006 (Jones & Dangl). The first layer of innate plant immunity is triggered by the perception of microbe- or pathogen-associated molecular patterns (MAMPs or PAMPs) by transmembrane pattern-recognition receptors (PRRs), leading to MAMP- or PAMP-triggered immunity (MTI or PTI). Well-known PRRs include the *Arabidopsis* leucine-rich repeat (LRR) receptor kinase FLAGELLIN SENSITIVE2 (FLS2) that is activated upon binding bacterial flagellin and initiates PTI. The receptor-like kinase (RLK) consists of a predicted signal peptide, an extracellular domain containing LRRs, a transmembrane domain and a protein kinase domain, typical structures for receptor kinases (Chinchilla et al. 2006). In a second layer, effector proteins secreted by specialised pathogens can overcome PTI and trigger a process called effector-triggered susceptibility (ETS). On the contrary, resistance (R) proteins in the plant can recognise effectors and induce effector-triggered immunity (ETI). The ETI is mostly mediated by nucleotide-binding domain leucine-rich repeat (NLR) proteins, cytosolic PRRs with a variable N-terminal domain, a central nucleotide-binding domain and a C-terminal LRR domain, that detect effectors of invading pathogens (Jones & Dangl 2006). Activation of immune receptors in PTI and ETI trigger a cascade of immune

responses such as the release of reactive oxygen species (ROS), a rapid influx of Ca+ into the cytosol, as well as the activation of kinase and hormone signalling. Immune responses triggered by PTI are effective against non-adapted pathogens and therefore classified as non-host resistance, whereas ETI is effective against adapted pathogens (Dodds & Rathjen 2010).

For example, the transcriptional immune response of pea towards *A. euteiches* and *P. pisi* infection has been compared by Hosseini *et al.* (2015) using differential gene expression analysis. Transcriptional modifications in response to both oomycetes included cell wall reinforcement and regulation of hormone signalling pathways. During early infection, jasmonic acid (JA) biosynthesis was downregulated and upregulated with progressing infection. Ethylene (ET) biosynthesis was upregulated in response to *A. euteiches* and *P. pisi*, whereas the expression of auxin-related genes and the induction of chalcone synthases were specific to *A. euteiches* (Hosseini et al. 2015).

2. Objectives and research questions

The main objective of this thesis was to provide a better understanding of the *A. euteiches* - pea pathosystem. Thesis work includes studies on the genetic diversity and virulence phenotype in European *A. euteiches* strains (**Paper I and IV)** as well as experiments on evaluating partial resistance in pea towards ARR and the role of single genes during the infection (**Paper II and III).** All aspects and findings of the thesis contribute to the improvement and practical application in pea breeding.

- To understand the genetic diversity and population structure of *A. euteiches* in Europe and investigate the correlation between genotypic variation and virulence in pea (**Paper I and IV**)
  Hypotheses:
  - Genetic populations of *A. euteiches* are mainly clonal and diversity is low
  - There is little to no correlation between neutral genetic variation in *A. euteiches* and virulence on pea

- To evaluate the use of PI180693 partial resistance in pea breeding (**Paper II**)
  - New breeding lines derived from crosses with PI180693 are significantly more resistant than their susceptible parent Linnea
  - Breeding lines perform consistently in greenhouse, growth chamber and field trials

- To identify ARR candidate disease resistance genes specific to PI180693 (**Paper III**)
    - Sets of differentially expressed genes (DEGs) differ between the resistant and susceptible pea genotypes
    - Upregulated DEGs upon *A. euteiches* infection are located in resistance QTL
    - The transcriptional immune response in pea depends on virulence levels of *A. euteiches* strains

- To investigate possible speciation in the *A. euteiches* strains belonging to genetic group S (Italy) (**Paper IV**)
    - Strains of group S form a phylogenetically separate clade
    - Strains of group S differ in gene content and gene family evolution compared to other strains
    - Strains of group S show differences in morphology and virulence phenotype

3. Materials and Methods

3.1 Plant material

All plant genotypes used in the thesis were provided by Nomad Foods Findus Sverige AB. The commercial pea cultivar Linnea was included in all the studies **(Paper I-IV**). The cultivar is highly susceptible to ARR and has been used in commercial pea breeding programs since 2010. When infected by *A. euteiches*, Linnea shows severe ARR symptoms, including browning of roots, a highly reduced root system and lack of lateral root formation (**Figure 4**).

The old landrace PI180693 was used as resistant pea genotype in three thesis projects (**Paper II-IV**). Its growth phenotype includes long internode length, round seeds with a pale seed coat and its peas lack the desirable sweet taste. However, PI180693 shows far less severe disease symptoms when infected with *A. euteiches* and forms bigger root systems with more lateral roots, compared to a susceptible pea genotype **(Figure 4**).

Besides Linnea, the pea genotypes Lumina and MN313 were used to assess pathogenicity and virulence of European *A. euteiches* strains (**Paper I**). Lumina is described to be susceptible to all *A. euteiches* pathotypes and MN313 showed susceptibility to pathotype I isolates but partial resistance to pathotype III (Wicker & Rouxel 2001). Seeds of both genotypes were provided by the French National Research Institute for Agriculture, Food and Environment (INRAE) and Terres Inovia, France.

For the greenhouse, growth chamber and field trials in **Paper II**, several new pea breeding lines were screened for disease resistance to ARR and other breeding traits. The lines Z1654-1 and Z1656-1 were backcrossed to Linnea once after an initial cross between Linnea and PI180693 and selfed

in the eighth generation, whereas lines Z1701-1, Z1701-2, Z1707-1 and Z1707-02 were second backcrosses to Linnea in the sixth generation selfed (Kälin *et al.* 2023).

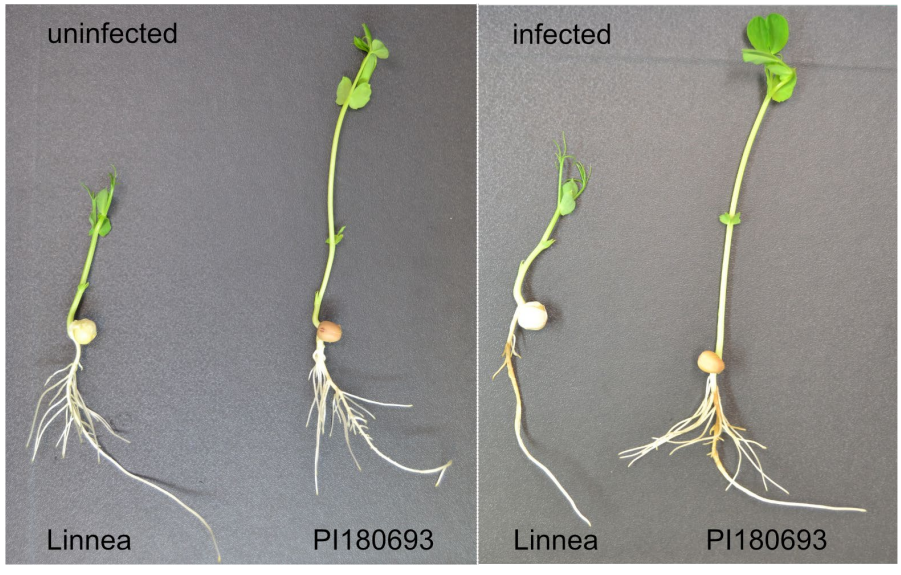

Figure 4. Root phenotype of Linnea and PI180693 uninfected (left) and under *A. euteiches* infection with highly virulent strain UK16 (right).

3.2 *Aphanomyces* strain collection

3.2.1 Geographic origin

The *A. euteiches* strain collection used in the thesis projects comprised a total of 85 strains from six European countries: Norway (3), Sweden (17), Finland (11), the United Kingdom (38), France (11) and Italy (5). Nomad Foods Findus Sverige AB provided the majority of strains from their vining pea production sites across Europe. The eleven strains from Finland were isolated from soil samples provided by the Novia University of Applied Sciences, Ekenäs, Finland. Ten previously genotyped French *A. euteiches* strains were provided by INRAE and Terres Inovia and included as a reference in **Paper I** (Moussart *et al.* 2007; Quillévéré-Hamard *et al.* 2018).

3.2.2 Strain baiting, cultivation and DNA extractions

The *A. euteiches* strains were collected either as soil samples or from roots of infected plants from different European vining pea cultivation sites. Soil samples were collected at 20-25 cm depth and stored in sealed plastic bags at 6 °C to maintain humidity until culturing (Kälin *et al.* 2022). The susceptible pea genotype Linnea was grown in the collected soil to bait present *A. euteiches* strains following the protocol described by (Olofsson 1967). The subsequent procedure is identical to diagnostic soil testing prior to sowing and includes root washing and grading discoloration and ARR symptoms visually and microscopically. Root pieces that were classified as infected with *A. euteiches* were then moved to a selective agar medium (Larsson & Olofsson 1994) for 2-3 days before being re-cultured on corn meal agar (CMA; BD Biosciences) for up to 10 days at 20 °C, and then moved to 4 °C for long-term storage. The entire strain collection was re-cultured on fresh CMA plates twice a year.

To extract DNA for PCR, 3% hexadecyl-trimethyl-ammonium bromide (CTAB) extraction (Nygren *et al.* 2008) was used (**Paper I**). For whole-genome sequencing of the *A. euteiches* strain collection, however, a combination of several DNA extraction procedures, including Genomic-tips (Qiagen, Hilden, Germany) and NucleoBond High Molecular Weight DNA (Takara Bio, USA) kits, as well as CTAB extractions was used to achieve high enough DNA quality and yield to pass quality standards for Illumina sequencing (**Paper IV**).

3.2.3 UK16 and SE51

The *A. euteiches* strain SE51, isolated in Skåne, Sweden, has been used in Findus breeding programs as a low-virulence strain when screening pea lines for resistance to ARR. Strain UK16 from the vicinity of Kingston upon Hull in the United Kingdom has shown to be highly virulent on pea and was also used for controlled infections in Findus breeding programs. In this thesis, the two strains were used as representatives for low and high virulence pressure on pea in **Paper II** and **III** and in phylogenomic and population genomic studies in **Paper I** and **IV**.

3.3 *A. euteiches*-pea infection systems

Throughout the thesis, several methods for reproducing *A. euteiches* infections and root rot disease on pea were used, depending on whether the focus of the respective experiment was on the pathogen or plant side. This section describes the different infection systems that have been used to answer our research questions.

3.3.1 Screening for *A. euteiches* virulence

Controlled inoculations using pot assays in a growth chamber or greenhouse with controlled light and humidity parameters were used to assess the pathogenic potential and virulence levels of *A. euteiches* strains on several pea genotypes with varying levels of resistance (**Paper I** and **Paper IV**).

For this, pea seeds were surface sterilized by several washing steps with 70% ethanol, 1% sodium hypochlorite and autoclaved water to avoid contamination by other pathogens. The air-dried seeds were then pre-germinated on 0.8% water agar for four days at 25 °C (Kälin *et al.* 2022). Square plastic pots filled with vermiculite (Sibelco, Antwerpen, Belgium) and one agar plug (approx. 10 mm diameter) with 2-3 week-old *A. euteiches* mycelia was applied to each 4 cm deep hole together with one pea seedling. Pots inoculated with different *A. euteiches* strains, as well as an uninfected mock control, were kept on separate water-filled trays to maintain moist growth conditions and prevent cross-contamination. For every treatment, 3-5 biological replicates (pots) and 4-5 technical replicates (seedlings) were scored after two weeks in the greenhouse or growth chamber. To assess *A. euteiches* virulence, the roots were washed in water and visually scored for disease symptoms. As a proxy for strain virulence, we used a disease index (DI) scoring scale from 0 (completely healthy) to 100 (completely dead) in steps of 10 as described in (Kälin *et al.* 2022).

3.3.2 Screening for pea resistance

When investigating disease resistance levels of new pea breeding lines to ARR, we relied on a combination of greenhouse, growth chamber and field experiments (**Paper II**). The inoculations in controlled conditions (growth chamber and greenhouse) were carried out using pot assays as described above, but with minor adjustments to facilitate the inoculation process. Seeds

were surface sterilized as previously described but not pre-germinated on water agar. Square plastic posts were filled with a layer of vermiculite (Sibelco, Antwerpen, Belgium), covered with the *A. euteiches* inoculum (in this case CMA discs with mycelia), and again covered with a layer of vermiculite to distance the seed from the inoculum for the germination. To compensate for the avoidance of pre-germination, the growth period was prolonged to three weeks, instead of two, prior to disease scoring using the same disease index scoring scale (Kälin *et al.* 2023). Additionally, the dry weight of the root systems for every biological replicate (pot) was recorded and correlated with the DI.

Three field trials were performed in southern Sweden (Skåne) in the years 2020 and 2022. The presence of *A. euteiches* and *P. pisi*, respectively, was confirmed prior to sowing by soil testing. Plants were sown in randomized 1 m^2 or 12 m^2 plots during spring and ten plants per plot were scored just before flowering, based on root discoloration using the DI scoring scale (Kälin *et al.* 2022). Local climate and breeding traits such as emergence rates, plant height, yield, ratio of green peas compared to the total plant biomass, as well as the number of pods per plant and average length of the second node pod were measured and reported (**Paper II**) (Kälin *et al.* 2023).

3.3.3 Controlled inoculations for transcriptomics

To assess the transcriptomic immune response of pea in Linnea and PI180693 in **Paper III**, a previously described water infection system using pipette tip boxes (Hosseini *et al.* 2012) was specifically modified for this experiment. The system allowed for controlled infections with little variation between biological replicates, as one biological replicate contained both pea genotypes, which were infected simultaneously. Three-day-old, surface sterilized and pre-germinated Linnea and PI180693 seedlings were placed on the racks of 200 µl pipette tip boxes and their roots were dipped in *A. euteiches* zoospore solution at $5x10^4$ spores/ml concentration for 30 seconds to allow zoospore encystment on the root surface. They were then transferred to boxes with autoclaved water where they were kept in a growth cabinet until root harvests at three time points. To confirm infection in the UK16 and SE51 treatments and absence of infection in mock (water) treatments, four additional seedlings of susceptible Linnea were left in every biological

replicate for several days after harvesting until visual ARR symptoms were visible.

3.4 Transcriptomics and differential gene expression

In **Paper III**, RNA was extracted from the roots of infected Linnea and PI180693 seedlings and, after passing quality control, sequenced at the NGI sequencing facility (SciLifeLab, Uppsala). We received the raw sequencing data of 54 libraries (three replicates per treatment and time point) and proceeded with adapter removal and quality trimming. To avoid mismapping, a combined genome index and combined genome for the reference genomes of the *P. sativum* genotype 'Caméor' (Kreplak *et al.* 2019) and the *A. euteiches* strain ATTCC201684 (Gaulin *et al.* 2018) were used to map our reads using STAR with default parameters (Dobin *et al.* 2012). A count table obtained with featureCounts (Liao *et al.* 2013) was analysed for differential gene expression in R using the package DESeq2 version 1.32.0, considering genes differentially expressed with absolute values of log2FC > 1 and FDR adjusted p-values < 0.05, compared to the mock treatments. A combination of sequence similarity searches using InterProScan (v. 5.48) and BLAST, as well as gene ontology (GO) enrichment analysis, was used to further characterize genes of interest.

3.5 *A. euteiches* population genomics

3.5.1 Population structure and genetic diversity

In **Paper I**, neutral SSR (or microsatellite) markers that were specifically developed for genotyping *A. euteiches* (Mieuzet *et al.* 2016; Quillévéré-Hamard *et al.* 2018) were used to identify unique multilocus genotypes (MLGs) per country of origin and genetic groups in the entire strain collection. The Simpson's diversity index (λ) was used to calculate the diversity within countries (Simpson 1949). Linkage disequilibrium was described using the adjusted index of association ($\overline{r}d$) (Agapow & Burt 2001). Additional analyses comprised covariance standardized principal coordinate analysis (PCoA), minimum spanning networks (MSNs) and the construction of a neighbour-joining (NJ) tree based on Bruvo distance

(Bruvo *et al.* 2004), as well as the estimation of common ancestry between samples using LEA, the R package for landscape and ecological association studies (Frichot & François 2015).

In **Paper IV**, the sequenced *A. euteiches* genomes were assembled using SPAdes v. 3.15.0 (Bankevich *et al.* 2012). The assemblies were done using funannotate v. 1.8.15 (https://github.com/nextgenusfs/funannotate/tree/master) which integrated both de novo and homology-based gene prediction. The PacBio reference genome of *A. euteiches* strain ATTCC201684 (Gaulin *et al.* 2018) and available expressed sequence tag (EST) data on AphanoDB (Madoui *et al.* 2007), together with *Aphanomyces* RNA reads from **Paper III** were used to optimize the annotation. SNPs were scored between our sequenced *A. euteiches* strains and the reference genome of strain ATTCC201684. The program STRUCTURE was used to identify the number of genetic clusters in the population via model-based Bayesian clustering (Pritchard *et al.* 2000), which was further confirmed by principal component analysis (PCA).

3.5.2 Phylogenies

In **Paper IV,** a genealogical concordance phylogenetic species recognition (GCPSR) analysis approach was followed to describe strain relatedness and species boundaries. The approach defines species limits as observed conflicts between phylogenetic trees based on recombination within a lineage (Taylor *et al.* 2000). A set of Benchmarking Universal Single-Copy Orthologs (BUSCOs) for Stramenopiles was used for the identification of BUSCOs in our sequenced *A. euteiches* strains. The genomes of *A. invadans*, the opportunistic pathogen *A. stellatus*, as well as the phytopathogenic *A. cochlioides,* were included as outgroups in our analyses. Phylogenetic trees were then constructed using IQ-TREE v. 2.1.3 (Minh *et al.* 2020) including ModelFinder (Kalyaanamoorthy *et al.* 2017) and evaluated by 1000 bootstrap replicates.

3.5.3 Gene family evolution

A species tree, based on concatenated BUSCOs, including the three outgroups (*A. cochlioides*, *A. invadans* and *A. stellatus*) and the genomes of two *A. euteiches* strains representing different phylogenetic species based on GCPSR served as a basis for the gene family evolution analysis in **Paper IV**. The analysis included gene families defined by Orthofinder (Emms & Kelly

2019), carbohydrate-active enzyme families (CAZymes) defined by CAZy (Cantarel *et al.* 2009) and protease families defined by Merops (Rawlings *et al.* 2012).

Significant gene copy number expansions and contractions in gene families were identified using computational analysis of gene family evolution (CAFE) (Mendes *et al.* 2021). Additional phylogenetic and sequence-based analyses, including predicted modular structure, were performed on selected CAZyme and protease gene families with expanded gene sets in *A. euteiches*.

4. Results and discussion

The thesis' main objective was to gain insights on genetic diversity and virulence on the pathogen side and on genes connected with partial resistance to ARR on the plant side. Here, the key findings of **Papers I-IV** are summarized and presented in a cohesive manner.

4.1 *A. euteiches* population structure and comparative genomics

4.1.1 Genetic diversity in Europe

Initially, the entire European *A. euteiches* strain collection was genotyped using 22 neutral SSR markers (**Paper I**). All 85 strains were diploid, with no more than two alleles per locus and strain, and the number of markers was sufficient to capture the actual genetic differentiation of *A. euteiches* in Europe. Three monomorphic, non-informative, loci were removed from further analyses.

Strains from Norway exhibited the highest prevalence of unique MLGs, characterized by an absence of clones. Within all countries, the genotypic diversity was high and the prevalence of clones rather low. The Simpson diversity index (λ) ranged from 0.667 in Norway to 0.971 in the United Kingdom. The latter contributed the largest share of genotyped strains, accounting for 38 out of 85, and demonstrated the highest genetic diversity. The adjusted index of association ($\overline{r}_d$) described no linkage between alleles, an indication for limited clonal reproduction (Kälin et al., 2022).

We observed an overall low genetic diversity within populations that increased with geographical distance, as expected for soil borne pathogens

with restricted possibilities to spread and predominant homothallic reproduction. The broad host range of *A. euteiches* and frequent crop rotations in agricultural practices, together with the high genotypic diversity observed within each population, led to the assumption that outcrossing is likely to occur (Kälin *et al.* 2022).

4.1.2 Genetic structure along a north-to-south gradient

Out of 67 MLGs, only five were shared between countries (**Paper I**). Most of them were shared between two geographically close countries. Two were shared between Sweden and the United Kingdom and one between Sweden and Finland. Another two MLGs were shared between the United Kingdom and France. One MLG was shared between the United Kingdom, France and Norway, indicating movement between these three countries (**Figure 5**).

Both analyses of population structure, using neutral SSR markers (**Paper I**) and SNP markers derived from whole-genome sequencing (**Paper IV**), confirmed the genetic clustering of *A. euteiches* strains into three groups. All strains from Finland, together with two strains from eastern Sweden formed a north-eastern (NE) group and all remaining strains (from Sweden, Norway, the United Kingdom and France) clustered in a central European (CE) group (**Paper I**). Strains from Italy, representing the most southern (S) sampling area, formed a genetically very distinct third group (**Figure 5**). They were lacking shared ancestry with other strains, indicated low migration rates between Italy and the other European countries (**Paper I and IV**). Given that that increased genetic differentiation from the central European group was observed at the most northern and southern sampling regions, climatic factors might be important drivers of local differentiation through increased rates of sexual reproduction.

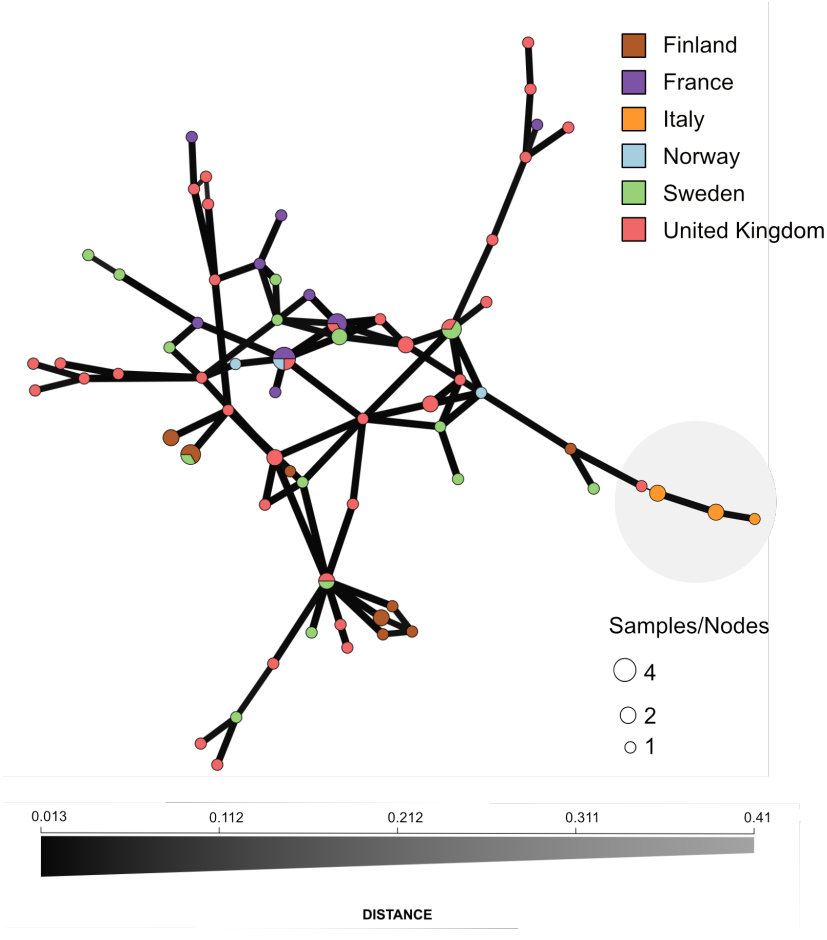

Figure 5. Genetic relationship of European *A. euteiches* strains. Minimum spanning network based on Bruvo distance. Figure modified from **Paper I**.

Local differentiation in strains from Italy - possibility of speciation?

The genetic distinctness and lack of shared common ancestry with other *A. euteiches* strains led to the question if the observed local differentiation in the southern genetic group is in fact local speciation. In **Paper IV**, we followed-up on this question by whole-genome sequencing 68 European *A. euteiches* strains, including all five strains from Italy (group S), for comparative genomics analyses.

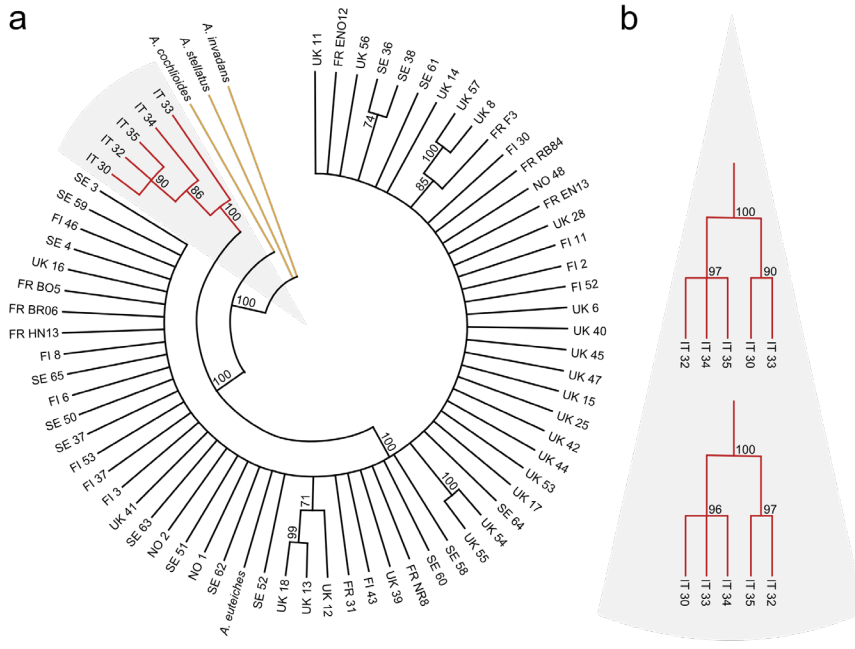

Figure 6. Phylogenetic tree based on concatenated BUSCOs including 68 European *A. euteiches* strains, the *A. euteiches* reference strain ATCC201684 and *Aphanomyces* outgroups *A. invadans*, *A. stellatus* and *A. cochlioides* (**a**). Two representative examples of incongruence within the clade of group S strains (**b**). Figure modified from **Paper IV**.

We followed a GCPSR approach based on the comparison of phylogenetic trees constructed with BUSCOs for Stramenopiles. In the phylogenetic analysis, we included the fish pathogen *A. invadans*, the opportunistic pathogen *A. stellatus* and the sugar beet infecting *A. cochlioides* as outgroups. In 79 phylogenetic trees, all five strains from Italy formed a separate clade, as reflected in a phylogeny based on concatenated BUSCOs (**Figure 6a**), which was also as previously observed in neighbour joining trees in **Paper I**. Within this clade of genetic group S, we observed incongruence between phylogenetic trees, an indication for possible recombination and genetic exchange between strains (**Figure 6b**). Further, the *Aphanomyces* phylogeny based on concatenated BUSCOs showed closest relatedness of clade S to other *A. euteiches* strains (**Figure 6a**). However, in a phylogeny based on genome-wide SNPs, the strains from Italy

grouped as a sister clade to *A. cochlioides*. These findings emphasize the genetic distinctness of the southern strains, which may indicate introgression or hybridization with *A. cochlioides,* or that the strains of group S form a separate phylogenetic species (phylogenetic species 1 [PS1]).

Nevertheless, the small sampling size of strains from Italy (5) and the discontinuous sampling strategy with no representative strains from southern France, Switzerland and Germany, it is not possible to classify strains from Italy as a new *Aphanomyces* species with complete certainty. In addition, all strains of the collection were isolated via strain baiting from infected pea roots and initially classified as *A. euteiches* by visual and microscopic inspection of morphological characteristics.

4.1.3 Variation in strain virulence and morphology

Although all strains were initially classified as *A. euteiches* based on morphology, cryptic species can occur. For this reason, we further explored the phenotypic variation of the strains and genetic groups. In **Paper I**, ten *A. euteiches* strains representing the three genetic groups (NE, CE and S) were chosen for a phenotyping assay on pea using pot trials. Three strains from Sweden, Finland and Italy, as well as the French strain Rb84 were used to infect and score the DI on the pea genotypes Linnea, Lumina and MN313. The results confirmed the resistance levels of the pea genotypes, but showed no correlation between virulence levels and genetic groups based on genotyping with neutral SRR markers.

To get a better insight into the pathogenic potential and differences in virulence levels between *A. euteiches* strains of the European collection, a large-scale virulence screening on the susceptible pea genotype Linnea and the partially resistant PI180693 was performed (**Paper IV**). Virulence screening of 56 *A. euteiches* strains revealed a large variation in virulence levels between single strains. On a genetic group level, DI scores were higher on Linnea than on PI180693 for all groups, with the S group displaying highest DI on Linnea, and the CE group being most virulent on PI180693 (**Table 1**). The only significant ($p < 0.05$) difference in virulence between genetic groups was observed between the CE and S group on the partially resistant PI180693.

Table 1. Average disease indices (DI) and oospore sizes for the genetic groups of *Aphanomyces euteiches.*

Genetic group	Average DI on Linnea	Average DI on PI180693	Average oospore size [mm^2]
Central European	65^{a} +/- 33.3	45^{a} +/- 26.4	0.022^{a} +/- 0.00669
North-eastern	52^{a} +/- 34.9	36^{ab} +/- 27.3	0.026^{b} +/- 0.00596
Southern	72^{a} +/- 32.3	28^{b} +/- 18	0.017^{c} +/- 0.00247

* Letters indicate FisherLSD significant differences between genetic groups for average DI ($p < 0.05$) and oospore size ($p < 0.001$), +/- standard deviation

The production of asexual survival structures, such as thick-walled chlamydospores, was shown to correlate with invasiveness in regions with cold winters for invasive *Phytophthora* spp. (Redondo *et al.* 2018). As oospores are known to be the primary survival structure in *A. euteiches*, the oospore sizes of 31 strains representing the three genetic groups were measured microscopically to assess differences between genetic groups established in distinct climates. The three strains representing the S genetic group had the smallest oospores with an average of 0.017 mm^2, whereas largest oospores were measured in strains representing the NE genetic group (**Table 1**). Posthoc pairwise comparisons (FisherLSD) showed significant ($p < 0.001$) differences in oospore size between all genetic groups.

4.1.4 Evolution of virulence-related gene families

We selected five *Aphanomyces* strains for CAFE analysis to gain further insight into expansions and contractions of virulence-related gene families in non-plant pathogenic *A. stellatus* and *A. invadans*, and the plant pathogens *A. euteiches* and *A. cochlioides*, as well as between *A. euteiches* and PS1. In *A. euteiches*, strain SE50 represented the genetic groups CE and NE, and strain IT32 the group S (PS1). A species tree including the five *Aphanomyces* species showed significant ($p < 0.05$) expansions and contractions of proteases and CAZymes, as well as hierarchical orthogroups ($p < 0.001$) (**Figure 7a**, **Paper IV**). Various CAZymes are known to be released by phytopathogenic fungi and oomycetes during host infection, and act predominantly in host cell wall and tissue degradation (Lyu *et al.* 2015). In *A. euteiches*, the secretome is largely made up of CAZymes and proteases (Kiselev *et al.* 2022), consequently non-random evolution of these gene families were of particular interest in our analysis.

Within non-plant pathogenic *Aphanomyces* species, we observed 123 gene families expanded and three contracted in *A. stellatus* whereas seven families were expanded and 28 contracted in *A. invadans* (**Figure 7a**). More specifically, several protease families underwent significant contractions in *A. invadans* but were expanded in *A. stellatus*.

At the branch representing the ancestor to all three plant-pathogenic species, we detected the expansion of eight gene families, among them a family of adenosine 5'-triphosphate (ATP)-binding cassette (ABC) transporters with a predicted function in multidrug resistance. Two oxidoreductase families and a metalloprotease family were among the contracted families in the same branch (**Figure 7a**).

The ancestral branch to *A. euteiches* (SE50) and PS1 (IT32) included 18 expansions of gene families, including four oxidoreductases, three of them being cytochrome p450 oxidases, ABC-, major facilitator superfamily (MFS), and aa-transporters families and one family of M20 peptidases.

In *A. euteiches* (SE50), we found that glutathione S-transferases (GSTs), oxidoreductases as well as sugar and ABC transporter families underwent significant expansions. Glutathione S-transferases were shown to act in detoxification in fungal as well as oomycete pathogens, defending the pathogen cells from plant-derived toxic metabolites and ROS that are released during infection (Bryant *et al.* 2006; Rahmanpour *et al.* 2009).

In PS1, represented by strain IT32, we found expanded endo-1,3-beta-glucanases, folate/biopterin and sugar transporter families and M20D carboxypeptidases, whereas two ABC transporter families with predicted functions in the efflux of cholesterol and phospholipids, as well as in pleiotropic drug resistance underwent significant contractions (**Paper IV**). Notably, we found the family of carbohydrate esterases 1 (CE1) sifnificantly ($p < 0.001$) expanded in *A. euteiches*, compared to PS1. In plant-pathogen interactions, CEs act in the de-acetylation of hemicellulose and pectin units of plant polysaccharides, enabling the pathogen to pass physical barriers such as plant cell walls (Sista Kameshwar & Qin 2018). A domain analysis of all CE1 homologs in the five *Aphanomyces* species identified two major groups: one group of orthologs of predicted S-formylglutathione hydrolases and a group of predicted C or B/C/D-type feruloyl esterases (**Figure 7b**). As feruloyl esterases act in decoupling plant cell wall polysaccharides and lignin (Fry 1982; Ralph *et al.* 1995; Ralph *et al.* 1998; Caffall & Mohnen 2009), it is possible that they allow *A. euteiches* to access energy from plant cell walls.

Also, no feruloyl esterase domains were present in non-plant pathogenic *Aphanomyces* strains, whereas *A. cochlioides* comprised three members, *A. euteiches* seven, and PS1 two members. Most of them had signal peptides and a cellulose binding module. Two feruloyl esterase genes were recently duplicated in *A. euteiches* and *A. cochlioides,* indicating a beneficial role in the evolution of plant pathogenic *Aphanomyces* species (**Figure 7b**).

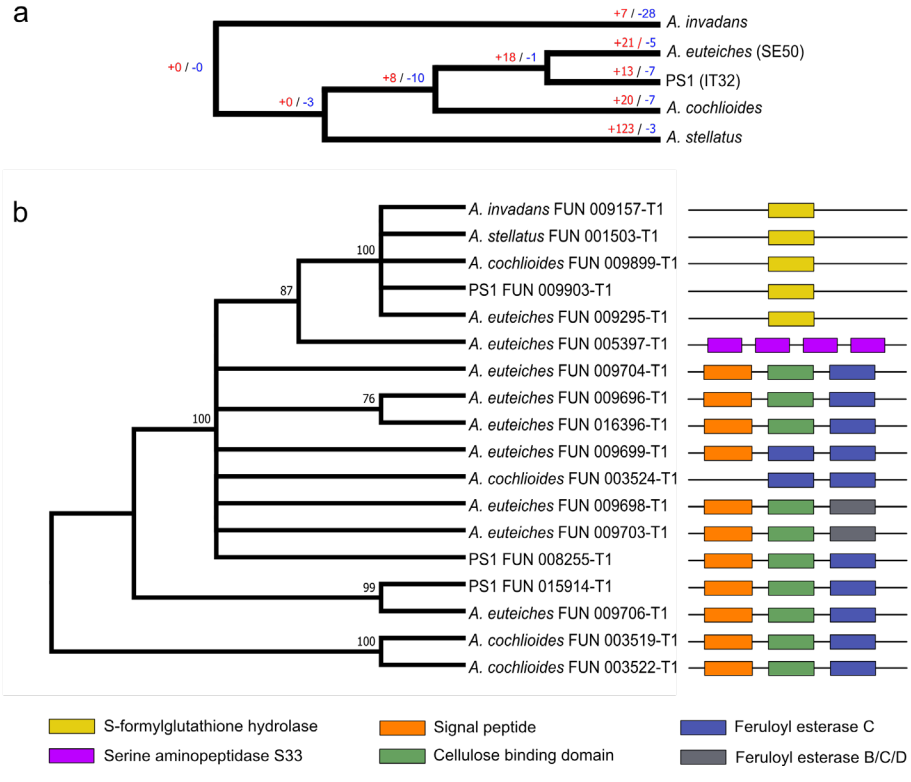

Figure 7. (**a**) *Aphanomyces* species tree used in CAFE analysis with combined significant expansions and contractions of phylogenetic orthogroups ($p < 0.001$), CAZymes ($p < 0.05$) and proteases ($p < 0.05$). (**b**) Structural domains of members of the CE1 family in *A. invadans*, *A. stellatus*, *A. cochlioides*, *A. euteiches* (SE50) and PS1 (IT32). Figure modified from **Paper IV.**

In conclusion, the expansion of ABC transporters acting as multidrug resistance exporters in *A. cochlioides*, *A. euteiches* and PS1 could indicate an adaptation to the exposure of host-derived antimicrobial secondary metabolites in plant pathogenic lifestyles. The adaptation to the pea host in *A. euteiches* and PS1 was associated with the expansion of cytochrome p450 oxidases and various transmembrane transporters, further indications for non-random evolution of detoxification mechanisms. However, detoxifying GSTs underwent contractions in the ancestral branch of plant pathogenic species as well as in PS1 and showed to be expanded only in *A. euteiches*. The significant ($p < 0.001$) expansion of feruloyl esterase C and B/C/D in *A. euteiches,* together with the exclusive presence of these enzymes in plant pathogenic species, suggest a role of feruloyl esterase enzymes in disease induction on pea.

4.2 Deciphering resistance in PI180693 and the pea immune response to *A. euteiches* infection

4.2.1 The potential of PI180693 partial resistance in pea breeding

In **Paper II**, the potential of PI180693 partial resistance against ARR in pea breeding crosses was evaluated by a combination of greenhouse, growth chamber and field trials. Six new back-crossed pea breeding lines and their parental lines Linnea and PI180693 were screened for their resistance to ARR. The *A. euteiches* strains used for infections in growth chamber and greenhouse trials differed in their levels of virulence. Strain UK16 scored highest disease index scores on all lines, strain SE51 displayed intermediate virulence and strain SE58 was least virulent on all lines (Kälin et al. 2023).

Significant negative correlation between root dry weight and disease index in controlled conditions

In growth chamber trials, the five breeding lines Z1654-1, Z1656-1, Z1701-1, Z1701-2 and Z1707-2 displayed significantly ($p < 0.05$) lower disease indices than the susceptible parent Linnea upon infection with UK16 and SE51. Upon infection with the least virulent SE58, only one of these

lines was still significantly ($p < 0.05$) more resistant than Linnea (**Table 2**). The lowest root dry weight was observed in pea genotypes infected with the most virulent *A. euteiches* strain UK16 and the highest root dry weight was scored in plants infected with the least virulent SE58. This negative correlation of disease index and root dry weight was in agreement with the virulence assays in **Paper I**.

Table 2. Disease index and root dry weight scores for six breeding lines, Linnea and PI180693.

Pea line	DI UK16	Rdw UK16 [g]	DI SE58	Rdw SE58 [g]	DI SE51	Rdw SE51 [g]
Linnea	91.6 ^{a}	0.022 ^{c}	46.4 ^{ab}	0.156 ^{c}	79.3 ^{a}	0.05 ^{e}
Z1707-1	84.4 ^{ab}	0.028 ^{c}	54.2 ^{a}	0.152 ^{c}	73.8 ^{ab}	0.08 ^{cd}
Z1701-2	84.0 ^{b}	0.043 ^{bc}	44.0 ^{ab}	0.26 ^{ab}	69.6 ^{bc}	0.105 ^{bc}
Z1656-1	83.6 ^{b}	0.027 ^{c}	45.8 ^{ab}	0.24 ^{ab}	66.8 ^{cd}	0.063 ^{de}
Z1654-1	78.6 ^{bc}	0.042 ^{bc}	48.4 ^{a}	0.223 ^{b}	61.8 ^{de}	0.101 ^{bc}
Z1707-2	77.4 ^{bc}	0.065 ^{b}	41.6 ^{ab}	0.221 ^{b}	65.1 ^{cde}	0.115 ^{b}
Z1701-1	75.0 ^{c}	0.034 ^{c}	19.4 ^{c}	0.27 ^{a}	60.0 ^{e}	0.102 ^{bc}
PI180693	56.4 ^{d}	0.113 ^{a}	32.0 ^{bc}	0.162 ^{c}	37.6 ^{f}	0.173 ^{a}

* Letters indicate FisherLSD significant ($p < 0.05$) differences between pea lines for each trait

In greenhouse trials (**Paper II**), the strain SE51 with intermediate virulence was used for infections. Only line Z1654-1 was significantly ($p < 0.05$) more resistant than the susceptible parent, based on DI scores. Even though the other breeding lines did not differ in resistance from Linnea, the lines Z1656-1, Z1701-1, Z1654-1 and Z1707-2 displayed significantly ($p < 0.05$) higher root dry weight measurements (**Table 2**). These four lines showed highest resistance levels to *A. euteiches* infection in the growth chamber trials, suggesting that root dry weight can be used as a measure for disease resistance under intermediate or low virulence pressure. It has been shown that resistance QTL for partial resistance to ARR are linked with increased root volume (Desgroux *et al.* 2018; Sivachandra Kumar *et al.* 2020). However, the natural variation in root architecture and volume between pea lines should be taken into account when comparing absolute numbers of root dry weight with Linnea.

The impact of co-occurrence of other soil pathogens and climate on observed disease resistance in the field

Two field trials including the same eight pea lines were performed in the year 2020. In field A, a field with confirmed presence of *A. euteiches*, all breeding lines were significantly ($p < 0.05$) more resistant (i.e. scored lower DI) than Linnea, comparable to resistance level in PI180693. In field B, with confirmed co-occurrence of both *A. euteiches and P. pisi*, only breeding line Z1656-1 was significantly ($p < 0.05$) more resistant than Linnea. Whereas seedling emergence was negatively correlated with DI in field A, no correlation was observed in field B with both pathogens present.

The field trial in the year 2022 revealed overall lower DI than in the previous field trials. Remarkably, PI180693 showed most severe disease symptoms while maintaining the highest score of seedling emergence. For field R-22-10-91, several breeding traits were measured to compare the phenotype of breeding lines with the commercial parent Linnea. In general, higher DI were correlated with low yield. But several breeding lines still differed significantly in measured breeding traits such as the ratio of green peas versus total plant biomass, the length of the second node pod or plant height. However, the 2022 field trial started with high levels of soil moisture during sowing, providing optimal conditions for *A. euteiches* infection, which were then followed by a dry summer, not favourable for ARR development. Therefore, results from the 2022 field trial should be interpreted with care.

In conclusion, our results confirmed the effectiveness of partial resistance in PI180693 against *A. euteiches*, especially upon infection with strains exhibiting high or intermediate virulence. The PI180693 partial resistance was less effective against root rot symptoms caused by *P. pisi.* Whereas several breeding lines displayed higher resistance levels to *A. euteiches* than Linnea, only few did not differ significantly from Linnea in measured breeding traits. Our results further highlight the difficulty with breeding for robust resistance in pea and the need for fine mapping techniques to break linkages between ARR resistance and unfavourable breeding traits.

4.2.2 Pea immune response to *A. euteiches* infection in susceptible and resistant genotypes

In **Paper III**, we used controlled infections of Linnea and PI180693 seedlings with *A. euteiches* strains UK16 and SE51 to investigate the pea immune response in a susceptible and resistant genotype to high and low virulence pressure. The pea roots were harvested at 6 hours post inoculation (hpi), 20 hpi and 48 hpi for RNA sequencing followed by differential gene expression analysis.

Host resistance drives pea immune response during advancing infection

Our results very clearly confirmed a host-specific immune response depending on the host's quantitative resistance rather than the virulence level of the pathogen. A PCA based on gene expression showed distinct clustering according to pea genotype and only a subtle separation by *A. euteiches* strain and sampling time point (**Figure 8**).

An exponential increase in reads mapping to *A. euteiches* was observed along with increasing sampling time point. In this context, the percentage of reads mapping to the *A. euteiches* reference genome was regarded as a proxy for pathogen biomass. Upon infection with the highly virulent strain UK16, more *A. euteiches* biomass could be detected on both pea genotypes, again increasing with progressing infection. In general, less pathogen biomass was observed on the resistant genotype. The partial resistance in PI180693 has previously been shown to inhibit the production rate of *A. euteiches* oospores, possibly slowing down the infection process and appearance of symptoms (Kraft & Boge 1996).

An exponential increase was also observed in the number of DEGs in interactions with UK16, with more defence-related genes differentially expressed in the susceptible genotype. Therefore, further analyses focused mainly on the later stage of infection (48 hpi).

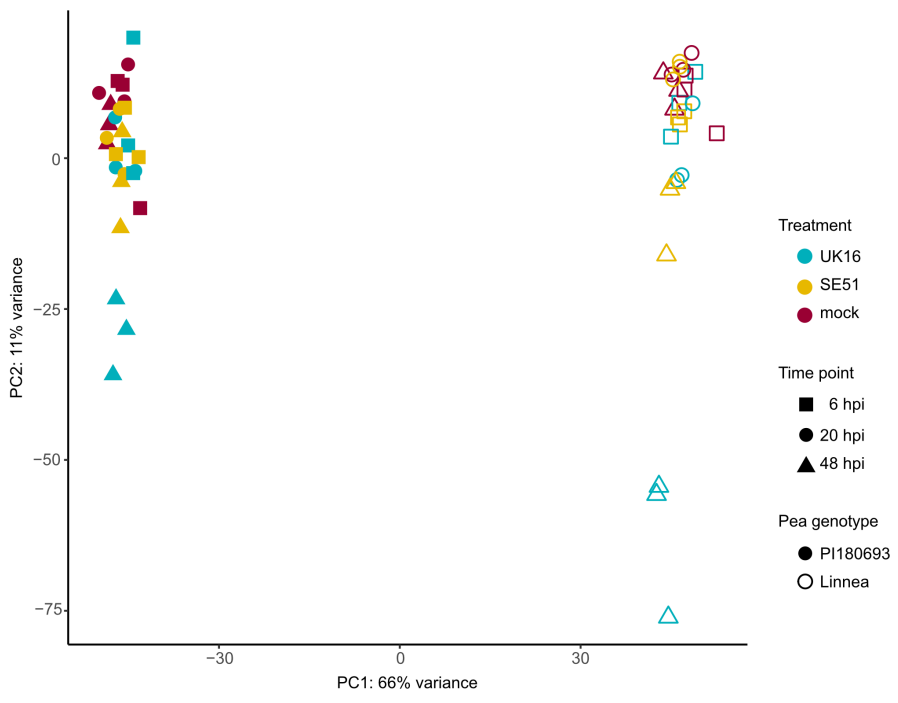

Figure 8. Principal component analysis (PCA) of transcriptomics data (**Paper III**). The data sets includes three biological replicates for the susceptible pea genotype Linnea and resistant PI180693, for the *A. euteiches* treatments with the highly virulent strain UK16, lowly virulent SE51, as well as the mock control, and for the three root harvesting time points (6 hpi, 20 hpi and 48 hpi). Figure reproduced from **Paper III**.

Differential gene expression analysis reveals genes involved in hormone signalling and transcription factors

At the earliest root harvesting time point (6 hpi), only few genes were differentially regulated with no apparent pattern of genotype- or strain-related gene regulation. We found seed linoleate 9S-lipoxygenase-3-like genes to be downregulated in Linnea and PI180693 at 6 hpi and 20 hpi respectively but upregulated in the resistant genotype at 48 hpi. 9S-lipoxygenases generate oxylipins, which can act as precursors to the plant hormone jasmonic acid (JA) that is involved in defense reactions to necrotrophic plant pathogens (Hwang & Hwang 2010; Laluk & Mengiste

2010; Carvalhais *et al.* 2013; Singh *et al.* 2022). This is of particular interest, since *A. euteiches* is known to undergo a shift from biotrophy to necrotrophy on its host (Kiselev *et al.* 2023).

At 20 hpi, we found the putative disease resistance proteins Pi176 and Pi49 upregulated exclusively in PI180693. The genes have been associated with induced expression in pea upon infection with *Fusarium solani* (Riggleman *et al.* 1985; Fristensky *et al.* 1988). Genes involved in abscisic acid (ABA) signalling pathways were amongst DEGs between the pea genotypes. As ABA is known to be involved in the induction of lateral root formation in legume species (Liang & Harris 2005), ABA signalling might be of importance for the PI180693 partial resistance to ARR.

At 48 hpi, we found myeloblastosis (MYB)-like transcription factors among the most strongly DEGs in Linnea and PI180693, almost exclusively upon infection with the highly virulent strain UK16. The upregulation of MYB transcription factors, as well as the differential regulation pattern of two specific WRKY transcription factors, suggest an involvement in ABA signalling and root development in the pea-*A. euteiches* interaction.

Thirty-nine candidate disease resistance genes

Cross-referencing DEGs at 48 hpi with genes known to segregate with partial resistance to ARR (Wu *et al.* 2021; Wu *et al.* 2022), resulted in a set of 39 candidate disease resistance genes (**Figure 9**). Linnea and PI180693 share more upregulated than downregulated genes segregating with partial resistance to ARR and no such genes were specifically downregulated in the resistant cultivar. The robust immune response (DEGs in both genotypes) involved two upregulated receptor-like protein kinases and three downregulated oxylipin biosynthesis genes. Four genes, upregulated only in PI180693, were of particular interest. They included a predicted resistance to *Uncinula necator* 1 (RUN1)-like disease resistance protein, a predicted *P. sativum* defensin 2 (Psd2), a seed linoleate 9S-lipoxygenase-3-like gene and the putative leucine-rich repeat (LRR) receptor-like kinase (RLK) Psat7g091800.1.

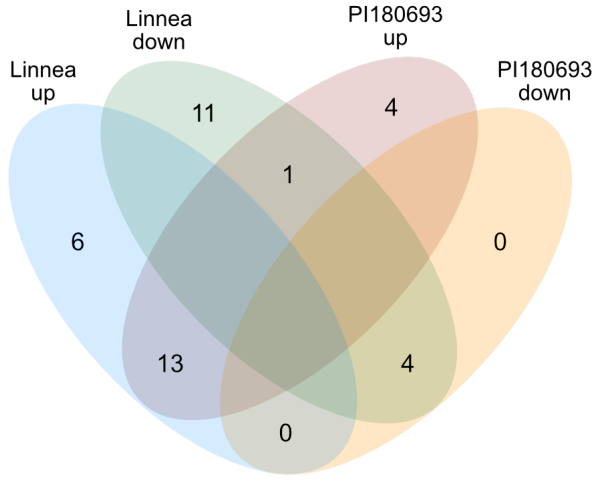

Figure 9. Thirty-nine candidate disease resistance genes at 48 hpi. Differentially expressed genes (absolute value of log2FC > 1 and adjusted p-value < 0.05, compared to mock treatment) in the susceptible pea genotype Linnea and the resistant PI180693 upon response to *A. euteiches* strain UK16 or SE51, split by up- and downregulation. The DEGs were cross-referenced with genes known to be segregating with partial resistance to ARR (Wu *et al.* 2021; Wu *et al.* 2022). Figure reproduced from **Paper III**.

The LRR-RLK is polymorphic between Linnea and PI180693

The gene Psat7g091800.1 has an exon-intron-exon structure and is located on chromosome seven of the pea genome. It is predicted to encode a 1157 aa long protein with a nucleotide-binding domain leucine-rich repeat (NLR) immune receptor structure (**Figure 10**). Sanger sequencing from genomic DNA of both Linnea and PI180693 and subsequent alignment to the reference sequence of the cultivar Caméor revealed no SNPs between Linnea and Caméor. This could be due to the cultivars' shared history in pea breeding, undergoing similar genetic selection steps for commercial breeding. Between PI180693 and Linnea however, 39 SNPs leading to 17 non-synonymous mutations were detected. Four of them were located in LRRs, possibly modifying the function as immune receptor in Linnea (**Figure 10**).

Furthermore, domain searches on the protein revealed a FLAGELLIN SENSING 2 (FLS2)-like domain, reminiscent of the *Arabidopsis thaliana* receptor (Chinchilla *et al.* 2006). Phylogenetic analyses however, showed that the Psat7g091800.1 encoded a distinct LRR-RLK protein, sharing only 53.4% sequence identity with *A. thaliana* (**Paper III**). The putative NLR immune receptor encoded by Psat7g091800.1 might be involved in the recognition of pathogen effectors and induction of host cell death in the pea immune response. However, to properly assess the usefulness of the gene for future pea breeding programs, it will have to be better characterized. Deletion and overexpression experiments could provide a better understanding on the effect the RLK has on ARR resistance. Synthesizing the gene with individual SNPs and/or combinations of SNPs, followed by overexpression would allow to identify specific structural domains that are required to maintain gene functionality.

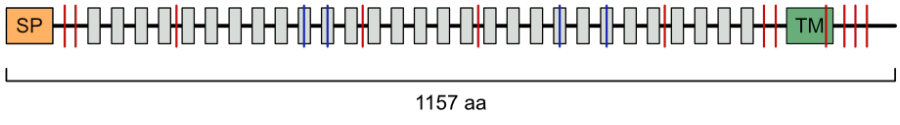

Figure 10. Schematic figure of the putative nucleotide-binding domain leucine-rich repeat (NLR) immune receptor encoded by Psat7g091800.1. Signal peptide (SP), transmembrane domain (TM) and leucine-rich repeats (LRRs, grey boxes) are indicated. Nonsynonymous single nucleotide polymorphisms (SNPs) are marked in red and SNPs within LRRs in blue.

5. Conclusions and perspectives

This thesis provides new insights into the genetic and virulence diversity of *A. euteiches* in Europe and into pea resistance and immune response to ARR. The main findings can be summarized as follows:

- European *A. euteiches* strains cluster in three genetically differentiated groups along a north-to-south gradient. (**Paper I**)

- Despite the limited dispersal capacity of *A. euteiches*, shared MLGs between geographically distant regions indicate human-aided movement between countries. (**Paper I**)

- The PI180693 partial resistance was successfully employed in crosses with the commercial cultivar Linnea and new breeding lines displayed enhanced resistance to ARR. (**Paper II**)

- Exposure to other pathogens and soil microbes as well as changing climate conditions highlight the difficulty of predicting pea breeding line performance in the field by experiments in controlled conditions. (**Paper II**)

- The pea immune response to *A. euteiches* infection is defined by the resistance level of the host and the infection progress rather than the virulence level of the pathogen. (**Paper III**)

- A set of 39 candidate disease resistance genes was identified upon *A. euteiches* infection, including a polymorphic NLR specifically upregulated in PI180693. (**Paper III**)

- Strains of the *Aphanomyces* genetic group S display lower virulence on PI180693 and smaller oospores compared to groups CE and NE. Based on phylogenetic recognition, group S could be considered a separate *Aphanomyces* species (PS1). (**Paper IV**)

- The CAZyme family CE1 is non-randomly expanded in *A. euteiches* compared to PS1. Homologs in plant pathogenic species comprise feruloyl esterase C and B/C/D domains. (**Paper IV**)

Improving pea breeding programs with PI180693 partial resistance and candidate disease resistance genes

With an increasing demand for plant-based protein and the ability of pea to tolerate cold climates, it can be assumed that pea production and subsequent yield losses due to ARR will remain a focus in Swedish agriculture.

In **Paper II** we showed how the partial resistance of PI180693 was successfully introduced in new Swedish breeding lines, which showed enhanced resistance to ARR than their commercial parent. Results of the large-scale virulence assay in **Paper IV**, showed how PI180693 displayed lower DI than Linnea when exposed to a vast range of European *A. euteiches* strains belonging to different genetic groups. Based on these findings, the pea genotype PI180693 provides a valuable source of robust resistance against current Swedish and European *A. euteiches* strains. However, the introduction of resistance into new crosses is still associated with the introduction of unfavorable breeding traits. Finer mapping techniques with higher resolution in the detection of disease resistance markers are required for breaking this linkage.

In the characterization of the pea transcriptomic immune response to *A. euteiches* (**Paper III**) we revealed fundamental differences in gene expression between a resistant and susceptible pea genotype. Strain virulence levels had a lower effect on gene regulation patterns and we were able to identify candidate disease resistance genes in a successful defense response in PI180693. More specifically, we provide a set of 39 candidate genes for the development of resistance markers in future pea breeding programs. Based on genetic characterization, the Psat7g091800.1 NLR presents a

particularly interesting candidate for functional validation in overexpression or knock-out experiments.

Optimizing breeding targets in pea by identifying A. euteiches key virulence factors

The genetic basis of virulence in *A. euteiches* is still poorly understood but increased knowledge on *A. euteiches* disease development and virulence factors is crucial for the improvement of specific breeding targets in pea. Previous work on linking *A. euteiches* genotype and virulence using neutral markers have been unsuccessful, but large-scale GWAS may prove valuable in the identification of markers for *A. euteiches* virulence in the future.

In **Paper IV** we followed an approach of comparing the evolution of virulence-related gene families in *Aphanomyces* species. The observed expansion of ABC multidrug resistance exporter families and the presence of feruloyl esterase domains in CE1s homologs of exclusively plant pathogenic species could represent key virulence factors of a plant pathogenic lifestyle. More specifically, the pea-infecting species *A. euteiches* and PS1 displayed expansions of several oxidoreductases and membrane transporter families. Detoxification and tolerance to toxic plant-derived secondary metabolites during infection seem to be evolutionary beneficial to *A. euteiches* during host infection. On the other hand, pea breeding programs could aim at the selection for specific traits such as optimized cell wall composition or increased secondary metabolite and enzyme production. Whereas conventional plant breeding techniques can face limitations in the genetic diversity available in existing germplasm collections, targeted mutagenesis and genome editing technologies offer the possibility to specifically improve desirable traits.

A. euteiches in a changing climate

In **Paper I** we presented the first study of the genetic diversity of *A. euteiches* in Europe covering six countries and different climatic zones. Since the genetically most differentiated groups were found at the northern and southern sampling border, we hypothesize that *A. euteiches* populations are affected by local climate. Local climate can have direct effects on *A. euteiches* growth and progressing disease severity on pea by providing more or less favorable temperature and humidity conditions during a growing

season. In the 2022 field trial (**Paper II**), we saw how high levels of soil moisture during sowing, followed by a dry and warm growing season could result in unexpectedly high DI on partially resistant pea lines. This observation is of particular importance in regard to global climate change. A general increase in temperature and drought most likely impedes *A. euteiches* growth and ability to infect. On the other hand, more frequent extreme weather conditions, such as heavy rainfalls and consequent flooding, can increase *A. euteiches* zoospore production, disease dispersal and infection. How *A. euteiches* adapts to changing climate and the possible effect on its virulence on pea are unclear and need further investigation.

Importance of monitoring gene flow, reproduction mode and outcrossing in A. euteiches populations

Shared multilocus genotypes between countries indicate gene flow between geographically distant regions (**Paper I**). This movement is likely supported by human activity due to the soilborne nature of the pathogen and its limited mode of dispersal. It could occur through the transport of contaminated materials and equipment between production fields. The spread of *A. euteiches* should be kept at a minimum to avoid the introduction of invasive strains and new genetic material for recombination. This might require stricter regulations in the transportation of infested soil, infected plant material or contaminated agricultural equipment. Monitoring the reproductive mode of *A. euteiches* populations provides valuable information for ARR risk assessment. An early detection of signs of sexual recombination, compared to the more common selfing nature of the pathogen, could indicate a risk of the pathogen population to acquire virulence traits that can break the partial resistance in the locally used pea cultivars. Further, it is possible that crop rotations with various *A. euteiches* host crops contribute to the pathogen's specialization and adaptation on respective hosts, subsequently enabling outcrossing between genetically distinct strains. The subsequent development of new genotypes increases the risk for new virulence alleles to establish in pathogen populations. In this thesis, specific information on the crop history of *A. euteiches* sampling regions was lacking but GCPSR analysis in **Paper IV** revealed both signs of sexual reproduction in the genetic group S and a close relatedness to *A. cochlioides* based on SNP markers. Further differentiation of group S to other European *A. euteiches* strains was observed in morphology (oospore

size) and virulence on a partially resistant host, providing polyphasic support of possible speciation in *Aphanomyces* populations. The effects of gene flow, reproduction mode, outcrossing and possible speciation on strain virulence are largely unknown but should be monitored in future studies on *A. euteiches* populations.

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Popular science summary

Aphanomyces root rot (ARR) in pea is caused by the oomycete pathogen *Aphanomyces euteiches* Drechs. Oomycetes, also known as water molds, resemble fungi in their growth and appearance but are more closely related to algae. The first report of *A. euteiches* as a threat to pea production goes back to 1925 but the pathogen has since become a major constraint in all pea growing regions with temperate climate. Infected plants display typical root rot symptoms, such as brown and water-soaked lesions and a general reduction of the root volume. Mitigation methods against the pathogen are scarce, as many commercial fungicides have no effect on suppressing the oomycete and additionally, *A. euteiches* produces thick-walled survival structures (oospores) that can remain in the soil for long periods. Crop rotation and avoidance of fields with high occurrence of *A. euteiches* in the soil are therefore the most efficient available measures against the disease. The deployment of resistant cultivars would be the economically and environmentally most beneficial way to reduce yield losses caused by ARR. Currently, there is no commercial pea cultivar available that carries full resistance to ARR but several genetic individuals (genotypes) carry partial resistance and are used in breeding programs. It is known that resistance to ARR is linked with unfavourable breeding traits. An example for a pea genotype with resistance to ARR is the old German landrace PI180693. It carries partial resistance to *A. euteiches* but also undesirable breeding traits such as long stems, a dark seed coat and smooth seeds that are associated with a starchy flavour. The line is currently used in Swedish pea breeding programs and crossed with the commercial pea cultivar Linnea. Linnea is a cultivar that carries all the desirable breeding traits such as wrinkly seeds, a sweet flavour, a normal growth length but is also highly susceptible to ARR.

Classical breeding programs focus on crosses between the two genotypes and could benefit from available genetic markers which allow for screening for desirable genes at an early growth stage. The goal of this thesis was to identify genes that are associated with ARR resistance and thereby gaining new insights into the genetic diversity of *A. euteiches* and its degree to cause infection (virulence) in pea.

In this thesis, I confirmed the potential of PI180693 partial resistance in growth chamber, greenhouse and field trials and found how crosses with the commercial cultivar Linnea displayed higher levels of resistance to ARR than their susceptible parental line. Controlled infections of Linnea and PI180693 with *A. euteiches,* followed by analyses on the differential regulation of genes revealed a very distinct immune reaction of the two pea genotypes. The immune reaction was both host- and time-dependent. We cross-referenced differentially regulated genes with genes situated in genomic regions associated with resistance to ARR and identified a set of 39 candidate disease resistance genes that can be used for the development of genetic markers in future breeding programs.

To further support ARR resistance breeding, more detailed knowledge about the pathogen diversity and population structure across pea growing regions is essential. The thesis work includes genetic analyses on a collection of European *A. euteiches* strains from six different countries, spanning from north to south. Three genetically differentiated groups were identified: a central European, a northeastern, and a genetically very distinct group in the south. We found signs of genetic recombination in the mostly clonally reproducing pathogen, as well as evidence for genetic movement of *A. euteiches* between countries. The southern group of strains shared no common ancestry with the other groups and differed in oospore size and virulence on pea – all of them possible indications to view the southern group as a separate *Aphanomyces* species.

The thesis results contribute to future resistance breeding programs with a better understanding of the genetics underlying ARR resistance in pea, and new insights into genetic diversity, population structure and virulence of European *A. euteiches* strains.

Populärvetenskaplig sammanfattning

Aphanomyces rotröta (ARR) på ärter orsakas av algsvampen *Aphanomyces euteiches* Drechs. Algsvampar liknar svampar i sin tillväxt och morfologi men är närmare besläktade med alger. Den första rapporten om *A. euteiches* som en patogen på ärter är från 1925. Patogenen har sedan dess blivit ett stort problem i alla regioner med tempererat klimat där ärter odlas. Angripna växter uppvisar typiska symptom på rotröta, såsom bruna och vattensjuka sår och en allmän minskning av rotvolymen. Det finns få metoder att bekämpa sjukdomen, och många kommersiella fungicider har inte någon effekt på patogenen. Dessutom producerar *A. euteiches* tjockväggiga oosporer som kan finnas kvar i jorden under långa perioder. En varierad växtföljd och att undvika odling av ärt på fält med högt inokulum av *A. euteiches* i jorden är för närvarande de mest effektiva åtgärderna mot sjukdomen. Att använda sjukdomsresistenta sorter skulle vara det ekonomiskt och miljömässigt mest fördelaktiga sättet att minska skördeförluster orsakade av ARR. För närvarande finns det dock ingen kommersiell ärtsort tillgänglig med full resistens mot ARR, men flera genotyper bär på partiell resistens och används för närvarande i en del förädlingsprogram. Det är känt att resistens mot ARR är kopplad till andra, ogynnsamma egenskaper. Ett exempel på en ärtgenotyp med partiell resistens mot ARR är den gamla tyska landrasen PI180693. Den bär på partiell resistens mot *A. euteiches* men också oönskade egenskaper som långa internoder, släta frön, mörka frön och en stärkelserik smak. Genotypen används för närvarande i svenska ärtförädlingsprogram och har korsats med den kommersiella ärtsorten Linnea. Linnea är en sort som har många önskvärda egenskaper såsom skrynkliga frön, en söt smak, en normal tillväxtlängd men är också mycket mottaglig för ARR. Klassiska förädlingsprogram fokuserar på korsningar mellan de två genotyperna och

kan dra nytta av tillgängliga genetiska markörer som möjliggör för snabbare identifiering av önskvärda egenskaper i ett tidigt tillväxtstadium.

I denna avhandling bekräftade jag värdet av den partiella resistensen från PI180693 i tillväxtkammare, växthus och fältförsök och fann hur korsningar med den kommersiella sorten Linnea uppvisade högre nivåer av resistens mot ARR än deras mottagliga förälder. Kontrollerade infektioner av Linnea och PI180693 med *A. euteiches*, följt av en genuttrycksanalys, avslöjade ett mycket olika immunsvar hos ärtgenotyperna. Immunsvaren var både genotyp- och tidsberoende. Genom att studera uttrycket av gener i områden i ärtgenomet som associerar till resistens mot ARR kunde vi identifiera 39 kandidatgener för sjukdomsresistens som kan användas för utveckling av genetiska markörer i framtida förädlingsprogram.

För att ytterligare stödja förädling för ARR-resistens behövs mer detaljerad kunskap om patogenens diversitet och populationsstruktur i olika regioner. Avhandlingsarbetet inkluderar därför genetiska analyser av en samling av europeiska *A. euteiches* från sex olika länder, som sträcker sig från norr till söder. Tre genetiskt differentierade grupper identifierades: en centraleuropeisk, en nordostlig och en genetiskt mycket distinkt grupp i söder. Vi fann tecken på genetisk rekombination i den mestadels klonalt reproducerande patogenen, liksom bevis för förflyttning av enskilda *A. euteiches* individer mellan länder. Den södra gruppen som var genetiskt skild från de övriga två grupperna och skilde sig också åt i oosporstorlek och virulens på ärt – de här tre observationerna är möjliga indikationer för att den södra gruppen bör betraktas som en separat *Aphanomyces* art.

Avhandlingens resultat bidrar till framtida förädlingsprogram med en bättre förståelse av genetiken bakom ARR och nya insikter om genetisk diversitet, populationsstruktur och virulens hos europeiska *A. euteiches*-isolat.

Populärwissenschaftliche Zusammenfassung

Die Wurzelfäule der Erbse wird durch einen Komplex von im Boden vorkommenden Krankheitserregern verursacht, darunter der Oomycet *Aphanomyces euteiches* Drechs. Oomyceten, auch bekannt als Algenpilze oder Scheinpilze, ähneln in ihrem Wachstum und ihrer Morphologie den Echten Pilzen oder Schleimpilzen, sind aber enger mit Braunalgen und Goldalgen verwandt. Der erste Bericht über *A. euteiches* als Bedrohung für den Erbsenanbau stammt aus dem Jahr 1925, seither hat sich der Erreger jedoch als große Bedrohung in allen Erbsenanbaugebieten mit gemäßigtem Klima etabliert. Befallene Pflanzen zeigen typische Symptome, wie braune und wassergetränkte Läsionen und eine allgemeine Verringerung des Wurzelvolumens. Bekämpfungsmethoden gegen *A. euteiches* sind rar, da viele handelsübliche Fungizide keine Wirkung auf die Unterdrückung dieses Krankheitserregers zeigen. Außerdem produziert *A. euteiches* dickwandige Oosporen, die über lange Zeiträume im Boden verbleiben können. Wechsel in der Fruchtfolge beim Anbau und das Meiden von Feldern mit hohem *A. euteiches*-Vorkommen im Boden sind daher die wirksamsten verfügbaren Maßnahmen gegen Wurzelfäule der Erbse. Der Einsatz von resistenten Sorten wäre der wirtschaftlich und ökologisch vorteilhafteste Weg zur Verringerung des Ertragsverlustes durch Wurzelfäule. Derzeit gibt es keine kommerzielle Erbsensorte, die eine vollständige Resistenz gegen *A. euteiches* aufweist, aber mehrere Genotypen (genetische Individuen) tragen eine Teilresistenz und werden in Zuchtprogrammen verwendet. Es ist bekannt, dass die Resistenz gegen *A. euteiches* mit ungünstigen Zuchtmerkmalen gekoppelt ist. Ein Beispiel dafür ist die alte deutsche Landsorte PI180693. Sie trägt eine Teilresistenz gegen *A. euteiches*, aber auch unerwünschte Zuchteigenschaften wie sehr lange Stängelsegmente,

eine dunkle Samenschale, glatte Samen und den damit einhergehenden stärkehaltigen Geschmack. Die Landsorte wird derzeit in schwedischen Erbsenzuchtprogrammen verwendet und mit dem kommerziellen Erbsenkultivar Linnea gekreuzt. Linnea ist ein Kultivar, der alle erwünschten Zuchtmerkmale, wie z. B. faltige Samen, einen süßlichen Geschmack, eine normale Wachstumslänge, jedoch auch eine hohe Anfälligkeit für Wurzelfäule verursacht durch *A. euteiches* aufweist. Die klassischen Zuchtprogramme in Schweden konzentrieren sich auf Kreuzungen zwischen den beiden Genotypen und könnten von verfügbaren genetischen Markern profitieren, die eine Selektion auf erwünschte Gene in einem frühen Wachstumsstadium erlauben. Ziel dieser Arbeit war, mögliche Resistenzgene gegen *Aphanomyces* Wurzelfäule zu identifizieren und dabei neue Erkenntnisse über die genetische Vielfalt und das Krankheitspotenzial von *A. euteiches* zu erlangen.

Im Umfang dieser Arbeit habe ich das Potenzial der Teilresistenz von PI180693 in Gewächshaus- und Feldversuchen erforscht und bestätigt, dass Kreuzungen mit dem kommerziellen Kultivar Linnea ein höheres Maß an Resistenz gegen *A. euteiches* aufwiesen als Linnea selbst. Kontrollierte Infektionen von Linnea und PI180693 mit *A. euteiches*, gefolgt von einer Analyse zur unterschiedlichen Regulierung von Genen während des Infektionsverlaufs, zeigten eine sehr unterschiedliche Wirts- und zeitabhängige Immunreaktion der beiden Erbsengenotypen. Wir verglichen die unterschiedlich regulierten Gene mit Genen mit bekannter Resistenz gegen *A. euteiches* Wurzelfäule und identifizierten 39 Kandidaten für Krankheitsresistenzgene, die für die Entwicklung von genetischen Markern in zukünftigen Zuchtprogrammen verwendet werden können.

Zur weiteren Unterstützung der Resistenzzucht gegen Wurzelfäule der Erbse sind detailliertere Kenntnisse über die Diversität und Struktur von *A. euteiches*-Populationen in Erbsenanbauregionen von wesentlicher Bedeutung. Die Dissertationsarbeit umfasst genetische Analysen einer Sammlung von europäischen *A. euteiches*-Isolaten aus sechs verschiedenen Ländern, die sich von Norden nach Süden erstrecken. Es wurden drei genetisch differenzierte Gruppen identifiziert: eine mitteleuropäische, eine nordöstliche und eine genetisch sehr unterschiedliche Gruppe im Süden. Wir fanden Anzeichen für Genaustausch in dem meist klonal reproduzierenden Erreger sowie Anzeichen für Gentransfer zwischen Ländern. Die südliche Gruppe von Isolaten teilte keine Verwandtschaft mit anderen Isolaten und

unterschied sich auch in der Größe der Oosporen und des Krankheitspotenzials (Virulenz) auf Erbsen – zusammengenommen mögliche Anzeichen dafür, dass die südliche Gruppe eine differenzierte *Aphanomyces*-Spezies sein könnte.

Die Ergebnisse diese Dissertation tragen zu einem besseren Verständnis der genetischen Grundlagen von Resistenz bei der Erbse bei. Neue Erkenntnisse über die genetische Vielfalt, Struktur und Virulenz europäischer *A. euteiches* Isolate bieten eine wertvolle Grundlage in der künftigen Resistenzzucht gegen Wurzelfäule.

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I

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ORIGINAL ARTICLE

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Genetic diversity of the pea root pathogen *Aphanomyces euteiches* in Europe

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Abstract

The oomycete pathogen *Aphanomyces euteiches* causes root rot in various legume species. In this study we focused on *A. euteiches* causing root rot in pea (*Pisum sativum*), thereby being responsible for severe yield losses in pea production. We aimed to understand the genetic diversity of *A. euteiches* in Europe, covering a north-to-south gradient spanning from Sweden, Norway and Finland to the UK, France and Italy. A collection of 85 European *A. euteiches* strains was obtained, all isolated from infected pea roots from commercial vining pea cultivation fields. The strains were genotyped using 22 simple-sequence repeat markers. Multilocus genotypes were compiled and the genetic diversity between individual strains and population structure between countries was analysed. The population comprising strains from Italy was genetically different and did not share ancestry with any other population. Also, strains originating from Finland and the eastern parts of Sweden were found to be significantly different from the other populations, while strains from the rest of Europe were more closely related. A subset of 10 *A. euteiches* strains from four countries was further phenotyped on two susceptible pea genotypes, as well as on one genotype with partial resistance towards *A. euteiches*. All strains were pathogenic on all pea genotypes, but with varying levels of disease severity. No correlation between the genetic relatedness of strains and virulence levels was found. In summary, our study identified three genetically distinct groups of *A. euteiches* in Europe along a north-to-south gradient, indicating local pathogen differentiation.

KEYWORDS
Aphanomyces root rot, genetic diversity, pathogenicity, *Pisum sativum*, virulence

1 | INTRODUCTION

The oomycete pathogen *Aphanomyces euteiches* is the causative agent of Aphanomyces root rot disease in a broad range of various legume host species, including pea (*Pisum sativum*). Pea is one of the most important legumes in the world and with the global trend towards a more sustainable food production and consumption, peas are becoming increasingly high in demand as a valuable source of plant-based protein (Ge et al., 2020). Aphanomyces root rot is the major constraint for increased pea production in Europe and can cause very high yield losses and negatively affect quality. Vining peas are harvested as immature seeds (green peas) and consumed as a vegetable. They are cultivated worldwide in areas with a temperate climate, and worldwide production reached 19.87 million tonnes in 2020 (FAO, 2021).

A. euteiches is a diploid, homothallic (self-fertile) species, although there are clear indications of occasional outcrossing (Quillévéré-Hamard et al., 2018). Through sexual reproduction, *A. euteiches* produces highly resilient oospores that serve as the primary inoculum and can remain dormant in the soil for several years (Hughes & Grau, 2013). Once a suitable host plant is present, the oospore germinates to form a sporangium that in turn produces actively swimming zoospores that are typically responsible for the infection of host root tissue. Common symptoms of infection include root browning and reduction of root volume and function. Late-stage symptoms include leaf chlorosis, wilting and in extreme cases plant death (Hughes & Grau, 2013; Wakelin et al., 2002).

Due to the soilborne nature of *A. euteiches*, management of root rot disease in pea is difficult and relies mainly on avoidance of highly infested fields and inoculum build-up. Forecasting methods include soil tests prior to sowing, using susceptible pea genotypes in field soil (Hughes & Grau, 2013). Crop rotation with nonhost crops is another widely used control measure. However, due to the long survival of resilient oospores in the soil, crop rotation periods should span at least 6–8 years to minimize the risk of root rot disease (Wu et al., 2018). These long intervals in crop rotation pose a considerable constraint to the total production of peas, especially for cultivation of vining peas where production sites must be close to processing factories to keep short time spans between harvest and processing in order to maintain good quality of the final product. Seed treatment with chemical or biological products may provide a limited protection towards the disease. Currently, there are no commercial pea varieties with complete resistance against Aphanomyces root rot, although pea genotypes carrying partial resistance have been identified (Desgroux et al., 2016; Hamon et al., 2011; Lavaud et al., 2015).

Previous population genetic studies from major pea production regions in the United States revealed high genetic diversity within fields but rather low diversity among populations, and no population structure at a regional level (Malvick & Percich, 1998a; Malvick et al., 2008). Le May et al. (2018) showed that North American *A. euteiches* strains isolated from pea could be divided into three different populations, while strains from cultivated pea in France formed a single population with no substructure. In contrast, a study based on codominant simple-sequence repeat (SSR) markers of French *A. euteiches* strains described two distinct genetic groups (Quillévéré-Hamard et al., 2018). Strains from the Bourgogne region showed higher levels of heterozygosity compared with strains from other parts of France.

Earlier studies, both in the United States and Europe, have investigated the link between genetic diversity of *A. euteiches* strains and host range and disease severity. No association between genotypic diversity and disease severity was detected using single-zoospore progeny of North American *A. euteiches* strains (Malvick & Percich, 1998b). Likewise, no relationship between race phenotype and genotype was detected in alfalfa-infecting *A. euteiches* strains (Malvick et al., 2008). Wicker et al. (2001) investigated pathogenic diversity among *A. euteiches* isolates from France and described four pathotypes based on their host range and aggressiveness. They further confirmed the existence of two virulence phenotypes for pea-infecting isolates with host range "pea" or "pea/alfalfa" (Malvick et al., 1998). Quillévéré-Hamard et al. (2018) reported on high diversity in aggressiveness between strains, especially in the Bourgogne population, but a weak relationship between genetic structure and aggressiveness.

A better understanding of the genetic diversity of *A. euteiches* on a European level is important for future efforts in breeding for disease resistance and for long-term deployment of management strategies. Therefore, this study aimed to investigate the genetic diversity among *A. euteiches* strains sampled across Europe with the emphasis on a north–south gradient, using codominant SSR markers. More specifically, we investigated (a) the genetic diversity and population structure of *A. euteiches* across Europe, and (b) the correlation between genetic variation and virulence on pea.

2 | MATERIALS AND METHODS

2.1 | Sampling and isolation of European *A. euteiches* strains

Strains of *A. euteiches* were collected from different European vining pea cultivation sites (Table S1). Soil samples were collected at 20–25 cm depth during October and November in 2012, 2014, 2018, 2019, and during May and June in 2020, and stored at 6°C in sealed plastic bags to retain humidity until culturing. In addition, roots of infected plants in production fields were sampled in 2018, in the beginning of May in Italy and in the beginning of June in France, at plant growth stage 35–60 according to the BBCH scale (Feller et al., 1995). *A. euteiches* was baited from each soil sample using the susceptible cultivar Linnea, as described by Olofsson (1967). All plant roots were washed in order to grade the characteristic colour and softness of roots caused by *A. euteiches*. After washing and microscopic investigation, root pieces of individual samples were placed on a filter under running water for 1 h and then moved to selective medium agar (Larsson & Olofsson, 1994). After 2–3 days, tips of hyphae growing out from the root pieces were cut and transferred to Petri plates with corn meal agar (CMA; BD Biosciences). Plates were incubated at 20°C for 10 days to initiate growth and then moved to 4°C for long-term storage in darkness. Strains were routinely transferred to new CMA plates twice a year. Ten previously genotyped *A. euteiches* strains from France (Moussart et al., 2007; Quillévéré-Hamard et al., 2018) were obtained on agar plates. For DNA extractions, strains were grown in glucose peptone broth (GPB; glucose 5 g/L, peptone 20 g/L) and incubated at room temperature and shaken at 120 rpm for 5–7 days. Mycelia were harvested by filtering through filter paper (grade 1003; Ahlstrom Munksjö) and immediately processed.

2.2 | DNA extraction and SSR amplification

Harvested mycelia were ground in 2 ml screw cap tubes with three 2 mm diameter glass beads per tube for 2 ×30 s at maximum speed, using a Precellys 24 Tissue Homogenizer (Bertin Technologies). Genomic DNA was extracted following a 3% hexadecyl-trimethyl-ammonium

bromide (CTAB) extraction protocol (Nygren et al., 2008) with an additional chloroform purification step. The DNA concentration and quality were measured using a NanoDrop 1000 spectrophotometer (Thermo Scientific) and by 1% agarose gel electrophoresis.

PCR amplification of the 22 SSR markers was conducted using primers with 5′ FAM/HEX modifications (Table S2) following the protocol by Mieuzet et al. (2016) with minor modifications. Each PCR contained 1 U *Taq* DNA polymerase (Thermo Scientific), PCR buffer, 0.2 mM dNTPs, 1 μM fluorescence dye-tagged forward primers, 1 μM reverse primers and 20 ng genomic DNA template, made up to a total volume of 10 μl with sterile distilled water. The PCRs were run on a Veriti 96-well thermal cycler (Applied Biosystems) with an initial denaturation at 96°C for 5 min, followed by 20 cycles at 95°C for 60 s, an annealing step at 58°C for 60 s and extension at 72°C for 90 s. Some primer pairs were run with adjusted annealing temperatures (Table S2). PCR product concentrations were measured with a NanoDrop 1000 spectrophotometer, and the fragment size was verified through 2% agarose gel electrophoresis. Negative PCR amplifications were rerun to confirm null alleles. PCR products for two markers with different florescent dyes were pooled together with concentrations adjusted to 50 ng/μl for each product prior to being air-dried overnight at room temperature and sent to Macrogen Europe B.V. (Amsterdam, Netherlands) for fragment analysis on a 3730xl DNA analyser using standard parameters and HD400 as the internal standard.

2.3 | Allele scoring and primer quality assessment

Allele scoring was done using the GeneMarker software v. 3.0.1 (SoftGenetics LLC) using standard parameters (Fragment Plant, default data process). The 10 *A. euteiches* strains from France were used as an internal control to allow comparison with previous studies (Quillévéré-Hamard et al., 2018). Multilocus genotypes (MLGs) were obtained by combining data from the 22 loci for each sample. The Excel (Microsoft) plugin GenAlEx v. 6.503 (Peakall & Smouse, 2006, 2012) was used for the initial quality assessment of the data. The R package poppr v. 2.9.3 (Kamvar et al., 2014, 2015) was used to check marker performance and basic overall quality assessment. A genotype accumulation curve was created with loci being resampled 1000× without replacement and dropping monomorphic loci. A locus table including number of alleles for each locus and missing data percentage were calculated prior to clone-correcting the data set and calculating evenness. Of the 22 loci, four were monomorphic. Three monomorphic loci were excluded in further analyses, while the fourth showed an uneven distribution of missing values (null alleles present in strains from Finland and in two strains from Sweden) and was thus retained.

2.4 | Analyses of population structure of *A. euteiches* in Europe

Population genetic analysis was performed on strains grouped by country of isolation (see Figure 1b for overview). The number of unique MLGs was determined for each country. Genotypic diversity for each country was calculated as the number of MLGs divided by the number of samples in each country. Because the number of samples differed substantially between the different countries, the Simpson index (λ) was used to calculate the within-country genetic diversity (Simpson, 1949). Similarly, the adjusted index of association ($\bar{r}_d$) was used to describe linkage disequilibrium, as it is less sensitive to uneven sample sizes (Agapow & Burt, 2001). The $\bar{r}_d$ can only be calculated on groups including more than five individuals, and thus Norway and Italy were excluded from this analysis. The initial visualization of genetic diversity between samples using distance-based, covariance standardized principal coordinate analysis (PCoA) was done in GenAlEx v. 6.503. Minimum spanning networks (MSNs) based on Bruvo distance (Bruvo et al., 2004) were calculated using the bruvo.msn function in the R package poppr to visualize the relationship among strains. In addition, a neighbour-joining (NJ) tree was created using the bruvo.boot function with 1000 bootstrap resamplings. To estimate common ancestry between samples, the snmf function in the R package for Landscape and Ecological Association Studies (LEA) was used (Frichot & François, 2015). For this analysis, the number of genetic clusters (*K*) was ml set to range between 1 and 10 and the number of ancestral populations was selected via a cross-validation technique enabling an entropy criterion to choose the best *K* value (Alexander & Lange, 2011; Frichot et al., 2014). Missing data were complemented based on an ancestry coefficients estimation, taking into account ancestral genotype frequencies (Frichot & François, 2015).

2.5 | Assessment of *A. euteiches* virulence on pea

In the current work, we define pathogenicity of *A. euteiches* strains as the ability to cause disease (a qualitative measure) and virulence as the severity of disease symptoms (a quantitative measure) on pea. Three pea genotypes with different levels of susceptibility were used in pot experiments to assess pathogenicity and virulence of *A. euteiches* strains: Lumina (susceptible), Linnea (susceptible) and MN313 (partly susceptible). We used a phenotyping protocol under controlled conditions that is similar to assays used in commercial breeding programmes. Pea seeds were surface sterilized by washing in 70% ethanol for 1 min, rinsed with sterile water, and subsequently washed with 1% sodium hypochlorite for 5 min, followed by several washing steps with autoclaved water. Air-dried seeds were aseptically placed on 0.8% water agar and incubated at 25°C for 4 days in darkness. Strains of *A. euteiches* were grown on CMA plates for 2 weeks at 20°C in darkness prior to their use in infection. Square plastic pots (0.254 L) were filled with vermiculite (Sibelco) and a single 10 mm-diameter agar plug of *A. euteiches* inoculum was added directly into holes (c.4 cm depth and 1 cm diameter) made in the vermiculite. To prevent cross-contamination, tools used for the inoculation of *A. euteiches* were sterilized with 70% ethanol between strains. Furthermore, pots inoculated with different *A. euteiches* strains were kept on separate trays until scoring. Four-day-old, germinated pea seedlings were transferred

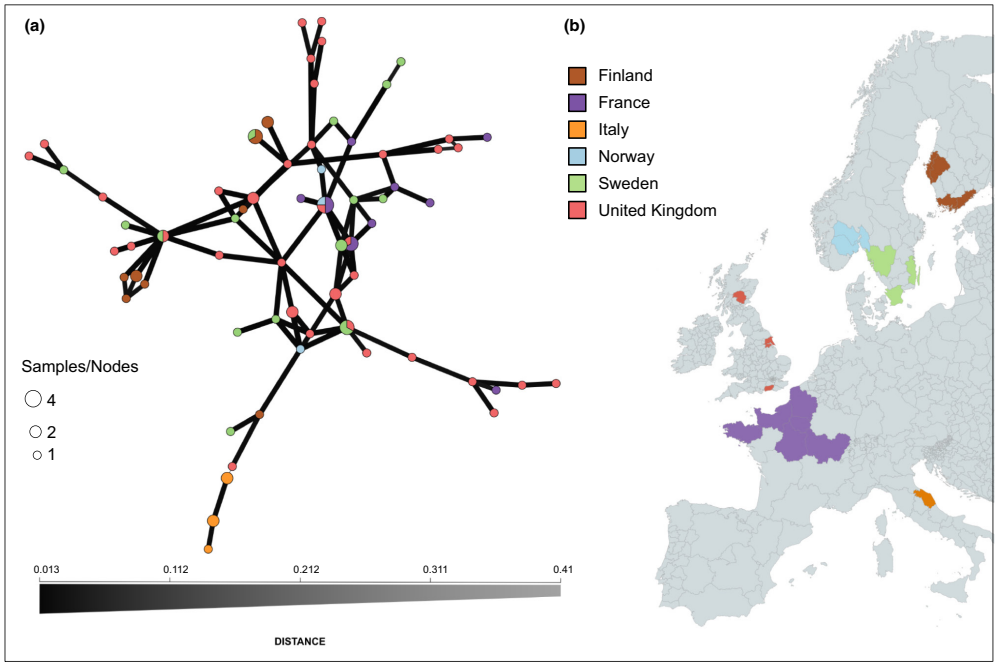

FIGURE 1 Genetic relationships and origin of European *Aphanomyces euteiches* strains. (a) Minimal spanning network based on Bruvo distance representing genetic distance between countries (colour) and number of strains (samples/node). Branch thickness represents genetic relatedness and shared multilocus genotypes between countries are indicated with split nodes. (b) Map showing *A. euteiches* strains originating from Finland (brown), France (purple), Italy (orange), Norway (blue), Sweden (green) and the UK (pink). The online tool MapChart was used for illustration (https://mapchart.net/europe-detailed.html). [Colour figure can be viewed at wileyonlinelibrary.com]

into the holes containing the inoculum, followed by incubation in a growth chamber (CMP6050; Conviron) at 22°C, 55% humidity and 150 μmol·m^{-2}·s^{-1} light intensity in a 12 h light/12 h dark cycle. The pots were kept on a tray filled with 2 cm of water to keep the vermiculite moist during the experiment. To account for unequal light or humidity conditions, the trays were randomly moved within the growth chamber at all watering occasions, every third day. The experiment was conducted with five pots (biological replicates) per treatment, and each pot contained four plants representing technical replicates. After 2 weeks of incubation, disease severity was assessed by washing the roots carefully in tap water and scoring them on a scale from 0 (completely healthy) to 100 (completely dead,) in steps of 10, by two different people.

2.6 | Statistical analysis

Disease scores were tested for normality followed by two-way analysis of variance (ANOVA) in R using the aov function (package stats v. 4.1.0; R Core Team, 2021) to assess the effects of scorer, cultivar and strain on disease scores. Cultivar × strain interactions were further analysed on their estimated marginal means using the emmip function (package emmeans v. 1.7.0, (Lenth, 2021) on the ANOVA output residuals. To analyse multiple pairwise comparisons, we further used the emmeans and pairs function on the fitted model with specified cultivar and isolate interactions.

3 | RESULTS

3.1 | Genetic diversity of *A. euteiches* strains

A total of 75 *A. euteiches* strains, originating from Sweden, Norway, Finland, the UK, France and Italy were isolated in pure culture (Table S1). Successful PCR amplification of 22 SSR marker loci was achieved for all 75 strains and 10 reference strains from France (Moussart et al., 2007; Quillévéré-Hamard et al., 2018), with the exception of markers Ae12, Ae45 and Ae63 where no amplification was found in the 11 strains from Finland and strains SE64 and SE65 from Sweden. These results were repeated twice and confirmed as true missing data. The number of identified alleles within loci varied from one (Ae04, Ae36, Ae63 and aph82) to four (Ae44; Table 1). Out of the four monomorphic loci, locus Ae63 had missing data exclusively in the Finnish population and two Swedish strains and was

TABLE 1 Characteristics of the simple-sequence repeat markers used in this study

Locus	No. of alleles	Missing data (%)	Evenness
Ae04[a]	1	0.0	n.a.
Ae12[a]	3	21.2	0.48
Ae13[a]	2	0.0	0.49
Ae17[a]	2	8.2	0.50
Ae32[a]	2	0.0	0.49
Ae34[a]	2	1.2	0.49
Ae36[a]	1	4.7	n.a.
Ae37[a]	3	0.0	0.56
Ae44[a]	4	1.2	0.49
Ae45[a]	3	15.3	0.43
Ae54[a]	3	7.1	0.84
Ae63[a]	1	15.3	n.a.
aph1[b]	4	1.2	0.64
aph4[b]	2	2.4	0.49
aph9[b]	4	1.2	0.44
aph20[b]	3	3.5	0.68
aph25[b]	2	4.7	0.49
aph32[b]	4	3.5	0.78
aph35[b]	3	3.5	0.77
aph50[b]	2	7.1	0.56
aph76[b]	2	1.2	0.49
aph82[b]	1	7.1	n.a.
Total	54	n.a.	mean 0.56

Abbreviation: n.a., not applicable.

[a]Locus described by Mieuzet et al. (2016).

[b]Locus described by Quillévéré-Hamard et al. (2018).

therefore kept in the data set for further analyses. Loci Ae04, Ae36 and aph82 were noninformative and excluded from the data in the genetic diversity and population structure analysis. Locus Ae54 had the most evenly distributed alleles (E_5 = 0.84), followed by aph32 and aph35 (Table 1). We observed no more than two alleles per locus and individual, indicating that the analysed strains were diploid. The genotype accumulation curve approached saturation and indicated that the number of markers included in this study was close to enough to differentiate the actual genetic differences in the sampled populations (Figure S1).

We found a total of 67 MLGs across all countries, with the highest proportion of MLGs in the strains collected in Norway, where the number of MLGs corresponded to the actual sample size (Table 2). However, in all other countries the number of MLGs was lower than the number of genotyped isolates, indicating the occurrence of clones in the respective populations. The genotypic diversity was high within all countries, with values of the Simpson diversity index (λ) ranging between 0.667 and − 0.971. Values of λ revealed the largest diversity within the UK, which also comprised the highest number of genotyped strains (Table 2). Values of $\bar{r}_d$ were not significantly different from zero for any of the countries, indicating no linkage between alleles and thus no recombination (Table 2).

3.2 | Genetic structure of *A. euteiches* in Europe

MSNs showed that only five MLGs were shared between countries (Figure 1a). More specifically, Sweden and the UK shared two MLGs (MLG.17 and MLG.22) while MLG.61 occurred in both Sweden and Finland. The UK and France shared two MLGs (MLG.37 and MLG.38) where the latter was also present in Norway. Based on a PCoA, *A. euteiches* strains were divided into three main genetic clusters: one cluster containing all strains from Italy, one cluster containing all strains from Finland and two strains from Sweden (SE64 and SE65), while the third cluster contained all other strains (Figure S2). The NJ tree confirmed the separation between the Italian strains and the other European strains (Figure 2). These results were confirmed by LEA analysis on ancestral genotype frequencies that indicated two main genetic clusters, where the Italian strains belong to a different ancestral population from all other strains ($K = 2$; Figure S3). PCoA and the NJ tree did not identify clustering of strains according to regions within a country or by year (data not shown).

3.3 | Virulence of *A. euteiches* strains on pea

Ten strains of *A. euteiches* were selected for virulence assays on pea representing different geographic origins and the three genetic clusters identified in the PCoA: strains SE51, SE58 and SE64 from Sweden, strains FI2, FI37, and FI46 from Finland, strains IT30, IT32 and IT35 from Italy and the Rb84 reference strain from France (Moussart et al., 2007). Disease score values corresponded to the percentage of roots with disease symptoms (Figure 3a). As the disease severity was assessed by two different people, an initial ANOVA was performed that proved the scorer effect to be nonsignificant ($p = 0.56$). For the following analyses, a two-way ANOVA was performed with cultivar and strain as factors as well as their interaction effect. There were significant effects of strain ($p < 0.001$), cultivar ($p < 0.001$) and their interaction ($p < 0.001$) on disease severity (Table S5). When it comes to differences in susceptibility between pea genotypes, MN313 was less susceptible ($p \leq 0.045$) than both Lumina and Linnea to strains FI2, FI37 and IT30, less susceptible than Lumina to strains FI46 and IT32, and less susceptible than Linnea to IT35 and SE51 (Figure 3b, Table S6). Linnea was more susceptible ($p \leq 0.035$) than Lumina when infected with strains IT35 and SE58. All 10 *A. euteiches* strains were pathogenic on all pea genotypes, with significantly ($p < 0.001$) higher disease severity scores compared with the corresponding mock-treated controls (Figure 3b). There were also significant ($p \leq 0.044$) differences in virulence between *A. euteiches* strains, mainly involving a lower virulence of the Swedish strains compared with other strains (Figure 3b, Table S6).

TABLE 2 Population genotypic and genetic diversity based on 19 loci

Country	N^a	G/N^b	λ^c	$\bar{r}_d^d$	$p(\bar{r}_d)^d$
Norway	3	1	0.667	n.a.	n.a.
Sweden	17	0.882	0.933	0.004	0.594
UK	38	0.921	0.971	0.017	0.131
Italy	5	0.600	0.667	n.a.	n.a.
France	11	0.818	0.889	0.048	0.155
Finland	11	0.727	0.875	−0.126	0.997
Total/average	85	0.890	–	–	–

Abbreviation: n.a., not applicable.

[a]Number of samples.

[b]Number of genotypes divided by number of samples.

[c]Simpson index.

[d]$\bar{r}_d$ adjusted index of association and its *p* value.

4 | DISCUSSION

As a first study covering a geographic north-to-south gradient in collection of strains, the current work revealed higher genotypic diversity within *A. euteiches* populations than previously reported. We found three separate genetic groups of *A. euteiches* in Europe; strains from Italy and Finland (together with two strains from Sweden) form two separate groups, which in turn are genetically distinct from a larger group consisting of the remaining strains from Sweden, Norway, the UK and France. The *A. euteiches* strains from Italy are clearly genetically separated from the other European populations, based on all phylogenetic analyses. In combination with the lack of shared ancestry between these two main groups, this differentiation suggests very low migration rates of *A. euteiches* between Italy and the other sampling sites in north-western Europe. Climatic factors may probably act as drivers of local *A. euteiches* differentiation and selection. It has previously been shown that the ability to produce resilient oospores in certain *Phytophthora* species correlates with their establishment in northern latitudes at lower temperatures (Redondo et al., 2018). Alternatively, the genetic differentiation and lack of shared ancestry may be interpreted as the result of a recent introduction of non-European *A. euteiches* in Italy. However, testing this hypothesis requires sampling of *A. euteiches* from a worldwide distribution.

Due to the predominating homothallic reproductive mode of *A. euteiches* and its limited dispersal capacity (Grünwald & Hoheisel, 2006), it can be expected that the genetic diversity of *A. euteiches* is low within populations from limited geographic areas and increases with geographic distance. Here, we confirm the pattern with an overall low level of genetic diversity found in previous studies of French and North American *A. euteiches* populations (Grünwald & Hoheisel, 2006; Le May et al., 2018; Malvick et al., 1998; Mieuzet et al., 2016; Quillévéré-Hamard et al., 2018; Wicker et al., 2001). In the Grünwald and Hoheisel (2006) study, it was thought that a relatively higher diversity between populations was due to the limited spread of the soilborne pathogen. This explanation is valid for our data as well, given the proximity of both cultivation and processing sites of vining pea and limited choices of alternating fields in crop production, in combination with large geographic distances between production sites in different countries. Concurrently, indications of genetically differentiated groups have been reported from both the United States and France (Grünwald & Hoheisel, 2006; Malvick et al., 1998; Quillévéré-Hamard et al., 2018).

Within the large group of non-Italian *A. euteiches* strains, there are also indications of a genetic differentiation of strains from Finland and parts of Sweden compared with the remaining strains. This is supported both by PCoA and by the fact that three SSR markers failed to amplify PCR products from the Finnish strains. Notably, the two Swedish strains (SE64 and SE65) have missing data at the same loci as the Finnish strains and cluster together with the Finnish population in the PCoA. They were both sampled in the region of Kalmar, in south-eastern Sweden. The fact that the Swedish strains SE64 and SE65 are genetically similar to the Finnish strains suggests a movement of *A. euteiches* between these neighbouring countries. We also identified one MLG (MLG.61) that is shared between Sweden and Finland.

Additional support for international movement of *A. euteiches* is indicated by identical MLGs that are shared between France and the UK, as well as between Sweden and the UK. Given the limited long-range dispersal capacity of *A. euteiches*, it can be speculated that these movements are aided by human activities.

Within each country, the genotypic diversity was high, and only a few clones were identified. The nonsignificant values of $\bar{r}_d$ indicate no linkage between markers and limited clonal reproduction within each country. We acknowledge that the unequal sample sizes, that is, number of strains sampled per country and region, and the generally low number of *A. euteiches* strains limits the analytical power of a population genetics study, in particular within the populations. The not entirely saturated genotype accumulation curve indicates that we were not able to catch and describe the genetic diversity with the number and selection of markers used in this study. At the same time, our results indicate that in combination with the high genotypic diversity within each population, outcrossing is likely to occur in all populations, despite the more common selfing nature of the pathogen. One reason for this could be the pathogen's broad host range within the legume family. It allows for the possibility of outcrossing and genetic exchange between strains that have adapted to different legume species, which might be promoted by crop rotations including multiple host plants.

When phenotyping 10 *A. euteiches* strains, we observed a partial resistance of pea genotype MN313, which has been previously described by Wicker et al. (2001), and a generally high susceptibility of both Lumina and Linnea. As expected, all *A. euteiches* strains were able to infect and cause root rot disease on all tested pea genotypes, although the level of virulence differed between strains. This difference is partly correlated with geographic origin, as the Swedish strains display lower virulence than most other strains. However, this result requires confirmation with a larger data set and possibly different climatic conditions for phenotyping, taking into account that

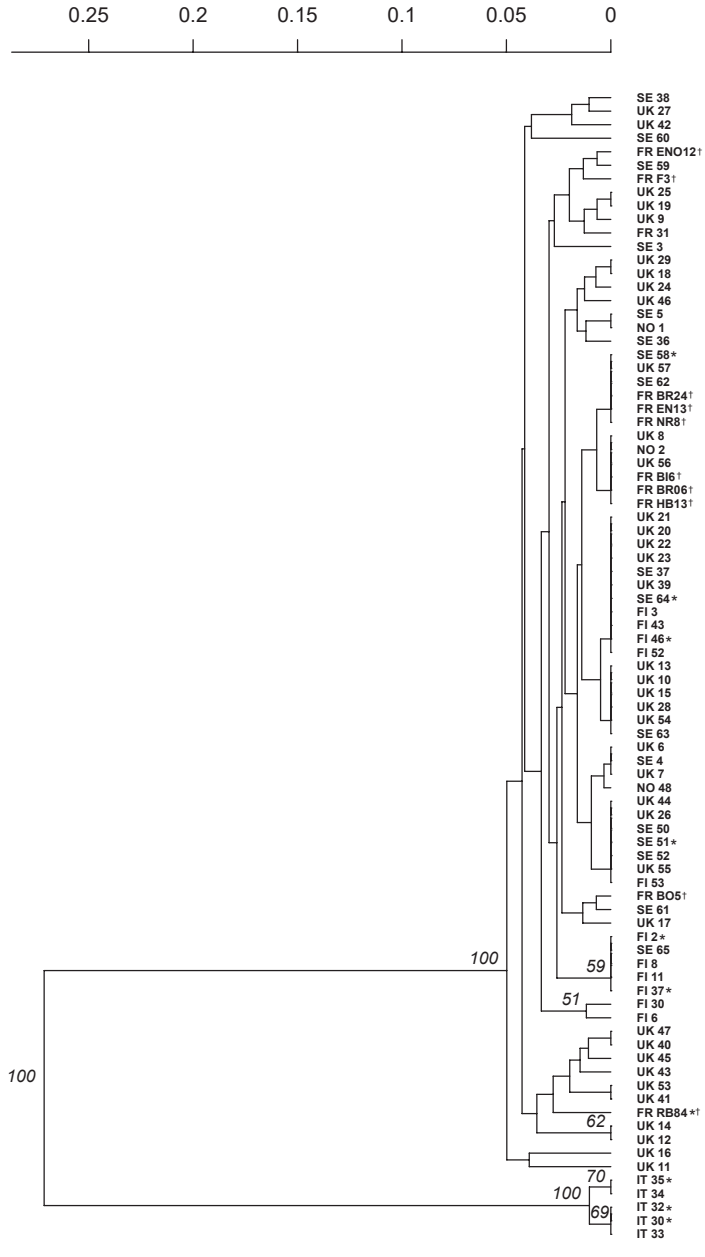

FIGURE 2 Neighbour-joining (NJ) tree showing genetic relatedness of *Aphanomyces euteiches* strains. An NJ tree comprising all 85 *A. euteiches* strains used in the study shows a clear separation of the Italian population from the other strains. The NJ tree was created with 1000 bootstrap resamplings and a cut-off value of 50. A two-letter country abbreviation was added to each strain ID. Strains marked with an asterisk (*) were phenotyped and the dagger symbol (†) marks the French reference strains.

disease severity caused by strains from latitudinal border regions might be climate-dependent. Differences in virulence are common between *A. euteiches* strains and have been observed by Malvick and Percich (1998a) as well as by Wicker et al. (2001), where virulence phenotypes were defined according to pathogenicity on different hosts, indicating host adaptation. Furthermore, there is no correlation between virulence and genetic structure in our data. This is shown by the fact that the reference strain Rb84 displayed a significantly higher virulence than the two Swedish strains (SE51 and SE58) that belong to the same genetic group. More support for this lack of phenotype–genotype correlation comes from the fact that even though the Italian strains were clearly genetically differentiated from

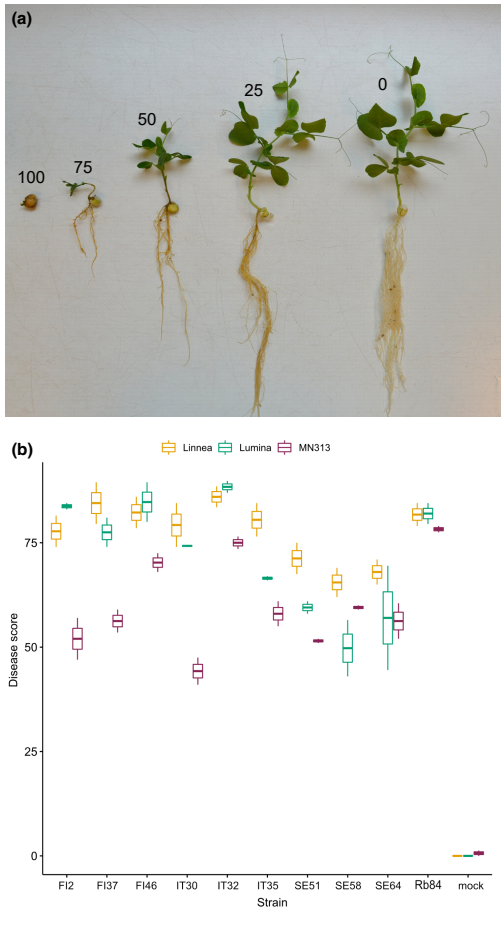

FIGURE 3 Assessment of *Aphanomyces euteiches* virulence on pea. (a) Five representative *Pisum sativum* plants show the range of disease severity caused upon infection with *A. euteiches*, 2 weeks postinfection. Disease severity was scored in steps of 10, with 0 being symptomless and 100 completely dead. (b) The boxplot shows the average disease score of 10 phenotyped *A. euteiches* strains (FI2, FI37, FI46, IT30, IT32, IT35, SE51, SE58, SE64 and Rb84) and the mock treatment (no infection) on three *P. sativum* cultivars Linnea (yellow), Lumina (blue) and MN313 (purple). [Colour figure can be viewed at wileyonlinelibrary.com]

the non-Italian strains, no consistent differences in virulence compared with strains from the other genetic groups were detected. This agrees with previous reports and is suggested to be partly due to the use of neutral markers, such as SSR, with limited genetic linkage to the loci encoding virulence factors (Quillévéré-Hamard et al., 2018).

Our results also highlight the importance of the experimental setup when performing virulence assays, and the risk of introducing biases in phenotypic assessment assays. The previously phenotyped French reference strain Rb84 was more virulent on the pea cultivar Lumina than on MN313 (Quillévéré-Hamard et al., 2018). However, in our virulence assay disease severity was high on all three pea genotypes upon infection with Rb84, with no significant differences between genotypes. One possible explanation for this difference might be related to the inoculum used in the different studies––an agar plug with mycelia in the current study compared with a zoospore solution used in the previous study. Our experimental approach further deviates in the method of allele amplification in PCRs. We tagged our forward primers directly with fluorophores instead of using an additional fluorescently labelled M13 primer. In the allele scoring, this resulted in a consistent base-pair shift when comparing with scored alleles of the 10 reference strains from Quillévéré-Hamard et al. (2018); however, this does not influence the overall results of the study.

In our study we found three genetically distinct groups of *A. euteiches* along a north-to-south gradient and signs of genetic differentiation between strains. Although no correlation between genotype and virulence was detected in the current work, the existence of genetic differentiation and a widespread capacity for occasional outcrossing among *A. euteiches* in Europe is a concern for future disease management strategies. Further, our results emphasize the need for complementing neutral genetic markers used in the current study with whole-genome sequencing and comparative genomics, in order to understand the genetic structure and virulence variation in *A. euteiches*.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are included in the current article and its associated files.

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Evaluation of pea genotype PI180693 partial resistance towards aphanomyces root rot in commercial pea breeding

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The cultivation of vining pea (*Pisum sativum*) faces a major constraint with root rot diseases, caused by a complex of soil-borne pathogens including the oomycetes *Aphanomyces euteiches* and *Phytophtora pisi*. Disease resistant commercial varieties are lacking but the landrace PI180693 is used as a source of partial resistance in ongoing pea breeding programs. In this study, the level of resistance and their interaction with *A. euteiches* virulence levels of six new back-crossed pea breeding lines, deriving from the cross between the susceptible commercial cultivar Linnea and PI180693, were evaluated for their resistance towards aphanomyces root rot in growth chamber and green house tests. Resistance towards mixed infections by *A. euteiches* and *P. pisi* and commercial production traits were evaluated in field trials. In growth chamber trials, pathogen virulence levels had a significant effect on plant resistance, as resistance was more consistent against *A. euteiches* strains exhibiting high or intermediate virulence compared with lowly virulent strains. In fact, line Z1701-1 showed to be significantly more resistant than both parents when inoculated with a lowly virulent strain. In two separate field trials in 2020, all six breeding lines performed equally well as the resistant parent PI180693 at sites only containing *A. euteiches*, as there were no differences in disease index. In mixed infections, PI180693 exhibited significantly lower disease index scores than Linnea. However, breeding lines displayed higher disease index scores compared with PI180693, indicating higher susceptibility towards *P. pisi*. Data on seedling emergence from the same field trials suggested that PI180693 was particularly sensitive towards seed decay/damping off disease caused by *P. pisi*. Furthermore, the breeding lines performed equally well as Linnea in traits important for green pea production, again emphasizing the commercial potential. In summary, we show that the resistance from PI180693 interacts with virulence levels of the pathogen *A. euteiches* and is less effective towards root rot caused by *P. pisi*. Our results show the potential use of combining PI180693 partial resistance against aphanomyces root rot with commercially favorable breeding traits in commercial breeding programs.

KEYWORDS

***Aphanomyces euteiches*, pea root rot, *Phytophthora pisi*, resistance, breeding**

1 Introduction

The production of pea (*Pisum sativum* L.) is globally on the rise as the easy-to-grow crop poses an important source for food and feed (https://www.fao.org). Peas are widely cultivated as an environmentally sustainable alternative to soybean in many plant-based products, due to their high nutritional value and protein content (Xiong et al., 2018; Wei et al., 2020). *P. sativum* can be grown worldwide in temperate to cool climates with Sweden being one of the northernmost regions of pea cultivation. In Sweden, different pea cultivars have been grown since Neolithic times and the plant has remained one of the country's most important crop species alongside cereals (Osvald, 1959; Hjelmqvist, 1979; Leino et al., 2013).

Root rot, a soil-borne disease caused by a complex of fungal and oomycete pathogens, poses a major threat to commercial pea production. Oomycetes resemble fungi in morphology and growth but are able to reproduce both asexually *via* motile zoospores and with the production of sexual oospores. The oospores are resilient to desiccation and can remain in the soil as inoculum for several years (Mitchell and Yang, 1966; Cannesan et al., 2011). Among these root rot pathogens, *Aphanomyces euteiches* is the main causal agent for pea root rot. Its symptoms include discoloration of roots and epicotyl, root damage, wilting and eventual severe yield losses (Malvick et al., 2001; Wu et al., 2018). Another emerging oomycete infecting pea roots is *Phytophthora pisi*, which was first shown to cause root disease in pea in Sweden. Disease symptoms in pea are similar between the two oomycete pathogens, but symptoms of *P. pisi* are rarely observed on the epicotyl (Heyman et al., 2013). Furthermore, oospores of *P. pisi* can be morphologically differentiated from *A. euteiches* oospores under the microscope (Heyman et al., 2013). Differences in virulence among *A. euteiches* strains are observed in controlled infection experiments (Quillévéré-Hamard et al., 2018; Kälin et al., 2022) but prove difficult for the prediction of cultivar performance in the field where soil microbial compositions are complex (Wille et al., 2020).

Agro-ecological factors have been shown to influence soil microbial abundance and community composition in other legume crops (Naseri and Ansari Hamadani, 2017). The co-occurrence of several pathogens in the pea root rot complex (PRRC) has been reported but their interactions remain largely uncharacterized (Baćanović-Šišić et al., 2018; Chatterton et al., 2019). However, the increased susceptibility to single pathogens of the PRRC in presence of other pathogen species has been shown in controlled greenhouse experiments. Using co-inoculation experiments with *A. euteiches* and several *Fusarium* spp., Willsey et al. (2018) reported a disease reinforcement effect in presence of multiple pathogens. Peters and Grau (2002) showed that co-inoculations of pea with a non-pathogenic *F. solani* strain and *A. euteiches* resulted in significantly more severe disease symptoms compared to single infections with *A. euteiches*. Further, other important factors such as the significant effect of sowing date and depth on fusarium wilt development in chickpea cultivars have been shown by Younesi et al. (2020). Historically, breeding for resistance towards aphanomyces root rot has been most successful combining results from plant-pathogen interactions in both growth chambers and field experiments (Moussart et al., 2001; Wicker et al., 2003; Pilet-Nayel et al., 2005; Abdullah et al., 2017).

In Swedish pea production, current control measures against root rot pathogens focus on diagnosis of occurrence in the field and prevention of high pathogen inoculum levels in fields. Soil testing prior to sowing has been a reliable method for the avoidance of highly infested fields and long periods of crop rotation can prevent inoculum accumulation in the soil (Moussart et al., 2009; Moussart et al., 2013). The production of vining peas for quick-freezing are especially challenging since crop production has to be carried out in proximity of factory sites. Breeding for increased resistance against *A. euteiches* remains the most promising approach in disease control. However, sources of partial resistance in pea are scarce, polygenically inherited and largely affected by environmental effects (Hamon et al., 2013; Desgroux et al., 2016; Lavaud et al., 2016). Pea cultivars with complete resistance to aphanomyces root rot are lacking, but several cultivars with partial resistance have been used in breeding programs. Among them, the landrace PI180693 has been identified as a source of resistance towards *A. euteiches* by Lockwood (1960) and has been used in several studies for its potential to tolerate *A. euteiches* infection (Pilet-Nayel et al., 2002; Wicker et al., 2003). Further, PI180693 has shown to maintain high levels of resistance towards fusarium root rot in both controlled and greenhouse conditions (Grünwald et al., 2003; Infantino et al., 2006; Coyne et al., 2019). However, the landrace is associated with unfavorable breeding traits, such as extremely long internode length (long haulm), pale peas, normal leaves and round seeds with a starchy flavor. In modern crop production, semi-leafless and shorter varieties are preferred, as they will remain more erect at harvest, which reduces the risk of picking up small stones and soil particles that can contaminate the produce. Further, peas for quick freezing should have a 'sweet flavor' as well as a uniform, bright and attractive green color. Therefore, PI180693's growth phenotype is unsuitable for commercial cultivation and quick-freezing.

Our study aimed at evaluating the usefulness of the partial resistance against aphanomyces root rot originating from PI180693 in practical pea breeding, with emphasis on disease range and intraspecific pathogen variation, effectiveness and consistency. We used six back-crossed pea lines from a cross between PI180693 and the commercial variety Linnea to investigate (i) variation in disease resistance between breeding lines, (ii) interactive effects between disease resistance and virulence of *A. euteiches* strains, and (iii) the predictive power of climate chamber and greenhouse pot bioassays for estimating pea field performance. We show that the partial resistance towards aphanomyces root rot derived from PI180693 is useful for applied, commercial breeding and how monitoring the presence and virulence levels of pathogen populations is important for development and deployment of durable root rot resistant cultivars.

2 Materials and methods

2.1 *Aphanomyces euteiches* cultivation and growth

The *A. euteiches* strains used in this experiment originate from Sweden (SE51 and SE58) and the United Kingdom (UK16). All strains have been used in commercial breeding experiments, as they

are known to differ in virulence on pea. Strain SE58 was previously included in a phenotyping assay and shown to be of intermediate virulence. All three strains were described to belong to the same genetic cluster in previous population genetic analyses and were maintained as described in Kälin et al. (2022). Prior to be used as inoculum, strains were grown for two weeks on corn meal agar (CMA, BD Biosciences, San Jose, CA) at 20°C in the dark.

2.2 Pea breeding material

Two BC1F8 lines (Z1654-1 and Z1656-1) and four BC2F6 lines (Z1701-1, Z1701-2, Z1707-1 and Z1707-02) were included in this study. These six lines where selected based on screening results of various lines in greenhouse tests (data not shown). The selected lines showed better agronomic performance (yield component parameters and morphology) and tolerance against *A. euteiches* compared to their sibling lines in initial large-scale screenings. The BC1F8 lines were backcrossed once to Linnea, after an initial cross between Linnea and PI180693, whereas BC2F6 lines represents second backcrosses to Linnea in the sixth generation selfed (Table 1).

2.3 Growth chamber and greenhouse assays and phenotyping

Seed surface sterilization was performed following the protocol described in (Kälin et al., 2022) with minor changes. Square plastic pots (0,254 l) were filled with a first layer of vermiculite (Sibelco, Antwerpen, Belgium), on which an agar plate discs (8,5 cm diameter) with *A. euteiches* mycelium were placed in all pathogen treatments. For the infections, only plates fully covered with mycelia were used. The pots were then filled up with vermiculite in which five holes (3 cm depth, 1 cm diameter) were made to place the sterilized seeds. Tools used for the inoculation of *A. euteiches* were sterilized with 70% ethanol between inoculations, to prevent cross-contamination. Pots inoculated with one *A. euteiches* strain were kept together on a separate tray throughout the incubation in the growth chamber (CMP6050, Conviron) at 22°C, 55% humidity and 150 µmol light intensity in a 12 h light, 12 h dark cycle. Uninoculated pots of each cultivar were used as controls. For maintaining optimal pathogen growth conditions, the trays were filled with 2 cm of water and randomly moved within the chamber to account for uneven light or humidity conditions. The experiment was conducted with five pots with five plants each (biological and technical replicates, respectively). Disease scoring was done after three weeks of incubation and root disease symptoms were graded on a scale from 0 (completely healthy) to 100 (completely dead), by two different persons for every plant and then averaged on pot level. Assays in the greenhouse followed the same protocol but with 10 seeds per pot, five replicates, and 16h light, 8h dark cycle at 20°C and 19°C, respectively. For root dry weight measurements, all roots were harvested per biological replicate (pot) and dried over two days at 60°C before weighing on a Precisa 360 ES (growth chamber trials) or Mettler AT261 Delta Range scale (greenhouse trials).

2.4 Field trials and phenotyping

In 2020, two field trials were sown on the 2nd of April (Z20EA) and on the 5th of May (Z20EB) in randomized 1 m^2 plots (two blocks), whereas a single trial in 2022 was sown on the 23rd of March (R-22-10-91) in randomized 12 m^2 plots (4 blocks). All trials were conducted in southern Sweden (Skåne) and the choice of fields was made based on information from biotest indicating moderate infection rate by *A euteiches*. The soil biotest test prior season showed disease index 34 for Z20EA, disease index 76 for Z20EB and disease index 36 for R-22-10-91 trials. At the location for Z20EB both *A. euteiches* and *P. pisi* were detected, see Supplementary Table 1 for field coordinates and soil test scores. For phenotyping, ten plants from each plot were taken to rate the infection on roots and provide a disease index score based on root discoloration, between 0 (completely healthy) to 100 (completely dead). The field Z20EA was scored on the 1st of July 2020, Z20EB on the 7th of July 2020 and field R-22-10-91 on the 7th of June 2022, just before flowering to avoid root darkening due to natural maturation processes. Plant emergence was recorded as the percentage of emerged plants in relation to sowed plants in both field trials in 2020 and as the absolute number of emerged plants per square meter in the 2022 field trial. In field R-22-10-91, plant height, yield (at TR100, kg/ha) and the ratio of

TABLE 1 Information about pea cultivars used in the study.

ID	Type of material	Earliness class*	Leaf type	Flower color	Seed shape
Z1654-1	Breeding line (BC1F8)	+12	semi-leafless	white	wrinkled
Z1656-1	Breeding line (BC1F8)	+12	semi-leafless	white	wrinkled
Z1701-1	Breeding line (BC2F6)	+12	semi-leafless	white	wrinkled
Z1701-2	Breeding line (BC2F6)	+12	semi-leafless	white	wrinkled
Z1707-1	Breeding line (BC2F6)	+12	semi-leafless	white	wrinkled
Z1707-2	Breeding line (BC2F6)	+12	semi-leafless	white	wrinkled
Linnea	Commercial variety (used for BC)	+12	semi-leafless	white	wrinkled
PI180693	Landrace (source of resistance)	+12	leaved	pink	Non-wrinkled

*Earliness class indicated the number of days the cultivar is delayed in green pea harvest relative to reference variety 'Cabree' (earliness class 0). BC, backcross number; F, selfing cycle.

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green peas compared to the total plant biomass as well as additional growth parameters were measured.

2.5 Statistical analyses

In the growth chamber experiment, all disease score values were treated as an average of the disease score values scored by the two scorers. Data were tested for normality and mock scores were excluded from further analyses to approach normal distribution. Two two-way analyses of variance (ANOVA) in R using the aov function (package stats ver. 4.1.0, R Core Team, 2021) were performed to assess the effects of the two factors cultivar and strain on disease index and root dry weight, including the factor's interactions. Data on root dry weight of uninfected plants was assessed separately using Fisher LSD test on one-way ANOVA residuals. For the analysis of greenhouse trials, we used one-way ANOVAs for disease index and root dry weight including cultivar as independent variable, with Fisher LSD *post-hoc* tests. The correlation coefficient for disease index and root dry weight in the growth chamber trials, and for disease index and germination in the field trials, was calculated using Pearson correlation for normal distributions in R (cor.test function). Field data was analyzed separately for each field. For 2020 fields, one-way ANOVAs on the interaction of disease index and emergence with cultivar were performed and Fisher LSD test was used for mean comparisons between groups. For the 2022 field trial, we performed a two-way ANOVA on disease index including cultivar and block effect and one-way ANOVAs were performed for the breeding traits. The correlations of yield with disease index and emergence for each cultivar were analyzed using linear regression modelling.

2.6 Climate data

For the duration of the 2020 field trials, data on temperature, rainfall and relative humidity were retrieved from the closest weather station (56°03'04" N, 12°76'28" E), publicly available on https://www.smhi.se/data/meteorologi/ladda-ner-meteorologiska-observationer. For the 2022 field trial, average air temperature, precipitation (rain) and relative humidity were measured using a mobile weather station installed next to the field (56°01'07.8"N 12° 58'16.1"E). In both cases, daily measurements were retrieved and the averages over two weeks were calculated and used in Supplementary Figure 3.

3 Results

3.1 Disease resistance in growth chamber trials

The growth chamber pot assay showed significant effects of strain ($p < 0.001$), cultivar ($p < 0.001$) and their interaction ($p < 0.01$), on disease index (Table 2). *A. euteiches* strains differed in virulence with UK16 being most virulent on all lines, SE51 was of

TABLE 2 Results from analyses of variance of growth chamber and field trials.

Factor	Growth chamber*		Field R-22-10-91*					Field Z20EA#			Field Z20EB#	
	DI ~cultivar	RDW ~cultivar	DI ~cultivar	Emergence ~cultivar	Yield ~cultivar	Pea biomass ~cultivar	Plant length ~cultivar	DI ~cultivar	Emergence ~cultivar	DI ~cultivar	Emergence ~cultivar
Strain	***	***									
Cultivar	***	***	*	**	*	*	***	*	***	*	**
Straincultivar	**	***									
Block			**	***	**	***	**				
Cultivarblock			.	x	.	x	x				

*Two-way ANOVA. #One-way ANOVA. DI, disease index; RDW, root dry weight; significance codes: '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1.

intermediate virulence while SE58 was least virulent on all lines (Figure 1A). With low pathogen virulence, i.e. infection with SE58, larger variation in disease symptoms between breeding lines was observed, compared with infection with more virulent strains. The disease index of PI180693 was more consistent upon infection with *A. euteiches* strains differing in virulence (Figure 1A). Using Fisher LSD test, breeding lines Z1654-1, Z1656-1, Z1701-1, Z1701-2 and Z1707-2 had significantly ($p < 0.05$) lower disease indices than Linnea upon infection with highly virulent strain UK16 (Supplementary Figure 1A; Supplementary Table 2). In response to intermediate virulence (strain SE51), the same breeding lines were also significantly more resistant than their susceptible parent (Supplementary Figure 1B). However, only line Z1701-1 showed significantly lower disease indices than in Linnea upon infection with the lowly virulent strain SE58 (Supplementary Figure 1C).

3.1.1 Root dry weight in growth chamber trials

We measured lowest root dry weight in cultivars infected with the most virulent *A. euteiches* strain UK16 and highest root dry weight in roots of cultivars infected with the SE58 low virulent *A. euteiches* strain (Figure 1B). In PI180693, however, the root dry weight was highest in plants infected with SE51 and the difference in root dry weight between roots infected with the three strains was lower compared to other cultivars. Both *A. euteiches* strains and pea cultivars, as well as their interaction, showed to have a highly significant ($p < 0.001$) effect on root dry weight in the growth chamber pot trials (Table 2). Fisher LSD tests on cultivar comparisons revealed that upon infection with highly virulent strain UK16, only line Z1707-2 had significantly higher root dry weight than Linnea (Supplementary Figure 2A; Supplementary Table 2). In response to intermediate virulence (strain SE51), breeding lines Z1654-1, Z1701-1, Z1701-2, Z1707-1 and Z1707-2 scored significantly higher root dry weight than the susceptible parent (Supplementary Figure 2B). The same breeding lines, with exception of Z1707-1, also scored higher root dry weight upon infection with the lowly virulent strain SE58, including line Z1656-1 (Supplementary Figure 2C)

Root dry weight measurements of the non-inoculated controls showed natural variation in root volume between cultivars. With an average root dry weight of 0.36 g per biological replicate, breeding line Z1654-1 showed to have non-significantly ($p > 0.05$) lower root dry weight scores than PI180693 (average 0.396g) whereas dried roots of line Z1707-1 did not differ from Linnea (0.237g and 0.19g, respectively). All other breeding lines had intermediate root dry weight scores compared to their parent cultivars (Table 3).

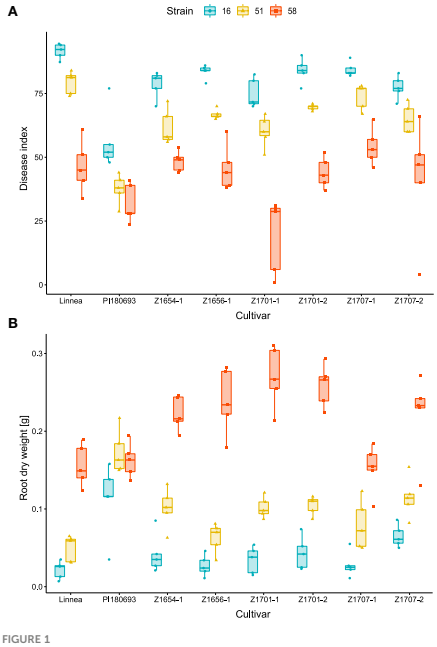

FIGURE 1
Virulence of *Aphanomyces euteiches* strains on pea cultivars. Disease indices **(A)** and root dry weight measurements **(B)** were assessed in growth chamber trials including six pea breeding lines and the two parental lines upon infection with *A. euteiches* strains UK16 (high virulence), SE51 (intermediate virulence) and SE58 (low virulence). Disease index scores (0 = completely healthy plant, 100 = completely diseased) and root dry weight measurements [g] are averages of five biological replicates.

3.2 Disease resistance and root dry weight in greenhouse trials

The effect of cultivar on measured disease indices showed to be highly significant ($p < 0.001$) in the greenhouse trials (Table 2). Fisher LSD tests on the ANOVA results showed that only breeding line Z1654-1 was significantly ($p < 0.05$) more resistant than Linnea upon infection with the intermediately virulent *A. euteiches* strain SE51 (Figure 2A). The effect on root dry weight was also highly significant ($p < 0.001$, Table 2). PI180693 displayed the highest root

TABLE 3 Root dry weight of uninfected pea cultivars in growth chamber experiments.

Cultivar	Root dry weight [g]*	Standard deviation	Fisher LSD#
Linnea	0.1894	0.04159086	e
PI180693	0.3962	0.07156256	a
Z1654-1	0.3598	0.03089822	ab
Z1656-1	0.3314	0.03415845	b
Z1701-1	0.3280	0.03205464	bc
Z1701-2	0.2698	0.04702871	cd
Z1707-1	0.2372	0.02060825	de
Z1707-2	0.2714	0.06148008	cd

*Roots were harvested after three weeks, and root dry weight values correspond to the average across five biological replicates (pots) with five plants each. #Fisher LSD test was applied on one-way ANOVA residuals. Letters a-e indicate significant ($p < 0.05$) different between group means.

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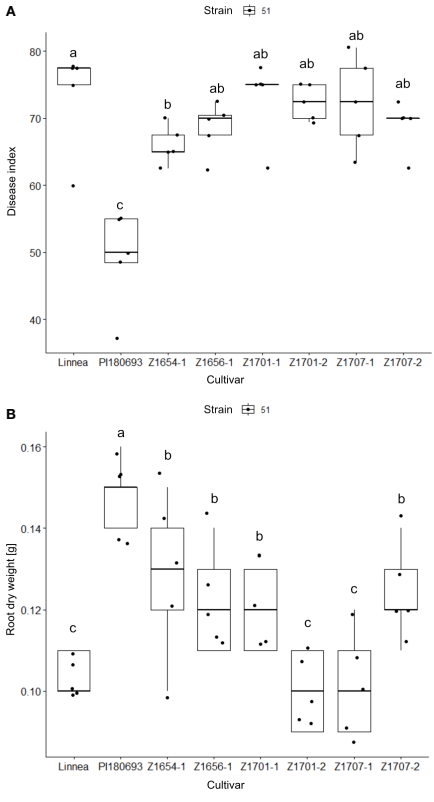

FIGURE 2
Disease index and root dry weight measurements in greenhouse trials. Disease index scores **(A)** and root dry weight measurements **(B)** in greenhouse trials, including six breeding lines and the two parental lines, upon infection with *A. euteiches* strain SE51 with intermediate virulence Disease index scores (0 = completely healthy plant, 100 = completely diseased) and root dry weight measurements [g] are averages of five biological replicates. Letters a-c indicate Fisher's significant ($p > 0.05$) differences between means of disease indexes and root dry weight between cultivars.

dry weight, whereas root dry weights of breeding lines Z1656-1, Z1701-1, Z1654-1 and Z1707-2 were significantly ($p < 0.05$) higher than Linnea and lower than PI180693 (Figure 2B).

3.3 Disease resistance and plant emergence in 2020 field trials

A. euteiches oospores were identified microscopically in fields Z20EA and Z20EB. In field Z20EB, *P. pisi* was also detected in soil tests and disease indices were higher on average. During the 2020 field seasons, air temperatures and relative humidity were lower than in year 2022 (Supplementary Figure 3).

In field Z20EA, Linnea was the most susceptible genotype with a significantly ($p < 0.05$) higher disease index compared with PI180693 and all breeding lines (Figure 3A). There were no differences in disease index between PI180693 and breeding lines. There was also a significant ($p < 0.001$) cultivar-effect on emergence in field Z20EA (Table 2), where Linnea showed a lower ($p < 0.05$) emergence compared with PI180693 and all breeding lines (Figure 3C). Disease index and emergence were significantly negatively correlated in field Z20EA (Pearson R = -0.637, $p < 0.01$).

In field Z20EB, where *P. pisi* co-occurred with *A. euteiches*, cultivar Linnea displayed the highest disease index, while PI180693 had the lowest ($p < 0.05$, Figure 3B). Only breeding line Z1656-1 had significantly ($p < 0.05$) lower disease index compared with Linnea (Figure 3B). Seedling emergence was significantly ($p < 0.05$) higher in breeding lines Z1707-2, Z1654-1 and Z1701-1 compared with Linnea (Figure 3D). Interestingly, no difference in seedling emergence was observed between PI180693 and Linnea (Figure 3D). Unlike in field Z20EA, there was no correlation between disease index and emergence in field Z20EB (Pearson R = 0.331, $p > 0.05$).

3.4 Disease resistance and plant emergence in 2022 field trial

As plots in field R-22-10-91 were larger than in fields Z20EA and Z20EB, we analyzed the effect of block size in our two-way ANOVA. Both cultivar and block had a significant effect on disease index ($p < 0.05$ and $p < 0.01$). The interaction effect of block and cultivar was not significant ($p > 0.1$, Table 2). Overall disease indices in field R-22-10-91 were lower compared with measured disease severity in the 2020 field trials but warmer average air temperature, less precipitation and higher relative humidity, especially during the sowing period, were measured in the 2022 field season (Supplementary Figure 3). Surprisingly, PI180693 scored the highest average disease index compared to all other cultivars ($p < 0.05$). Fisher comparisons between means of disease index per cultivar showed that no breeding line was significantly ($p < 0.05$) more resistant than the susceptible parent Linnea (Figure 4A).

Both cultivar and block had a significant effect on seedling emergence in field R-22-10-91 ($p < 0.01$ and $p < 0.001$, respectively, Table 2). Seedling emergence was significantly ($p < 0.5$) higher in PI180693 and breeding lines Z1707-2, Z1656-1, Z1701-1 and Z1654-1 than in Linnea (Figure 4B). In field R-22-10-91, the correlation between disease index and emergence was non-significantly negative (Pearson R = -0.308, $p > 0.05$).

3.4.1 Yield

In field R-22-10-91, block had a significant ($p < 0.01$) effect on yield, as well as cultivar ($p < 0.05$, Table 2). Breeding lines Z1701-2 and Z1707-2 had significantly ($p < 0.05$) lower yields than Linnea, but the yield of the other breeding lines did not differ from their commercially used parent. Interestingly, disease indices of lines Z1656-1 and Z1707-2 correlated positively with yield while all other cultivars showed a negative correlation (Figure 5A). The same two

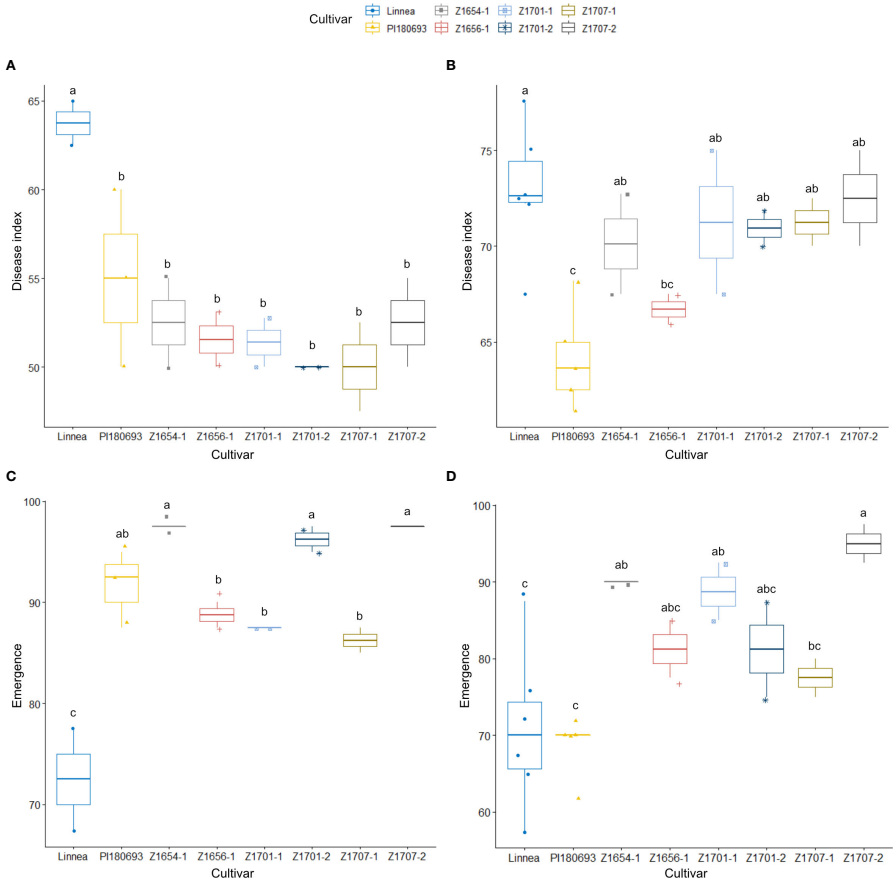

FIGURE 3
Disease index scores and emergence rates in 2020 field trials. Disease index scores **(A)** and emergence rates **(C)** for field Z20EA and field Z20EB with co-occuring *P. pisi*, **(B, D)**, respectively, are averages of two replicates for every breeding line and additional replicates for PI180693 and Linnea. Disease index is measured on a scale from 0 (completely healthy plant) to 100 (completely diseased) and emergence levels indicate the percentage of plants emerged compared to seeds sown. Letters a-c indicate Fisher's significant ($p > 0.05$) differences between means of disease indexes and emergence rates.

breeding lines also showed positive correlations between yield and emergence in linear regression analyses (Figure 5B).

3.4.2 Percentage of green peas compared to total plant biomass

Both cultivar and block had a significant ($p \leq 0.05$) effect on the amount of green peas per total plant biomass in field R-22-10-91 (Table 2). The percentage of peas versus total plant biomass in breeding lines Z1701-1 (17.7%) and Z1654-1 (17.3%) did not differ compared to 14.1% in Linnea (Supplementary Table 3). Interestingly, there was no correlation between disease index and the amount of peas versus the total plant biomass (Pearson correlation coefficient, R = 0.23, $p > 0.05$).

3.4.3 Plant height

In field R-22-10-91, both cultivar and block had a significant ($p < 0.001$ and $p < 0.01$, respectively) effect on the average plant height (Table 2). Cultivar PI180693 grew the tallest with an average plant length of 151 cm (Supplementary Table 3). The average length of other breeding lines was comparable to Linnea, except lines Z1654-1 and Z1656-1 that grew significantly ($p < 0.05$) taller than Linnea with average plant lengths of 77.6 cm and 81.8 cm.

3.4.4 Number of pods per plant and average length of second node pod

In the 2022 field trial, the number of pods per plant as well as the length of the second node pod were measured and compared to the

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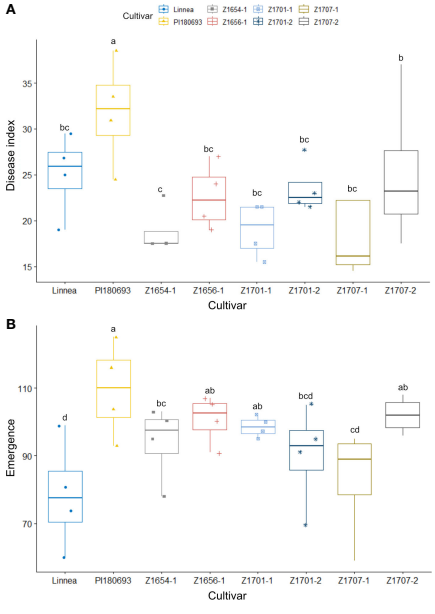

FIGURE 4
Disease index scores and emergence rates in the 2022 field trial. Disease index scores **(A)** and emergence rates **(B)** in field R-22-10-91. are averages of four replicates per cultivar. Disease index is measured on a scale from 0 (completely healthy plant) to 100 (completely diseased) and emergence levels indicate the percentage of plants emerged compared to seeds sown. Letters a-c indicate Fisher's significant ($p > 0.05$) differences between means of disease indexes and emergence rates.

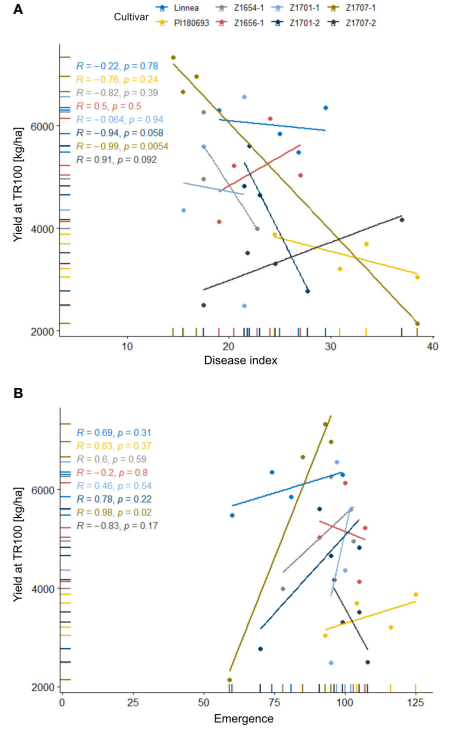

FIGURE 5
Correlations of yield with disease index scores and emergence rates in the 2022 field trial. Disease index scores **(A)** and yield and emergence rates **(B)** in field R-22-10-91are averages of four replicates per cultivar. Disease index is measured on a scale from 0 (completely healthy plant) to 100 (completely diseased) and emergence levels indicate the number of emerged plants per square meter. Lines represent linear regressions between the two factors, R values show Pearson correlation coefficients.

Linnea phenotype. Breeding line Z1707-2 had significantly less ($p < 0.05$, average 5.88) pods per plant than Linnea (average 7.5) while line Z1654-1 had more with an average of 9.12 (Supplementary Table 3). Comparing the average lengths of second node pods, breeding lines Z1656-1, Z1701-1 and Z1654-1 did not differ from the Linnea phenotype with an average length of 56.6 mm while the other breeding lines were comparable to the PI180693 phenotype with an average of 43.8 mm, (Supplementary Table 3).

4 Discussion

Taken together, our results show that the resistance from PI180693 can successfully be deployed in pea breeding line crosses. We found that some breeding lines are more resistant than their susceptible parent Linnea in field conditions and in growth chamber trials at low pathogen virulence levels. Line Z1654-1 scored lowest disease index on average (11.5% lower than Linnea) in both controlled experiments and scored on average 42% higher in root dry weight measurements compared to the susceptible parent. At lower pathogen pressure, line Z1701-1 showed to be significantly more resistant than both parents in the growth chamber trials with a 58.5% lower disease index than Linnea and 39.5% lower than PI180693. Interestingly, measured disease indices of PI180693 varied less in response to different virulence levels of *A. euteiches* compared with the breeding lines, indicating that the original source of resistance in PI180693 is more robust to varying pathogen virulence levels and partially lost during the breeding steps. This emphasizes the polygenic nature of the resistance and indicates that allele combinations for optimal disease resistance is yet to be achieved in the breeding lines. Along with this, we observed a negative correlation between pea root dry weight and disease index upon infections with *A. euteiches* across cultivars. Resistance QTLs in pea have previously been shown to be correlated with increased root volume and architecture (Desgroux et al., 2018). However, it remains to be investigated at which developmental stage the formation of roots is either fully inhibited or drastically reduced.

In our field experiments, the measured disease indices represented the overall plant health, including both root and shoot phenotype, and cannot be directly compared to disease indices in controlled conditions. Soil testing in fields Z20EA and Z20EB confirmed the presence of *A. euteiches* in the soil and in the latter the co-occurrence of *P. pisi*. We observed higher disease indices in field Z20EB compared to field Z20EA, indicating that presence of *P. pisi* enhanced disease levels. Comparing breeding line performance in field Z20EB, we did not find any indication that resistance in PI180693 is active against *P. pisi* infection. Whereas the genetic resistance in pea towards fusarium root rot caused by *Fusarium solani* f. sp. *phaseoli* is known to be inherited quantitatively (Mukankusi et al., 2011), little is yet known about the genes underlying the resistance to the emerging pathogen *P. pisi* (Heyman et al., 2013; Hosseini et al., 2014). In order to be able to make clearer predictions about the performance of the breeding lines upon infection with *P. pisi*, it will be essential to isolate virulent pathogen strains, and perform controlled single infections with the pathogen.

In field Z20EA where only *A. euteiches* was detected, all breeding lines had significantly higher emergence rates than Linnea, whereas in co-occurrence with *P. pisi* (field Z20EB), emergence rates were lower. We hypothesize that the additional presence of *P. pisi*, could have growth inhibiting effects in early plant growth stages and affect seed germination. When assessing emergence rates, natural variation in seed coat morphology must be taken into account, as for example PI180693 has shown to have a harder seed coat in seed germination tests (data not shown). In previous experiments we used pre-germinated pea seedlings that were able to germinate without pathogen pressure (Kälin et al., 2022). In these greenhouse and growth chamber trials we tried to spatially separate the inoculum from the seed, enabling the seeds to also germinate without pathogen pressure. In field conditions, however, seeds are subjected to *A. euteiches* and other root rot causing pathogens from the moment of sowing, which can lead to lower emergence rates. This emphasizes the importance of optimal timing of sowing within a growing season to reduce root rot disease in legume production (Nazer Kakhki et al., 2022).

In our 2022 field trial design, the size of blocks showed to have a significant effect on all analyzed parameters, which also corresponds to the typical patchy occurrence of *A. euteiches* in agricultural fields. Remarkably, PI180693 scored both highest disease indices and emergence rates in field R-10-22-91. None of the breeding lines showed disease index values that were significantly different from Linnea in this field trial, but four lines showed higher emergence rates than their susceptible parent. However, the 2022 season was very different compared with 2020, with moist soil conditions during sowing, followed by a very dry field season with high temperatures and low precipitation that were not conducive for root rot disease. It is known that levels of high soil moisture, due to heavy precipitation, poor drainage or high soil compaction, favor disease development in *A. euteiches* infections (Grath and Håkansson, 1992; Allmaras et al., 2003; Karppinen et al., 2020) and could therefore explain the observed patterns of lower average disease indices in field R-22-10-91, combined with a significant variation in emergence between cultivars.

With exception of two breeding lines, higher disease indices in field R-22-10-91 were associated with lower yield whereas four out of six breeding lines did not differ in yield compared to Linnea. Two of them (Z1701-1 and Z1654-1) were also comparable to Linnea in the ratio of green peas versus total plant biomass and average length of second node pod. Line Z1654-1 even scored more pods per plant than Linnea but inherited PI180693's tall growth phenotype. Our results confirm how breeding for robust resistance in pea is facing major challenges as resistance towards root rot is polygenically inherited and often associated with unfavorable breeding traits. Positive and negative associations between alleles controlling plant morphological traits, and resistance, suggesting pleiotropic genes involved in underlying resistance QTLs (Poland et al., 2009; Hamon et al., 2013). Desgroux et al. (2016) have reported a broken linkage between the traits of flower coloration and disease resistance against root rot in pea and recommend finer mapping techniques in future resistance breeding.

Our results further highlight the difficulty of predicting breeding line performance in the field based on results from experiments in controlled environments. In growth chamber experiments pressure from other pathogens is removed and only single or controlled co-infections at known virulence levels are assessed. In field conditions, however, the plants are exposed to a variety of PRRC pathogens with potential synergistic or antagonistic effects, as well as to a variety of other microbes (Wille et al., 2020). In summary, we showed the potential use of combining PI180693 partial resistance against aphanomyces root rot with commercially favorable breeding traits in commercial breeding programs.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Author contributions

CK, MK, MD, AKB and ME planned and designed, and CK and MD carried out the growth chamber experiments. Field experiments were planned and performed by AKB and A-KA. Data analysis was done by CK and MK. All authors contributed to the article and approved the submitted version.

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Conflict of interest

Authors AKB and A-KA are employed by Nomad Foods Ltd., Findus Sverige AB.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2023.1114408/full#supplementary-material

SUPPLEMENTARY TABLE 2
Combined Fisher LSD test results for growth chamber, greenhouse and field trials.

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RESEARCH

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Transcriptomic analysis identifies candidate genes for Aphanomyces root rot disease resistance in pea

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Abstract

Background *Aphanomyces euteiches* is a soil-borne oomycete that causes root rot in pea and other legume species. Symptoms of Aphanomyces root rot (ARR) include root discoloration and wilting, leading to significant yield losses in pea production. Resistance to ARR is known to be polygenic but the roles of single genes in the pea immune response are still poorly understood. This study uses transcriptomics to elucidate the immune response of two pea genotypes varying in their levels of resistance to *A. euteiches*.

Results In this study, we inoculated roots of the pea (*P. sativum* L.) genotypes 'Linnea' (susceptible) and 'PI180693' (resistant) with two different *A. euteiches* strains varying in levels of virulence. The roots were harvested at 6 h post-inoculation (hpi), 20 hpi and 48 hpi, followed by differential gene expression analysis. Our results showed a time- and genotype-dependent immune response towards *A. euteiches* infection, involving several WRKY and MYB-like transcription factors, along with genes associated with jasmonic acid (JA) and abscisic acid (ABA) signaling. By cross-referencing with genes segregating with partial resistance to ARR, we identified 39 candidate disease resistance genes at the later stage of infection. Among the genes solely upregulated in the resistant genotype 'PI180693', Psat7g091800.1 was polymorphic between the pea genotypes and encoded a Leucine-rich repeat receptor-like kinase reminiscent of the *Arabidopsis thaliana* FLAGELLIN-SENSITIVE 2 receptor.

Conclusions This study provides new insights into the gene expression dynamics controlling the immune response of resistant and susceptible pea genotypes to *A. euteiches* infection. We present a set of 39 candidate disease resistance genes for ARR in pea, including the putative immune receptor Psat7g091800.1, for future functional validation.

Keywords Abscisic acid, *Aphanomyces euteiches*, Candidate disease resistance genes, Differential gene expression, Immune receptor, Pea breeding, *Pisum sativum*, Transcriptomics

Background

Green pea (*Pisum sativum* L.) belongs to the Fabaceae family (or Leguminosae), and is cultivated worldwide in cool temperate areas [1]. The legume poses a valuable source of plant-based protein for food and feed [2], and the global production has been increasing steadily [3]. However, pea cultivation faces several biotic and abiotic constraints, most notably soil-borne pathogens causing root rot [4, 5]. Root rot in pea is caused by a complex of fungal and oomycete pathogens, whereas Aphanomyces

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root rot (ARR) is the most devastating threat to pea production in main vining pea production areas with temperate climate [6, 7].

The causative agent of ARR is *Aphanomyces euteiches*, which is a homothallic (self-fertile) oomycete with a broad host range on various legume species. The pathogen has a hemibiotrophic lifestyle, completing a shift from a biotrophic to necrotrophic growth phase on its host plant. An infection cycle starts with oospore germination and the production of asexual bi-flagellate motile zoospores, which detect root exudates and continue to encyst and penetrate the root system [7, 8]. In the first six days of infection, the biotrophic phase, the pathogen colonizes the cortex root tissue of the host plant. The necrotrophic growth phase is initiated by the invasion of the stele and vascular tissues, leading to the typical browning of the roots and premature plant death [9, 10]. The cycle ends with the production of sexual oospores in declining host tissues [11]. Oospores are particularly problematic in pea cultivation, as they can remain resilient in the soil for a long time [12]. Long periods of crop rotation and avoidance of highly infested fields are often the only effective measures in the mitigation of ARR [13, 14]. Understanding the molecular basis of host resistance in pea to ARR and the integration of resistant pea varieties would be the economically and ecologically most beneficial strategy in the mitigation of ARR.

There is currently no commercial pea variety with complete resistance to ARR, but the landrace 'PI180693' has been used as a source of resistance in commercial breeding programs [15]. However, 'PI180693' is unsuitable for commercial cultivation due to poor green pea quality (pale seed coat color, mealy and hard texture, lack of sweetness) as well as agronomic properties unfit for modern large scale crop cultivation (e.g. long internodes, susceptibility for powdery and downy mildew). The pea cultivar 'Linnea' on the other hand, bears favorable agronomic and green pea quality traits and has been used in commercial production in Sweden since 2010. However, 'Linnea' is highly susceptible to ARR. The levels of susceptibility of both pea genotypes to ARR have previously been evaluated in the field, and controlled greenhouse trials [16, 17].

The *P. sativum* genome is among the largest in legumes as its haploid size corresponds to 4.45 Gb on seven paired chromosomes. For the first annotated chromosome-level assembly for *P. sativum*, the French cultivar 'Caméor' was sequenced by Kreplak et al. [18] and has since been facilitating the development of genetic markers. Resistance to ARR in pea is quantitative and polygenic. Several consistent Quantitative Trait Loci (QTL) associated with partial resistance to ARR have been identified and validated in pea, paving the way for marker-assisted selection in breeding programs [19–25]. A cross between a susceptible and resistant pea cultivar was used to identify QTL for partial resistance to ARR based on greenhouse and field experiments, and ultimately identified the gene content in the ARR resistance QTL [26]. Genes segregating with ARR resistance were further expanded using bulked segregant RNA-seq (BSR-seq) analysis and used for cross-referencing with differentially expressed genes (DEGs) [27].

The use of transcriptomics in controlled host–pathogen infections allows the identification of candidate disease resistance genes and has been employed successfully in the field of legume-microbe interactions [28, 29]. In many studies, the legume model species *Medicago truncatula* is used to study the immune response towards *A. euteiches*. Badis et al. [30] for example, used a transcriptomics approach to identify genes involved in defence and signaling pathways that are associated with partial resistance to *A. euteiches* in *M. truncatula*. Hosseini et al. [31] investigated the transcriptional immune response in pea towards two oomycete pathogens, *Phytophtora pisi* and *A. euteiches*, and identified chalcone synthases and genes active in the auxin pathway to be specifically upregulated upon *A. euteiches* infection. Williamson-Benavides et al. [32] identified induced immune response genes in a susceptible *P. sativum* host upon infection with *Fusarium solani* f. sp. *pisi* compared to a partially resistant host. However, limited information is available about the genetic interaction between *A. euteiches* and the resistance level of its pea host during infection or how varying levels of *A. euteiches* virulence affects the pea immune response. Although *A. euteiches* strains are assigned to races based on their pathogenicity against alfalfa cultivars [33], little is known about how the transcriptomic immune response in their respective host is affected.

In the current study, we performed a transcriptomic analysis of two different pea genotypes with varying levels of ARR resistance, upon infection with two different *A. euteiches* strains with varying levels of virulence. Virulence was defined as the severity of disease symptoms after inoculation with *A. euteiches*. We hypothesized that i) partial resistance towards ARR is associated with different sets of DEGs in the susceptible and resistant pea cultivar, ii) genes that are differentially regulated upon *A. euteiches* infection are preferentially located in ARR resistance QTL, and that iii) there is an *A. euteiches*

virulence-dependent transcriptional response in the two pea genotypes upon infection.

Results

Immune response in pea is determined by quantitative resistance in the host rather than the virulence level of *A. euteiches*

Seedlings of 'Linnea' and 'PI180693' were inoculated by dipping into a zoospore solution of *A. euteiches* strains UK16 or SE51, consistently shown to differ in virulence on 'Linnea' and 'PI180693' in climate chamber trials [17]. The 'Linnea' seedlings serving as infection control were left in the open pipette boxes for several days and confirmed successful disease development by visual inspection in seedlings treated with *A. euteiches* strains UK16 and SE51, and the absence of disease symptoms in the mock treatments. The average number of million reads per sample ranged from 47.1 to 77.6, representing sufficient amount of sequence data for analyzing differential gene expression (Table S2). Principal Component Analysis (PCA) of the entire dataset showed a clear clustering according to pea genotypes, but not to treatment with *A. euteiches* strains (Fig. 1). Further, PCAs split by pea genotype showed a separation by time point but no clear separation by *A. euteiches* virulence levels, except for 'PI180693' at 48 h post-inoculation (hpi), inoculated with the more virulent UK16 (Figure S2).

Exponential increase in *A. euteiches* biomass and DEGs in 'Linnea' upon infection with strain UK16

To confirm an increasing presence of *A. euteiches* biomass during the infection process, we assessed the percentage of reads that mapped to the *A. euteiches* reference genome, as a proxy for biomass. The highest percentage of reads mapping to the *A. euteiches* genome was observed at 48 hpi in 'Linnea' upon inoculation with the highly virulent strain UK16. For all time points, more *A. euteiches* reads mapped in interaction with the susceptible pea genotype compared to the partially resistant 'PI180693' and strain UK16 accounted for more biomass in all conditions (Fig. 2a). This difference was most apparent at time point 48 hpi, where 9.5 times more reads were assigned to *A. euteiches* when infecting 'Linnea' as compared to 'PI180693' (Table S2). We observed low numbers of differentially expressed genes (DEGs, absolute value of log2FC > 1) at the early time points 6 hpi and 20 hpi with either *A. euteiches* strains and in both pea genotypes (Fig. 2b). Most DEGs were scored in 'Linnea' upon infection with the more virulent strain UK16 at 48 hpi. At the same time point, and at 20 hpi, we observed more DEGs in 'PI180693' compared to 'Linnea' upon infection with strain SE51. Overall, numbers of DEGs as well as *A. euteiches* reads were increasing with time and higher in the treatments with the highly virulent strain UK16.

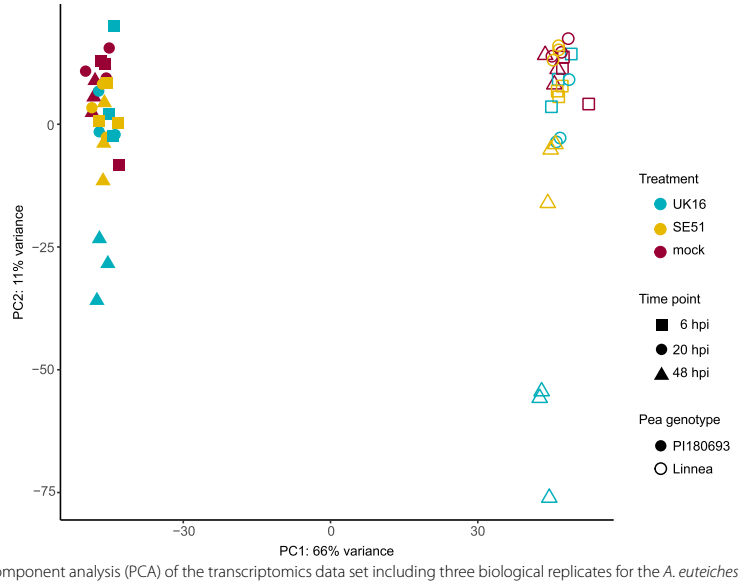

Fig. 1 Principal component analysis (PCA) of the transcriptomics data set including three biological replicates for the *A. euteiches* treatments (highly virulent UK16 and lowly virulent SE51) and mock control, root harvesting time points (shapes) and the pea genotypes 'PI180693' (filled shapes) and 'Linnea' (empty shapes)

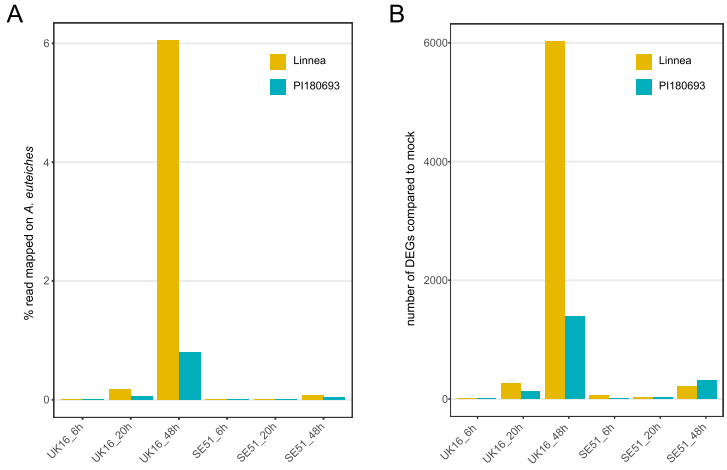

Fig. 2 **A** The percentage of reads that mapped on the *A. euteiches* reference genome for the pea genotypes 'Linnea' (yellow) and 'PI180693' (blue) for every *A. euteiches* treatment (highly virulent strain UK16 and lowly virulent SE51) and time point. **B** Increasing number of differentially expressed genes (DEGs) with absolute value of log2FC > 1 and adjusted *p*-value < 0.05, compared to mock treatment for 'Linnea' (yellow) and 'PI180693' (blue), separated by *A. euteiches* treatment and time point

Numbers of DEGs at all time points and conditions, as well as normalized read counts are listed in Table S3.

The transcriptional immune response of pea to *A. euteiches* is time-dependent

We identified 75 DEGs at 6 hpi and 375 DEGs at 20 hpi (Table S4) and retrieved the available information of the corresponding genes from the pea genome database (https://urgi.versailles.inra.fr/download/pea/), including gene ontology (GO) terms for all genes (Tables S5 and S6). At the earliest time point, we identified three seed linoleate 9S-lipoxygenase-3-like genes that were previously associated with partial resistance to Aphanomyces root rot (ARR) and predicted to be involved in oxidation–reduction processes and jasmonic acid (JA) biosynthesis [27]. Additionally, Psat2g149200.1, Psat5g289880.1 and Psat5g291320.1 were all downregulated in 'Linnea' upon infection with SE51 (Table S5).

At 20 hpi, more genes associated with the GO term "defense response to other organisms" (GO:0009814) were upregulated in 'Linnea' (eleven) than in 'PI180693' (four). A similar pattern was observed for predicted receptor-like kinases, where 17 were upregulated in 'Linnea', two of which were also upregulated in 'PI180693'. We identified seven genes putatively involved in disease resistance responses to be upregulated at 20 hpi. Disease resistance response proteins Pi176 and Pi49 have GO terms connected to abscisic acid (ABA) binding and were both upregulated in 'PI180693' but not in 'Linnea'. Psat2g115400.1 was upregulated in both pea genotypes, while Psat2g013480.1, Psat7g028600.1, Psat7g029960.1 and Psat7g028560.1 were upregulated only in 'Linnea'.

Among other upregulated genes in 'Linnea' we found ethylene-responsive transcription factors (TFs, Psat6g137360.1, Psat6g054800.1), an auxin-responsive, as well as ABA-responsive ABR18-like gene (Psat7g037160.1 and Psat6g217920.1). Additionally, we found four myeloblastosis (MYB)-like and six WRKY TFs (Table S5). Two chitinases (Psat1g150520.1, Psat1g148600.1) were among downregulated genes in 'Linnea' at 20 hpi. In 'PI180693', we found TFs *myb14*-like and *myb15*-like genes (Psat6g137320.1 and Psat6g105240.1) and gene Psat1g157240.1, encoding the disease resistance response protein Pi176, among the most upregulated DEGs. Upon infection of 'PI180693' with either *A. euteiches* strain, we found TF *myb102* (Psat1g209120.1) and abscisic acid and environmental stress-inducible protein encoding gene Psat2g026840.1 to be downregulated (Table S5).

Five of the differentially regulated genes at 20 hpi were located in genomic regions segregating with partial resistance to ARR. Psat4g140440.1, a probable leucine-rich repeat (LRR) receptor-like serine/threonine-protein kinase and Psat7g083880.1, a leaf rust 10 disease resistance locus receptor-like protein kinase homolog [26], were both upregulated in 'Linnea' upon infection with strain UK16. The other three genes were associated with hormone metabolism where Psat3g026920.1 was predicted to be part of methylsalicylate degradation. Genes

Psat5g289880.1 and Psat5g291320.1 were associated with oxidation–reduction processes and JA biosynthesis and were among the most downregulated genes in 'PI180693' [27] (Table S5).

Specific immune response differing between pea genotypes becomes apparent with progressing *A. euteiches* infection

At the later stage of infection, 48 hpi, we identified a total of 6036 DEGs in 'Linnea' and 1499 DEGs in 'PI180693' (Tables S3 and S4). At 48 hpi, we counted considerably more DEGs in both pea genotypes upon infection with the highly virulent strain UK16 than with strain SE51 (Figs. 3A, B). In 'Linnea', 196 DEGs were upregulated upon infection with either *A. euteiches* strain, comprising the majority (94.2%) of upregulated genes in the interaction of 'Linnea' and SE51 (Fig. 3A). In 'PI180693', 180 DEGs were upregulated in a non-strain specific manner, which accounted for 78.3% of genes upregulated upon infection with SE51 and only 15.6% of genes upregulated upon infection with UK16 (Fig. 3B).

In response to the more virulent strain UK16, the susceptible genotype 'Linnea' displayed more DEGs enriched ($p < 0.05$) for GO terms "defense response" than the resistant 'PI180693'. In response to the less virulent strain SE51, GO terms associated with upregulated DEGs in both 'Linnea' and 'PI180693' comprise "responses to biotic stimuli", "(protein) phosphorylation", and in 'PI180693' specifically "responses to (oxidative) stress" (Table S6).

Due to the great number of DEGs at 48 hpi, we focused on the 25 most strongly regulated genes upon infection in both pea genotypes for every condition and Table 1 shows a selection of genes with predicted defense-related gene functions and their closest characterized homolog. In general, interactions involving UK16 but not SE51 were very frequent among these strongly DEGs including a strongly downregulated seed linoleate 9S-lipoxygenase-3-like gene, Psat4g185080.1 (Table 1). Two more seed linoleate 9S-lipoxygenase-3-like genes, Psat5g289880.1 and Psat5g291320.1, were found among the strongly DEGs (Table 1). The allene oxide synthase

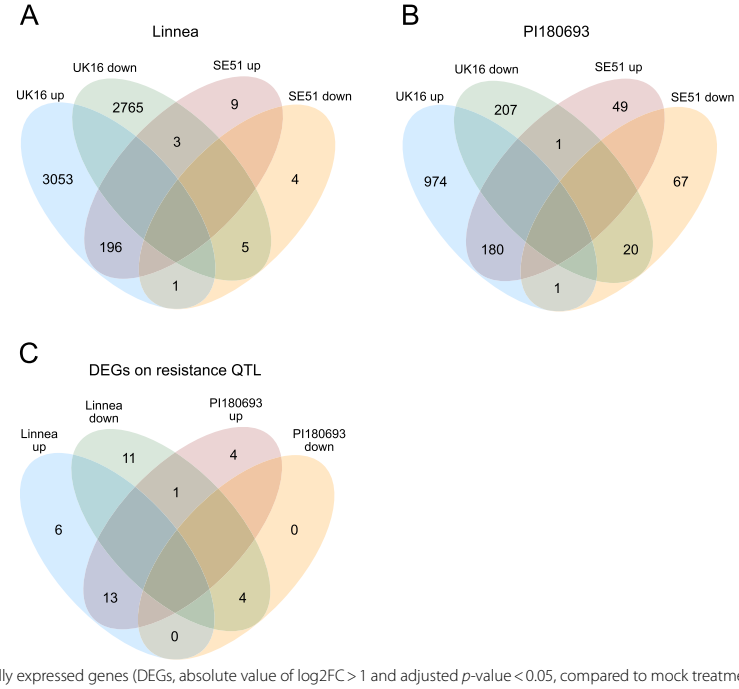

Fig. 3 Differentially expressed genes (DEGs, absolute value of log2FC > 1 and adjusted *p*-value < 0.05, compared to mock treatment) in the susceptible pea genotype 'Linnea' (**A**) and resistant 'PI180693' (**B**), split by *A. euteiches* strains UK16 (high virulence) and SE51 (low virulence) and up- and downregulation. **C** Non-strain specific DEGs segregating with loci for partial resistance to Aphanomyces root rot as previously described by Wu et al. 2021 and 2022

Table 1 Differentially expressed genes among 25 most up- and downregulated genes in 'Linnea' and 'PI180693' at 48 hpi associated with predicted defense response

Gene id	Closest BLAST hit	Linnea UK16	Linnea SE51	PI180693 UK16	PI180693 SE51
Psat0s1622g0080.1	NDR1/HIN1-like protein 10	2.501	n.s	n.s	-2.073
Psat2g109600.1	MLP-like protein 423	-3.685	n.s	-2.466	n.s
Psat4g185080.1*	Oxidation–reduction process, 9S-lipoxygenase-3-like	-6.734	n.s	-2.567	n.s
Psat4g182840.1	Disease resistance protein RPV1-like	4.218	1.175	2.261	n.s
Psat5g289880.1*	Oxidation–reduction process, 9S-lipoxygenase-3-like	-1.507	n.s	n.s	5.411
Psat5g242640.1	Pathogenesis-related protein	2.412	n.s	2.839	5.576
Psat5g291320.1*	Oxidation–reduction process, 9S-lipoxygenase-3-like	n.s	n.s	n.s	5.741
Psat6g146200.1	Pathogenesis-related protein PR-4-like	9.274	5.081	10.142	6.400
Psat6g109120.1	Pathogenesis-related protein PR-4-like	8.936	n.s	7.875	n.s
Psat6g042680.1	MLP-like protein 34	n.s	n.s	-2.448	n.s
Psat7g035720.1	Putative thaumatin	8.733	4.801	8.258	5.198
Psat7g029960.1	Disease resistance response protein 206-like	8.082	n.s	7.144	5.338
Psat7g036280.1	Thaumatin-like protein	8.585	4.371	8.915	5.439

* Genes segregating with partial resistance to Aphanomyces root rot described in Wu et al. 2022, *n.s.* Non-significant, differential gene expression compared to mock treatments, absolute value of log2FC > 1, adjusted *p*-value < 0.05

1 gene (Psat0s2724g0160.1) was induced in the interactions between 'Linnea' and UK16 and 'PI180693' and the low virulent strain SE51, but no significant induction was observed in the other two conditions (Table S5).

Fourteen genes were among the most upregulated genes across all interactions (Table S5). This group included several genes with similarity to known PR-protein genes (e.g. Psat1g211480.1, Psat6g146200.1, Psat7g035720.1 and Psat7g036280.1). An interesting set of genes in this analysis consisted of 24 genes that were highly upregulated in all interactions, except between 'PI180693' and the less virulent strain SE51. Among these genes were three transcription factor genes, two encoding WRKY transcription factors (Psat6g026680.1 and Psat5g236440.1) and one gene with similarity to the *rax3* MYB transcription factor (Psat4g080720.1) (Table S5). Eleven genes were strongly differentially regulated in all interactions except for the interaction between 'Linnea' and SE51, where no significant difference was found. This group of genes frequently lacked similarity with characterized genes but the strongly upregulated Psat6g137320.1 was similar to *myb14* transcription factors, while Psat1g001480.1 was upregulated in the interaction between 'PI180693' and the less virulent strain SE51 and was similar to 9-cis-epoxycarotenoid dioxygenase *nced1* (Table S5).

Thirty-nine candidate disease resistance genes at 48 hpi were previously associated with partial resistance to ARR

Differentially regulated genes at 48 hpi were cross-referenced with genes localized in genomic regions segregating with ARR resistance [26, 27]. The 39 genes displayed in Fig. 3C and Table 2 represent the non-strain specific immune response of 'Linnea' and 'PI180693'. Among the genes upregulated only in 'Linnea', Psat1g156920.1, encoding an ABR17-like protein, and Psat4g025040.1, a possible nodulin-13-like protein, were associated with the ABA-activated signaling pathway. Downregulated DEGs in the quantitative trait locus (QTL) specific to the susceptible 'Linnea' comprised three major latex protein (MLP)-like genes, two genes encoding disease resistance proteins (RFL1-like and RPM1-like), as well as two LRR receptor-like tyrosine protein kinase genes (Table S5). Upregulated genes in the QTL in both 'Linnea' and 'PI180693' involved two receptor-like protein kinases, Psat4g140440.1 and Psat6g203640.1. Among the downregulated genes associated with the QTL regions in both pea genotypes were three genes associated to oxylipin biosynthesis, Psat4g184760.1, Psat4g185080.1 and Psat5g289880.1. Interestingly, four DEGs associated with the QTL regions were upregulated exclusively in 'PI180693' at 48 hpi in response to *A. euteiches* infection. These include Psat2g013520.1, a predicted resistance to *Uncinula necator* 1 (RUN1)-like disease resistance protein, involved in signal transduction and originally described in the grapevine species *Muscadinia rotundifolia* for its resistance to powdery mildew [34, 35]. The second gene, Psat5g242600.1, a predicted *P. sativum* defensin 2 (Psd2), with associated GO terms "killing of cells of another organism" and "defense response to fungus". Additionally, a seed linoleate 9S-lipoxygenase-3-like gene was also among the genes exclusively upregulated in 'PI180693', as well as Psat7g091800.1, a

Table 2 Differentially expressed genes in 'Linnea' and 'PI180693' at 48 hpi previously described to be segregating with partial resistance to Aphanomyces root rot

Gene id	Putative biological role[a]	Linnea UK16	Linnea SE51	PI180693 UK16	PI180693 SE51
Psat1g105280.1	Methylsalicylate degradation	3.91	-0.30	2.18	1.53
Psat1g156920.1	Abscisic acid-activated signaling pathway	2.03	0.00	0.33	0.17
Psat2g013520.1	Signal transduction	n.s	n.s	3.13	1.15
Psat2g056400.1	Unknown	-1.26	-0.61	-0.23	2.23
Psat2g132720.1	Regulation of defense response	-1.53	0.12	-0.25	-0.22
Psat2g133040.1	Enhance wheat FHB resistance	5.19	0.40	3.92	0.66
Psat3g072480.1	Regulation of defense response	-1.56	-0.15	-0.09	0.35
Psat3g126560.1	Unknown	3.49	0.25	1.67	-0.84
Psat3g126600.1	Signal transduction	3.51	0.53	1.67	-0.85
Psat3g156760.1	Unknown	-1.32	-0.13	-0.91	0.74
Psat4g025040.1	Abscisic acid-activated signaling pathway	1.38	0.07	0.01	-2.58
Psat4g136120.1	Enhance wheat FHB resistance	2.89	0.43	1.68	0.64
Psat4g138760.1	Plant stress tolerance	-1.77	-0.20	-0.22	-0.43
Psat4g140440.1	Regulation of defense response	2.91	0.82	2.45	1.28
Psat4g152600.1	Unknown	4.68	0.51	2.79	1.46
Psat4g180200.1	Defense response	-1.12	0.29	-0.42	0.15
Psat4g184760.1	Jasmonic acid biosynthesis	-3.64	-0.22	-1.13	0.73
Psat4g185080.1	Jasmonic acid biosynthesis	-6.73	-0.59	-2.57	-1.20
Psat4g186560.1	Defense response	-3.62	0.35	-1.74	1.11
Psat4g188320.1	Unknown	-1.56	0.23	-0.62	0.79
Psat4g201520.1	Unknown	6.75	1.32	4.44	1.39
Psat4g201600.1	Unknown	7.61	1.75	4.90	1.00
Psat5g066680.1	Unknown	1.31	0.52	0.48	1.07
Psat5g242440.1	Defense response	3.53	1.27	3.44	2.76
Psat5g242600.1	Defense response	2.90	-2.40	4.62	8.26
Psat5g289880.1	Jasmonic acid biosynthesis	-1.51	-0.11	-0.01	5.41
Psat5g291320.1	Jasmonic acid biosynthesis	-0.66	0.10	0.41	5.74
Psat6g011200.1	Unknown	1.75	0.21	2.52	-0.51
Psat6g042720.1	Defense response	-3.31	-0.52	-0.76	-0.51
Psat6g042840.1	Defense response	-3.19	-0.11	-1.52	-0.86
Psat6g043800.1	Defense response	1.68	2.36	n.s	n.s
Psat6g144560.1	Plant defense	-2.13	-0.01	-0.43	-0.48
Psat6g146320.1	Defense against ecrotrophic fungi and abiotic stress tolerance	-1.11	-0.01	-0.22	-0.07
Psat6g203640.1	Plant defense	3.07	0.08	1.76	0.11
Psat6g207920.1	Biotic, abiotic stress, plant growth	4.32	2.09	2.33	0.50
Psat7g067680.1	Unknown	-2.12	-1.00	0.07	1.09
Psat7g083880.1	Regulating defense response	4.35	0.98	2.80	0.59
Psat7g091800.1	Plant defense	0.15	0.41	1.26	-0.78
Psat7g094400.1	Plant defense	1.51	0.12	0.56	0.09

[a] Gene function, biological processes/pathways from Wu et al. 2021 & 2022, differential gene expression compared to mock treatments, absolute values of log2FC > 1, adjusted *p*-value < 0.05, *n.s.* = not significant

putative receptor-like kinase (RLK) involved in plant defense (Table 2, Table S5). Psat7g091800.1 segregated with the foliar wilt Fwt-Ps7.1 major-effect QTL on chromosome 7 that was detected in greenhouse experiments, as well as the minor- to moderate-effect QTL for ARR tolerance Ae_{MRCD1}Ps-7.1, detected in field experiments [27]. Out of these four DEGs specifically upregulated in 'PI180693', Psat7g091800.1 was chosen for further analysis.

The receptor-like kinase Psat7g091800.1 is polymorphic between the resistant and susceptible pea genotypes

Psat7g091800.1 was located on chromosome 7 in the genome of the pea reference cultivar Caméor with exact coordinates chr7LG7:153,683,713–153,687,363. The annotation of gene Psat7g091800.1 is therefore 3650 bp whereas in our data, reads aligned starting from the second start codon, indicating that the actual full gene length was 3645 bp in 'Linnea' and 'PI180693'. Moreover, in the existing annotation, the gene has a long 3'-UTR region that encompasses a neighboring gene, but our read alignment did not support this and therefore the gene annotation was corrected to end at base 153,687,363 of chromosome 7 (Figure S3). The alternative start codon and the shorter 3'-UTR sequence was supported by a de-novo assembly of the transcript based on our RNA sequencing data. The gene had an exon–intron-exon structure with a 131 bp long intron, which had a 24 bp deletion in 'PI180693'. The Sanger sequences from genomic DNA of 'Linnea' and 'PI180693', together with the RNAseq data revealed 39 single nucleotide polymorphisms (SNPs), with 17 leading to non-synonymous mutations (Fig. 4, Table S7). No polymorphisms were found between 'Linnea' and the reference sequence of the cultivar 'Caméor'. Psat7g091800.1 was predicted to have a 24 amino acid (aa) long signal peptide, a 21 aa long transmembrane domain and 29 LRRs. Four of the SNPs between 'Linnea' and 'PI180693' were located in LRR10, LRR11, LRR21 and LRR23, and one SNP in the transmembrane domain. Eight SNPs resulted in aa changes associated with changes in polarity in the protein (Fig. 4). Domain searches in Psat7g091800.1 using Interproscan revealed similarities to the FLAGELLIN SENSING 2 (FLS2)-like domain, previously characterized as a LRR transmembrane receptor kinase crucial for flagellin perception in *Arabidopsis thaliana* [36]. Phylogenetic analyses using the entire Psat7g091800.1 protein sequence from 'PI180693', as well as the FLS2-encoding domain only, in comparison to homologs in other crop species showed that evolution of the Psat7g091800.1 protein followed the evolution of the analyzed species. This is also reflected in the Psat7g091800.1 protein sequence sharing > 70% sequence identity with all other legume species. In fact, the 'PI180693' Psat7g091800.1 protein sequence shared only 53.4% sequence identity with the *A. thaliana* homolog and thus encoded a LRR-RLK protein phylogenetically distinct from FLS2 (Figure S4).

Discussion

Our study presented a reliable experimental setup for pea transcriptomics experiments for assessing early stages of *A. euteiches* infection. The infection controls in every biological replicate, as well as the observed exponential increase in reads mapped to the *A. euteiches* reference genome, indicated an increase in pathogen biomass during the infection process. Moreover, the estimated *A. euteiches* biomass increase correlated to an increase in number of differentially expressed genes (DEGs) over time points and higher numbers of DEGs upon infection with UK16 than SE51. The three root harvesting time points have previously been sampled in a study on the quantification of DNA and RNA transcripts of *P. pisi*, another root-rot causing oomycete pathogen of pea. During infection, *P. pisi* DNA was detectable by qPCR from 2 hpi and peaking at 48 hpi when hyphae had been accumulating in root tissue [37]. Not only were more DEGs counted in the susceptible genotype but also more defense-related genes such as predicted receptor-like kinases than in the resistant 'PI180693'. It has previously been shown how resistance in 'PI180693' inhibited the production rate of oospores on infected pea root tips, associated with slower lesion development and pathogen growth than in susceptible pea lines [38]. Lavaud et al. [21] used 'PI180693' as a donor line for the development of Near Isogenic Lines (NILs) in their experiments and showed how root colonization and symptom appearance by *A. euteiches* can be slowed down by single or multiple resistance quantitative trait loci (QTL). Reduced oospore colonization in resistant compared to susceptible lines was also observed in *M. truncatula* infections with *A. euteiches* [9]. The host-specific immune response due to quantitative levels of resistance in 'Linnea' and 'PI180693' was further reflected in the clear separation of samples according to pea genotype in PCA analysis, as well as the lack of clustering according to virulence levels of *A. euteiches* strains. In summary, inoculation with *A. euteiches* resulted in different transcriptomic responses between the two pea genotypes that may relate to differences in disease resistance.

We observed only a few DEGs at 6 hpi and were not able to observe a clear pattern of gene regulation between pea genotypes or in response to varying *A. euteiches* virulence levels. However, among downregulated genes in the susceptible pea genotype 'Linnea', we found three seed linoleate 9S-lipoxygenase-3-like genes that were associated with partial resistance to ARR [27]. At 20 hpi, two of these seed linoleate 9S-lipoxygenase-3-like genes (Psat5g289880.1 and Psat5g291320.1) were among the most downregulated genes in 'PI180693', indicating a non-host specific downregulation of these genes. Interestingly, at 48 hpi, Psat5g289880.1 and Psat5g291320.1 were among the most highly upregulated genes in the resistant pea genotype, 'PI180693', in interactions with the less virulent *A. euteiches* strain. One of them, Psat5g291320.1, was previously shown to segregate with partial resistance to Aphanomyces root rot (ARR)

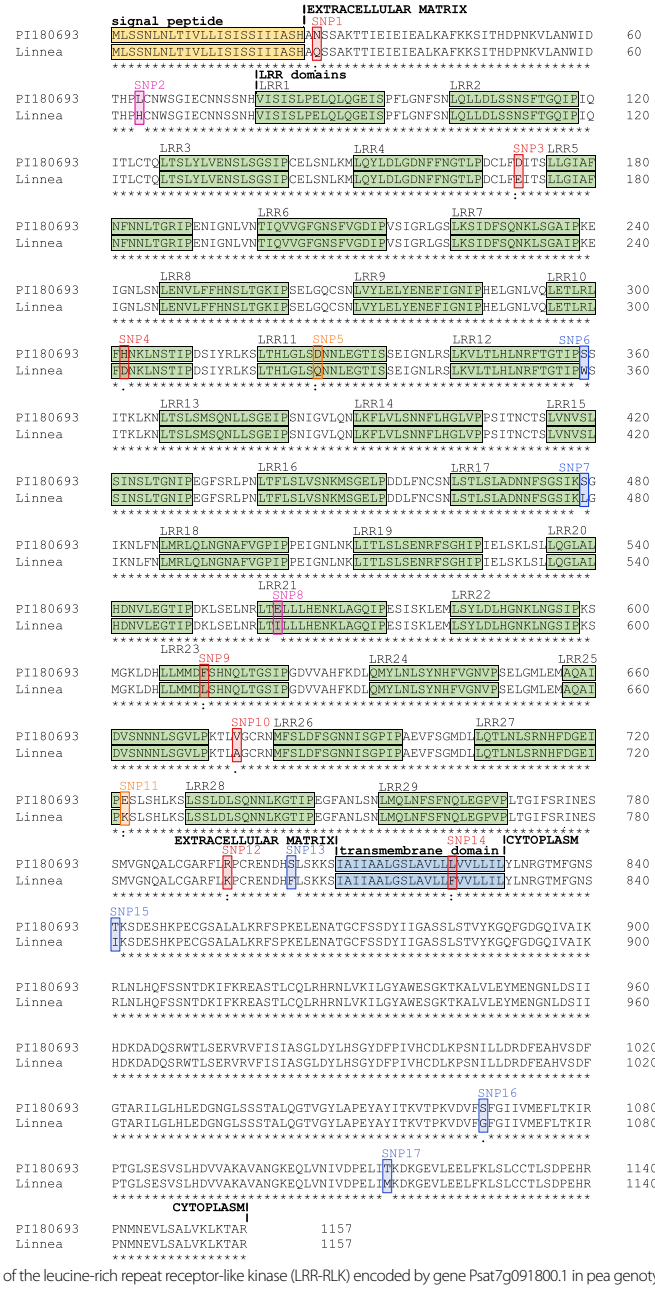

Fig. 4 Protein alignment of the leucine-rich repeat receptor-like kinase (LRR-RLK) encoded by gene Psat7g091800.1 in pea genotypes 'Linnea' and 'PI180693'. The protein sequence of 'Linnea' is identical to the reference sequence of the cultivar 'Caméor'. Amino acid substitutions altering polarity are highlighted in pink, changes in charge in orange and changes in both in blue. Nonsynonymous SNPs resulting in either change are marked in red

[26]. 9S-lipoxygenases oxygenate linoleic and linolenic acid in interactions with pathogens, generating various oxylipins including precursors to the hormone jasmonic acid (JA) [39, 40]. JA signaling has been associated with plant defense to necrotrophic pathogens [41, 42]. *Aphanomyces euteiches* undergoes a shift from a biotrophic to a necrotrophic lifestyle in later stages of infection [10]. In soybean roots, higher levels of JA were observed at later time points after inoculation with the oomycete *Phytophthora sojae* [43]. Furthermore, it was recently reported that soybean cultivars with different resistance levels to *P. sojae* accumulate different levels of oxylipins. In fact, the partially resistant cultivar generally increased the production of oxylipins upon attack, suggesting that production of oxylipins may be a critical component of the defense strategies used in resistant cultivars against *P. sojae* [44]. In this context and in light of the differentially expressed lipoxygenases it would be interesting to determine the accumulation of oxylipins in 'PI180693' during *A. euteiches* infection.

The putative disease resistance proteins Pi176 and Pi49 are highly similar in sequence and both genes were specifically upregulated in 'PI180693' at 20 hpi and were originally isolated as cDNAs in pea that showed a large induction of expression in tissue responding to infections with *Fusarium solani* [45, 46]. Pi49 was assigned to class 10 (PR10)-like abscisic acid (ABA)-responsive proteins and an ortholog was found to be significantly induced in *M. truncatula* upon infection with *A. euteiches* at 6 hpi. However, the induction correlated with *A. euteiches* infection development rather than host resistance responses [47–49].

In our experiment, we also saw a significant and specific upregulation of a 9-cis-epoxycarotenoid dioxygenase gene in 'PI180693' seedlings interacting with the low virulent strain. This gene encodes a key enzyme involved in the biosynthesis of ABA suggesting that 'PI180693' seedlings accumulate ABA. Liang and Harris [50] described the role of ABA in the induction of lateral root formation in all nodulating and non-nodulating legume species. Low doses of ABA and ethylene can stimulate lateral root formation in legumes. However, in this study, the pea seedlings were not grown longer than 48 hpi, which was too early to compare lateral root formation between the pea genotypes. From previous experiments with the same pea genotypes, we know that the resistant 'PI180693' is able to develop a bigger root system with more lateral roots upon *A. euteiches* infection, compared to 'Linnea' [17] and increased root volume and architecture has been correlated with resistance to ARR in pea [51]. Higher numbers of secondary roots were also observed in the *M. truncatula* line A17, resistant to ARR, when compared with more susceptible lines [9]. From our gene expression data, it is unclear which role ABA plays in the defense against *A. euteiches* and/or lateral root formation. In summary, we have evidence for differential regulation of ABA-responsive and biosynthesis genes between pea genotypes and hypothesize that the ABA signaling might be important for resistance in 'PI180693'.

The transcription factors myeloblastosis (MYB)14 and MYB15 were among the most strongly upregulated genes in both pea genotypes at 48 hpi. These genes belong to subgroup 2 of the MYB transcription factors that control phenylpropanoid metabolism. Members of this group are involved in stilbene biosynthesis in *Vitis vinifera* (VvMYB14 and 15), and isoflavonoid biosynthesis in *Lotus japonicus* in response to biotic and abiotic stress [52, 53]. Interestingly, we found *myb14* and *myb15* and other MYB-like transcription factors almost exclusively upregulated upon infection with the more virulent *A. euteiches* strain UK16. The *rax3* MYB transcription factor gene, which was strongly upregulated in all interactions except between 'PI180693' and UK16, is an ortholog of the *A. thaliana MYB84* gene. The *A. thaliana myb84* is a member of a network of MYB transcription factors that interact with ABA signaling to control suberin biosynthesis in root development and stress responses [54]. Another transcription factor with a similar expression pattern in this study is the pea ortholog of the *A. thaliana wrky18* gene. *wrky18* is quickly induced by ABA to inhibit root growth [55]. The ortholog of *wrky40*, an antagonist to *wrky18* [55], was significantly upregulated at 20 hpi in the interaction between 'Linnea' and UK16. This is further supporting a role of ABA signaling and root growth in the interaction between pea and *A. euteiches*.

By cross-referencing our DEG data set with genes located in genomic regions shown to segregate with ARR resistance in pea [26, 27], we arrived at 39 candidate disease resistance genes. The susceptible and resistant pea genotypes shared a higher proportion of commonly upregulated than downregulated genes and we found no genes specifically downregulated in 'PI180693' segregating with partial resistance to ARR. The four specifically upregulated DEGs in the resistant pea genotype were of special interest as they might reflect the genotype-dependent resistant phenotype. The gene Psat7g091800.1 presented an interesting candidate for further Sanger sequencing as it segregated with the ARR tolerance Ae_{MRCD1}Ps-7.2 QTL on pea chromosome 7 [27] and displayed a classical nucleotide-binding domain leucine-rich repeat (NLR) immune receptor structure. NLRs account for the largest family of plant resistance genes, and act by recognizing pathogen effectors delivered into the host and subsequently induce host cell death and resistance responses [56–58]. The Psat7g091800.1 allele in 'PI180693' displayed a number of potentially adaptive

amino acid (aa) substitutions compared to the allele in 'Linnea', as well as the pea reference genome from pea genotype 'Caméor' [18]. This is likely due to the fact that 'Caméor' as a bred cultivar (released in 1973) had been undergoing similar genetic selection steps as other commercial cultivars, resulting in a more similar genome than the old landrace 'PI180693' [15]. As four nonsynonymous single nucleotide polymorphisms (SNPs) were located within LRRs, the functionality of the immune receptor during pathogen defense in 'Linnea' might be compromised. However, to make further assumptions about the functionality of the immune receptor and its use in pea breeding, functional validation is required. The pattern recognition receptor (PRR) FLS2 was originally described in *A. thaliana* as being involved in the perception of the microbe-associated molecular pattern (MAMP) flagellin [36, 59]. In our analysis, the FLS2-like domain in Psat7g091800.1 showed to share only 58.2% sequence identity with the FLS2-encoding domains in *A. thaliana.* Moreover, phylogenies based on sequence homology reflected taxonomic differences between plant families rather than unique FLS2-like domains conserved in other plant species. In summary, Psat7g091800.1 encodes a putative NLR immune receptor that constitutes a candidate ARR disease resistance protein.

Conclusion

In conclusion, our work showed how transcriptomic data was successfully combined with available data on ARR resistance QTL to identify candidate disease resistance genes in pea. We gained insights on the transcriptomic immune response in pea to ARR, which has shown to be time-dependent. Differences in differential gene expression were clear between the resistant and susceptible pea genotype but much more subtle between *A. euteiches* virulence levels, representing a non-strain specific quantitative disease resistance mechanism in pea towards ARR. Furthermore, the 39 candidate disease resistance genes presented in this study pose a valuable resource for future marker-assisted selection in pea breeding programs. We were also able to identify a polymorphic, putative NLR immune receptor gene specifically induced in the partially resistant 'PI180693' pea genotype. Functional validation of this gene is required to assess its exact function in ARR disease resistance and its usefulness in pea resistance breeding programs.

Materials and methods

Aphanomyces euteiches cultivation and zoospore induction

For the *A. euteiches* infections in this study, we used strain SE51, from southern Sweden, and UK16 from the United Kingdom. Strain SE51 has been used for many years in Swedish pea breeding programs as a reference for low pathogen virulence. On the contrary, strain UK16 has been shown to be highly virulent on both 'Linnea' and 'PI180693' in growth chamber trials [17]. Both strains were included in a previous study on the genetic diversity of *A. euteiches* in Europe and were found to cluster together in a genetically similar, central European group [16]. Strains SE51 and UK16 were grown on corn meal agar (CMA; BD Biosciences) plates at 20 °C in the dark for two weeks. Inoculum preparation was performed following the protocol by Hosseini et al. [37], with few modifications. Five agar plugs (7 mm diameter) were used as inoculum in 200 ml V8 vegetable juice medium liquid cultures and grown in the dark at 25 °C for five days. For medium preparation, the vegetable juice (Eckes-Granini Group) was filtered through a miracloth (Merck Millipore) and diluted with sterilized water to a 20% solution, following addition of 0.3 g/L $CaCO_3$ and autoclaving. To induce zoospore production, the V8 medium was decanted, and the cultures were washed once with autoclaved river water (Fyrisån, Uppsala), followed by a three-hour incubation period in new river water at 25 °C in the dark for two days. The zoospore concentration was measured using a hemacytometer and adjusted with autoclaved tap water to 5×10^4 spores/ml.

Pea material and germination

In this experiment, the commercial pea cultivar 'Linnea' was used as a susceptible, and the partially resistant line 'PI180693' as a resistant genotype for *A. euteiches* infections [17]. Seeds were surface sterilized by several washing steps using 1% sodium hypochlorite and 70% ethanol as described in Kälin et al. [16] prior to pre-germination on 0.8% water agar plates at 20 °C for three days in the dark.

Experimental setup, inoculation and harvest

The experiment was conducted in a balanced replicated design with both pea genotypes represented in every of the five biological replicates (200 µl pipette tip boxes) as shown in Figure S1. Inoculation with *A. euteiches* strains SE51 and UK16 was performed simultaneously by placing the racks of the pipette boxes with protruding roots in respective zoospore solution (concentration 5×10^4 spores/ml) for 30 s, before transferring to new pipette boxes with autoclaved tap water. The replicates were kept open in a growth cabinet (20℃, 70–80% humidity, 12 h light, 12 h dark, 150 µmol per m^2/s) until sampling. Pea roots were sampled at 6, 20 and 48 h post inoculation (hpi), with two roots per genotype, treatment and biological replicate. The seedling development stages ranged from seedlings with only radicle and plumule at 6 hpi to the formation of scale leaves at 48 hpi (BBCH identification keys 07 to 09–10). The roots

were cut five mm from the proximal end and immediately frozen in liquid nitrogen and stored at -70 °C.

RNA extraction, quality control and sequencing

Three glass beads (2 mm diameter) were added to each 2 ml screw cap tube containing two frozen pea roots and extraction buffer. A Precellys 24 Tissue Homogenizer (Bertin Technologies) was used at 5500 rpm for 2×30 s. RNA was extracted using the Spectrum Plant Total RNA Kit (Sigma-Aldrich), following protocol A as described in the manufacturer's protocol. In brief, homogenized samples were incubated at 56 °C for 3 min and centrifuged at 17,000×g (Heraeus Pico 17 Microcentrifuge, Thermo Scientific). The lysate supernatant was collected and filtered through a column by centrifugation at 17,000×g. The clarified lysate was collected in a clean tube, added with 500 µl of the binding solution and mixed immediately. The mix was transferred into a binding column and centrifuged at 17,000×g for 1 min. After washing the column with wash solution, total RNA was eluted with two elution steps following procedures described by the manufacturers (Sigma-Aldrich). The column and solutions used in RNA extractions were provided in the kit. Extracted RNA was then diluted in nuclease-free water and measured with RNA Qubit RNA High Sensitivity (Thermo Scientific). Approximately 1000 ng RNA were used for subsequent DNase treatment in 10 µl reactions using DNase I (Thermo Scientific) with additional RNase inhibitor. DNase-treated RNA was run on an RNA Nano Chip on a 2100 Bioanalyzer System (Agilent Technologies) for quality assessment. Three biological replicates were chosen for sequencing and submitted to NGI sequencing facility (SciLifeLab, Uppsala) for library preparation for a total of 54 libraries (TruSeq Stranded Total RNA kit with Ribo-Zero Plant) and sequencing on a NovaSeq6000 S4 lane, 150 bp paired-end.

Transcriptomic analysis

Sequencing adapters removal and quality trimming was performed using Bbduk v. 38.90 [60] with the following parameters:

ktrim = r k = 23 mink = 11 hdist = 1 tpe tbo qtrim = r trimq = 10.

MultiQC v. 1.12 [61] was then used for checking the quality of the cleaned reads. To avoid mismapping, a combined genome index for the *A. euteiches* reference genome ATTCC201684 [62] and the sequenced pea genome of the French cultivar Caméor [18] was generated using STAR v. 2.7.9a [63] and the following settings:

The reads were mapped to the combined genome using STAR v. 2.7.9a with default parameters, and then read count tables were obtained using featureCounts v. 2.0.1 [64] with the following options:

−p − t exon − g Parent − B − C.

Differential gene expression analysis and visualization

The R package DESeq2 (ver. 1.32.0) was used with default parameters for differential gene expression analysis and principal component analysis (PCA) plots were generated with regularized log transformation. Contrasts were set comparing infection with *A. euteiches* strains, time points and genotypes to the same conditions, but mock treated. Genes with less than ten total read counts in a single contrast were dismissed from the analysis and genes were considered differentially expressed with log2FC values > <1 with FDR adjusted *p*-values of < 0.05. A list of genes segregating with partial resistance to ARR in pea as described in Wu et al. [26, 27] was used to further filter genes of interest. The online platform *Bioinformatics & Evolutionary Genomics* (https://bioinformatics.psb.ugent.be/links/credits) was used to illustrate DEGs in Venn diagrams. BAM files were loaded into the integrative genome viewer IGV (version 2. 12. 3 03) [65] for visualization of gene expression.

Gene ontology enrichment analysis and homology searches

The public annotation of the pea genome was downloaded from https://urgi.versailles.inra.fr/download/pea/ and gene ontology (GO) enrichment analysis was done through Fisher's exact tests with FDR-adjusted *p*-value of 0.05 as threshold. The Fisher tests were run using the agriGO online service [66] for simple enrichment analysis, and the enriched GOs were visualized using REVIGO with redundancy filtering [67]. The functional annotation available on the pea database was complemented with InterProScan (v. 5.48) and BLASTp analysis against the NCBI non redundant protein database, using a minimum ID of 60% and minimum query coverage of 80%.

Sequencing, SNP calling and analysis of Psat7g091800.1

Genomic DNA was extracted from roots of 'Linnea' and 'PI180693' using the DNeasy Plant Mini Kit (Qiagen) following the manufacturer's protocol. Primers were designed based on the reference sequences using

−sjdbOverhang 100−sjdbGTFfeatureExon CDS−sjdbGTFtagExonParentTranscript Parent−genomeSAindexNbases10.

DNASTAR (v. 17.2.1.61) software. PCR amplification of the Psat7g091800.1 gene were run on a Veriti™ 96 well Thermal Cycler (Applied Biosystems) using respective primers (Table S1). Each reaction contained 25 ng of template DNA and was conducted following the PCR protocol for Phusion Polymerase with 0.5 µM primer concentration in a total of 25 µl reaction volume. The initial denaturation was at 98 °C for 30 s, followed by 32 cycles at 98 °C for 10 s respective annealing temperature for 20 s and extension at 72 °C for 90 s. The concentrations of PCR products were determined with absorbance measurements on a NanoDrop 1000 Spectrophotometer and electrophoresis in 1.5% agarose gels was performed for verification of fragment size. The PCR products were purified using AMPure XP reagent (Beckman Coulter) and concentrations were adjusted to 50 ng/µl for each product prior to submitting to Macrogen Europe B.V. (Amsterdam, Netherlands) for Sanger sequencing. Contig assemblies were done using SeqMan Ultra (v. 17.2.1) and alignments were done in MEGA-X v. 10.0.5 [68] where single nucleotide polymorphism (SNP) calling was done manually. PhytoLRR [69], SignalP [70] and DeepTMHMM [71] were used for prediction of LRRs and functional domains. The variant effect predictor by EnsemblPlants (release 109) [72] was used to assess consequence types of SNPs, and the mapping of RNA reads on the gene were visualized through the Integrative Genomics Viewer (v. 2.15.4). To obtain a de-novo transcript sequence of the gene, the command "samtools faidx" [73] was used to isolate, from the bam files generated with STAR, the reads mapping within 2000 bp of the reported location of Psat7g091800.1. Said reads were then corrected using Rcorrector [74] with default parameters, the unfixable reads were removed, and the remaining ones were assembled through Trinity v. 2.11.0 [75] with default parameters.

Orthologs and phylogenetic analyses

The predicted Psat7g091800.1 protein sequence was compared against the NCBI protein database using the psi-BLAST algorithm [76] in a selection of representative cultivated organisms of different plant families, including pea, chickpea, soybean, white clover, *M. truncatula*, potato, tomato, wild cherry, rapeseed, *A. thaliana*, cucumber and melon. The protein sequence of the best hit for every species was used for a sequence alignment with the multiple sequence alignment program MAFFT (v. 7.453) [77] and the L-INS-I accuracy-oriented method with following options:

−localpair − maxiterate 1000.

Phylogenetic trees were computed using IQ-TREE (v. 2.1.3) [78] using the ModelFinder option [79] with following settings:

−seed 17 − st AA − m MFP − b 1000 − safe − T 1.

For the alignments of the entire protein and the FLS2-encoding domains, the best model according to Bayesian information criterion (BIC) scores was Q.plant + G4 for the construction of a maximum likelihood tree. Condensed trees were computed in MEGA-X v. 10.0.5 [68] with a bootstrap cutoff value of 70% (Figure S4).

Abbreviations

ARR	Aphanomyces root rot
QTL	Quantitative trait locus/loci
BSR-seq	Bulked segregant RNA-seq
DEGs	Differentially expressed genes
PCA	Principal component analysis
GO	Gene ontology
JA	Jasmonic acid
ABA	Abscisic acid
TF	Transcription factor
MYB	Myeloblastosis
LRR	Leucine-rich repeat
MLP	Major latex protein
RUN1	*Uncinula necator* 1
Psd2	*P. sativum* Defensin 2
RLK	Receptor-like kinase
SNP	Single nucleotide polymorphisms
aa	Amino acid
FLS2	FLAGELLIN SENSING 2
NLR	Nucleotide-binding domain leucine-rich repeat
PR	Pattern recognition receptor
MAMP	Microbe-associated molecular pattern
BLAST	Basic local alignment search tool

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12870-024-04817-y.

Additonal file 1: Figure S1.

Additonal file 2: Figure S2.

Additonal file 3: Figure S3.

Additonal file 4: Figure S4.

Additonal file 5: Table S1. Primer sequences, amplicon size and annealing temperatures for gDNA amplification of Psat7g091800.1.

Additonal file 6: Table S2. Read counts on Aphanomyces euteiches and Pisum sativum.

Additonal file 7: Table S3. Number of differentially expressed genes in every condition compared to the mock treatments.

Additonal file 8: Table S4. Significant results of the DESeq2 analysis. All combinations of pea genotype, strain and time point were compared to the mock inoculation in the same condition.

Additonal file 9: Table S5. DEGs at 6 hpi, 20 hpi, 48hpi and resistance DEGs.

Additonal file 10: Table S6. Enriched GO terms of 48 hpi DEGs.

Additonal file 11: Table S7. Positions of single nucleotide polymorphisms, including changes in amino acids in the gene Psat7g091800.1.

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Authors' contributions
CK, ABK, ME, MD, MK and SB designed the study. CK and MD performed the experiments. CK, EP, ME and SB analyzed the data. ABK provided the pea genotypes and *A. euteiches* strains. All authors read and approved the manuscript.

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Availability of data and materials
The transcriptome data is available on the European Nucleotide Archive (ENA, https://www.ebi.ac.uk/ena/browser/search) under Bioproject "PRJEB66187".

Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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The thesis aimed to study the genetics underlying aphanomyces root rot resistance in pea and the genetic and virulence diversity of European *Aphanomyces euteiches* strains. Three genetic groups of *A. euteiches* were identified on a north-to-south gradient, with the most southern group showing signs of genetic isolation. In pea, the transcriptional immune response showed to be highly dependent on the resistance level of the host and revealed several candidate disease resistance genes for the future application in pea breeding.

Carol Kälin received her graduate education at the Department of Forest Mycology and Plant Pathology, SLU, Uppsala. She received her M.Sc. in Plant Sciences from the University of Zurich, Switzerland.

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