DISCOVERING DISEASE RESISTANCE RESPONSES OF FIELD PEA TO MAJOR ROOT ROT PATHOGENS USING RNA-SEQ AND COMPARATIVE GENOMICS

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Master of Science in Environmental Studies, Kentucky State University, 2019

A thesis submitted
in partial fulfilment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

BIOMOLECULAR SCIENCE

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Lethbridge, Alberta, Canada

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DEDICATION

I dedicate this dissertation to my beloved parents, **Mr. Shivraj Pandit** and **Ms. Shanta Rijal Pandit**, whose love and upbringing instilled in me the confidence to pursue science with determination, and who taught me that being a kind human being is above all else.

ABSTRACT

Pea root rot complex (PRRC) is caused by a assemblage of soil-borne fungal and oomycete pathogens, including *Aphanomyces euteiches*, *Fusarium spp*., *Rhizoctonia solani*, and *Pythium spp.*, which collectively cause severe yield losses in pea production worldwide. Studies have confirmed *Aphanomyces euteiches* and *Fusarium avenaceum* as major and highly destructive contributors to the complex. Research on the pea root rot complex and its associated pathogens has improved understanding of pathogen diversity, host resistance mechanisms, and disease management strategies. However, the lack of fully resistant cultivars and the complexity of PRRC continue to pose challenges. With the ultimate aim to develop effective solutions for controlling this economically devastating disease through resistant breeding strategies and sustainable management practices, I studied pea defense responses involved in partial resistance to two major pathogens of PRRC, *A. euteiches* and *F. avenaceum*, using integrated genomic tools. I performed both field experiments and greenhouse experiments to validate multiple genotypes for their susceptibility and partial resistance to the respective pathogens. I also performed preliminary experiments to determine the pattern and timing of pathogen colonization. Finally, by performing RNAseq on roots inoculated with both *A. euteiches* and *F. avenaceum*, I found pathways and candidate defense genes related to both pathogens that may help develop resistant pea cultivars.

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Additional Contributions: Eric Amundsen, Anthony Erickson, and Christine Vucurevich assisted with some technical aspects of the project.

USE OF GENERATIVE AI

Use of Generative AI (ChatGPT, OpenAI) was limited to obtaining suggestions on formatting and idea organization. All scientific content, experimental design, data analysis, visualization, interpretation of results, and the writing of this thesis are entirely the authors' original work.

PREFACE

Chapter 1 is to be submitted for publication in a journal tbd.

Chapter 2 has been published in the Canadian Journal of Plant pathology, and permissions to include these works in this thesis have been obtained from the publishers and authors.

Pandit, S., Biscaglia-Horvath, K., Vucurevich, C., Amundsen, E., & Chatterton, S. (2025). Assessment of *Fusarium Avenaceum* Inoculation Methods for Consistent Development of Pea Root Rot under Greenhouse Conditions. Canadian Journal of Plant Pathology, 1-15. https://doi.org/10.1080/07060661.202537831

Chapter 3 is preliminary work done for Chapter 4 and Chapter 5.

Chapter 4 has been accepted for publication in the Legume Science. Permissions to include these works in this thesis have been obtained from the publishers and authors.

Pandit, S., Hodan Halane, H., Lim, N. Z-L., Singer, S. D., Dhaubhadel, S., Goyal, R., Zhang, W., Foroud, N. A., Schultz, E., & Chatterton, S. (2025). Transcriptomic and targeted metabolomic analyses of partially resistant and susceptible pea genotypes reveal differential defense responses during their interaction with *Aphanomyces euteiches*. Legume Science (Manuscript accepted).

Chapter 5 is to be submitted in a journal tbd.

ACKNOWLEDGEMENTS

First and foremost, I extend my heartfelt thanks to my supervisor, Dr. Syama Chatterton, for being an extraordinary mentor and an incredible role model. Your kindness, compassion, and understanding have made this journey not only productive but also deeply enriching. To my co-supervisors, Dr. Elizabeth Schultz and Dr. Ravinder Goyal, thank you for your invaluable guidance and support. Your expertise and encouragement have been instrumental in shaping this work. I am also deeply grateful to my committee members, Dr. Nora Foroud and Dr. Igor Kovalchuk, for their insightful contributions and feedback.

I would like to acknowledge the incredible support I received from AAFC Lethbridge and the members of the pulse pathology lab, especially Eric Amundsen, Christine Vucurevich, Anthony Erickson, Brooke Groenenboom, and all others who made my time in the lab both productive and enjoyable. Additionally, I am thankful to Dr. Foroud's lab members, particularly Daria Ryabova, for their collaboration and support. I am also grateful to Dr. Stacy Singer form AAFC Lethbridge, Dr. Sangita Dhaubadel from AAFC London, Wentao Zhang from NRC, Dr. Sabine Banniza and Dr. Tom Warkentin from the University of Saskatoon for their contributions and insights.

I express my deepest gratitude to my husband, Dr. Hari Poudel**,** for being my rock throughout this journey. Your unwavering support as an amazing husband, mentor, and best friend has been my greatest strength. You have stood by me through every challenge and celebrated every milestone, and for that, I am forever grateful. To my son, Reason, you are the sweetest gift I could ever ask for. Your boundless curiosity and joy inspire me to learn and grow every single day. You remind me of the beauty in discovery and the importance of perseverance.

To my darling siblings (Sujata, Sajana, Sandesh and Satkar), thank you for your love, respect, and trust. Your presence in my life has been a constant reminder of the importance of family and togetherness.

To my father in law Mr. Durga Prasad Poudel and mother in law Mrs. Bimala Poudel, thank you for teaching me perseverance and indirectly reflecting how any transformation in life can be embraced in a positive way.

Finally, I want to thank my amazing friends Salina, Deepa, Ambika, Roshani, and Sandhya for trusting me, standing by me, being family away from home and my pillars of strength. I have learnt a lot from all of you and your friendship has been a source of joy and comfort throughout this journey.

This PhD journey has been more than just an academic pursuit or a scientific endeavor; it has been a transformative experience that taught me to evolve as a person. I have learned that science is not a solitary pursuit, collaborative efforts amplify discoveries and drive progress. I am deeply thankful to everyone who has been directly or indirectly involved in this journey.

This thesis is a testament to the collective efforts, encouragement, and inspiration of all these incredible individuals.

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LIST OF ABBREVIATIONS

ABA	Abscisic acid
AM	Arbuscular Mycorrhizal
ARR	Aphanomyces root rot
BR	Brassinosteroids
CK	Cytokinin
DON	Deoxynivalenol
Dpi	Days post inoculation
ET	Ethylene
ETI	Effector triggered immunity
GO	Gene enrichment
Hpi	Hours post inoculation
HR	Hypersensitive response
JA	Jasmonic acid
MAPK	Mitogen-activated Protein Kinase
MAS	Marker Assisted Selection
PAMP	Pathogen-associated molecular pattern
PCD	Programmed cell death
PR	Pathogenesis related
PR	Partially resistant
PRR	Pattern recognition receptors
PRRC	Pea root rot complex
PTI	Pattern triggered immunity
qPCR	Quantitative PCR
QTL	Quantitative Trait Loci
ROS	Reactive oxygen species
RRC	Root rot complex
S	Susceptible
SA	Salicylic acid
SAR	Systemic Acquired Resistance
SSP	Small secreted Protein

CHAPTER 1 : INTRODUCTION AND LITERATURE REVIEW

1.1 PEA AS A GLOBAL PULSE CROP

Pea (*Pisum sativum* L.) is one of the most important legume crops globally, valued for its nutritional benefits, versatility in food systems, and role in sustainable agriculture (Tulbek et al., 2024). Peas contribute significantly to food security, human health, and environmental sustainability. After dry beans and chickpeas, peas are the third most widely grown pulse crop globally and are considered the second most important legume after soybean (*Glycine max*) (Shanthakumar et al., 2022). Dry peas are a nutrient-dense crop, containing 20-25 % protein, 1.5-2% fat, 60-65% total carbohydrates of which 40-50% is starch and 10-25% in dietary fiber on dry weight basis, and traces of vitamins and minerals. In 2022, global pea production was estimated at over 15 million tons, with Canada, Russia, and China being the top producers. Canada is the world's largest producer followed by Russia, China, Ukraine, United States, France, Australia (FAO, 2024) based on production of dry peas.

The growing demand for plant-based proteins and sustainable food systems presents significant opportunities to expand pea production and utilization (Rogers et al., 2024). This expansion has been driven in part by recent innovations in processing technologies and the development of value-added products such as pea protein isolates and textured pea protein (Grossmann, 2024; Maningat et al., 2022). In addition, peas contribute to a sustainable food system because of their ability to fix atmospheric nitrogen through symbiotic relationships with rhizobia, improving soil fertility and reducing the need for synthetic fertilizers (Faligowska et al., 2022; Hossain et al., 2017; Karkanis et al., 2016). However, pea production faces several challenges worldwide, ranging from biotic stresses (pests and diseases) to abiotic stresses (environmental factors) and socio-economic constraints (access to quality seeds, fluctuating

market prices, demand and supply). These challenges can significantly impact yield, quality, and profitability, posing barriers to sustainable pea cultivation. This review focuses on one of the major biotic challenges, known as root rot complex, and provides a comprehensive overview of its causes, impacts and potential management strategies.

1.2.1 PATHOGEN PROFILE OF ROOT ROT COMPLEX

Pea root rot complex (P/RRC) is a combination of diseases caused by soil or seed-borne pathogens that adversely affect root health resulting in substantial damage or even mortality of the plant (Chatterton et al., 2019; Grau, 1990; Tu & Findlay, 1986; Wu et al., 2018; A. G. Xue, 2003). Several soil-borne pathogen types are involved in RRC including *Aphanomyces euteiches, Fusarium* spp*., Pythium* spp*., Phythophthora* spp*.* and *Rhizoctonia solani* Kuhn (Bailey, 2003; Chang et al., 2013; Trenk et al., 2024). Each of these pathogens individually has the capability of causing root rot but the effect on disease severity is often synergistic when multiple pathogens are present together. Within *Fusarium* spp*., F. avenaceum* was the predominant causal agent associated with the RRC in Canada followed by other *Fusarium* spp*.,* especially *F. solani,* and other species such as *Pythium* spp. and *R. solani* (Chatterton et al., 2019; Feng et al., 2010; McLaren et al., 2019). Although long described as an important pathogen of pea, *A. euteiches* has only emerged as an important oomycete pathogen in the RRC of dry pea in the northern great plains of Canada and the United States in the past ten to fifteen years (Armstrong-Cho et al., 2014; Gaulin et al., 2007; Murphy et al., 2022; Sivachandra Kumar et al., 2021; Xue et al., 1998; Zitnick-Anderson & Pasche, 2016). High yield losses due to *A. euteiches* and *Pythium* spp. were reported in fresh processing peas in the United States during the early 1990s (Bowers & Parke, 1993) and the resurgence in dry pea crops in the 2010's was due to the expansion of dry pea acreage in the northern great plains that started in the mid-1990s. As pea cropping cycles

increased, there was likely a parallel increase in inoculum levels of *A. euteiches* in soil over time while the development and use of sensitive molecular assays facilitated more rapid and accurate detection (Chatterton et al., 2019; Murphy et al., 2022). Although symptoms of the RRC can include seed decay, damping off, and seedling blight, the major issue and cause of yield loss is root rot and decay which can cause plant death under severe conditions. Roots of affected plants display light brown to black lesions on primary and lateral roots which extends throughout the root system, and the epi- and hypocotyl, causing the root system to soften and decay. Under severe conditions, the aerial parts of plants exhibit yellowing, poor pod formation, or complete plant death, which in field conditions often appears as yellowing or vacant patches. Root rot ultimately affects nitrogen fixation and water and nutrient transport to the aerial parts of plants reducing the canopy and vigor of the plant (Xi et al., 1995). Thus, root rot caused by the RRC leads to poor shoot growth, yellowing of leaves and reduction of shoot branching causing poor or delayed canopy closure allowing high weed invasion. A combination of these factors results in poor pod development resulting in partial or complete yield losses. The impact that the RRC has on pea yield has been recognized as a significant threat to production in pea-growing regions worldwide since the early 1920s (Grau, 1990; Papavizas & Ayers, 1974; Tu & Findlay, 1986; A. Xue, 2003) while this has only emerged as a critical issue in the northern great plains growing regions since the early 2010's (Chatterton et al., 2019). Depending on the severity of disease and type of pathogen, yield reduction in field pea (*Pisum sativum* L) can be as high as 100% (Tu & Findlay, 1986; Willsey et al., 2018; A. G. Xue, 2003). The relative occurrence of different pathogens in the RRC and severity of the disease are affected by temperature, humidity, soil edaphic properties, soil moisture, and soil compaction (Feng et al., 2010; Ondrej et al., 2008; Tu, 1994; Zitnick-Anderson et al., 2020). *Aphanomyces euteiches* and *Fusarium avenaceum* are the

two major pathogens of the RRC in Alberta and Saskatchewan and given their prevalence and economic impact in these regions, they are the focus of this research and are reviewed in greater detail in the remainder of this chapter.

1.2.1 *APHANOMYCES EUTEICHES*

Aphanomyces euteiches belongs to the kingdom Stramenopila, sometimes classified under Chromista (Zitnick-Anderson et al., 2021), comprising over 100,000 eukaryotic species such as algae, water molds, diatoms, and oomycetes (Adl et al., 2019; Adl et al., 2012). Within this kingdom, *A. euteiches* is classified under oomycetes, a group often mistaken for fungi due to their filamentous growth, absorptive nutrition, and spore-based reproduction. However, unlike fungi which have chitin and β-glucan-based cell walls, oomycetes possesses cellulose and β-glucan-rich cell walls, have diploid coenocytic vegetative mycelium, and produce wall-less motile zoospores with two flagella, as well as thick-walled oospores during sexual reproduction (Badreddine et al., 2008; Becking et al., 2022; Spring et al., 2018).

The genus *Aphanomyces* comprises approximately 40 species, many of which are parasitic or saprophytic on fish, crayfish, and plants (Diéguez-Uribeondo et al., 2009; Grunwald, 2003). While most have broad host ranges, some, such as *A. cochlioides* (host: *Beta vulgaris*) and *A. iridis* (host: *Iris* spp.), are host-specific. *A. euteiches* infects multiple members of the Fabaceae family, including pea, lentil (*Lens culinaris*), alfalfa (*Medicago sativa*), snap and dry beans (*Phaseolus vulgaris*), white and red clover (*Trifolium repens*, *T. pratense*), and faba bean (*Vicia faba*) (Gaulin et al., 2007; Malvick et al., 2009; Papavizas & Ayers, 1974), but interestingly some species such as *Glycine max*, *Cicer arietinum*, and certain cultivars of *Vicia faba* exhibit high resistance or non-host status. This suggests that the degree of pathogenicity of

A. euteiches differs between host species (Malvick et al., 2009; Moussart et al., 2007; Wu et al., 2018) and *A. euteiches* strains differ in host specificity; some infect both pea and alfalfa, while others do not (Malvick & Grau, 2001; Moussart et al., 2024; Moussart et al., 2007; Wicker & Rouxel, 2001). Generally, pea and dry bean strains are not cross-pathogenic (Slusarenko 2004, Chatterton et al., 2022).

A. euteiches can infect their host plants at any growth stage, provided conditions are favorable. *A. euteiches* is a diploid and homothallic oomycete capable of completing its sexual cycle autonomously, without the presence of a mating partner, producing both oospores (sexual spores) and zoospores (asexual spores) (Gaulin et al., 2007; Lin & Heitman, 2007). Oospores persist in soil and plant debris during unfavorable conditions and germinate under favorable conditions to release motile asexual zoospores that disseminate the pathogen. Oospores are 15–18 μm in diameter, possess a thick cell wall, and store energy in the form of a central oil globule (Papavizas & Ayers, 1974; Pfender et al., 1984). Oospores can survive in the soil for over 10 years (Pfender et al., 1984) though their germination is often asynchronous (Shang et al., 2000). Remarkably resilient, they maintain viability even during extended unfavorable periods and can germinate in response to wild leguminous hosts, facilitating infection in the absence of cultivated pea or other host legume crops (Gaulin et al., 2007). However, the role that wild or weedy legume species play in maintaining inoculum levels in the soil is not well studied. Long distance dissemination of inoculum is caused by the transportation of soil, plant materials, and farm tools (Papavizas & Ayers, 1974; Trenk et al., 2024). In the presence of a host and in response to as-yet unknown host signals, oospores germinate to produce either mycelia or zoosporangia. Zoosporangia give rise to numerous primary pyriform motile zoospores, which are chemotactically attracted to host root exudates (Scott, 1961; Sekizaki et al., 1993; Zitnick-

Anderson et al., 2021). Upon reaching the host, the biflagellate zoospores adhere to the root surface and encyst in the presence of calcium ions (Deacon & Saxena, 1998; Gaulin et al., 2007). The cysts then germinate, penetrate host cells within hours, and develop rapidly into coenocytic hyphae that grow through the intercellular spaces of root tissues. After several weeks, sexual structures, the haploid antheridia and spherical oogonia (~25–35 μm in diameter) form. Antheridia fertilize the oogonia via fertilization tubes, producing diploid oospores (Gaulin et al., 2007; Scott, 1961). Infection and disease development take 10-14 days until oospores are formed within the roots of host plant (Kjoller & Rosendahl, 1998). The detailed life cycle of *A. euteiches* is depicted in Figure 1.1.

Symptoms of Aphanomyces root rot (ARR) include honey-brown discoloration of the roots, which can progress to complete root system collapse and plant death, depending on the severity of infection (Ali et al., 2025; Gangneux et al., 2014). Infected plants are often weakened, making them more susceptible to secondary invasion by other microbes and parasites (Willsey et al., 2018). In pea, *A. euteiches* is restricted to the roots and lower stem, indicating a tissue-specific response (Cannesan et al., 2011; Desgroux et al., 2016). The earliest visible symptoms appear on lateral roots, which initially become soft and water-soaked. These symptoms evolve into honey-brown lesions on cortical tissues, progressing upward to the hypocotyl and epicotyl. Extensive hyphal colonization destroys cortical tissues, leading to stunted growth, chlorosis, and premature plant death. In severe cases, lateral roots are entirely destroyed, and the epicotyl turns dark and collapses. The translocation of water and nutrients can be highly restricted in the severe diseased condition causing plants to stunt and turn yellow. Such plants wilt and die prematurely in the later stage of disease development (Gaulin et al., 2008; Muehlchen et al., 1990; Trenk et al., 2024). These symptoms apply both to pea and lentil however symptoms on lentil are complex

and difficult to understand due to patchy discoloration and cortex remaining intact (Chatterton et al., 2019). Pea plants infected at later growth stages may show no visible symptoms above ground and can still produce pods and seeds (Hughes & Grau, 2007; Papavizas & Ayers, 1974). These resilient and cryptic features make it a particularly difficult pathogen to manage.

1.2.2 DISTRIBUTION AND PATHOGENICITY OF *A. EUTEICHES*

Many studies have described the diversity of *A. euteiches* populations worldwide. *A. euteiches* populations isolated from pea are pathogenically, genotypically, and geographically variable (Malvick & Percich, 1998; Quillevere-Hamard et al., 2018). It has been reported in nearly all pea/lentil-growing regions globally and is considered one of the most destructive soilborne pathogens of legumes (Wu et al., 2018). The pathogen *A. euteiches* has been reported worldwide including in the United States, Canada, France, Australia, Asia, New Zealand and Scandinavia (Allen et al., 1987; Becking et al., 2022; Chatterton et al., 2015; Didelot & Chaillet, 1995; Jones & Drechsler, 1925; Levenfors, 2003; Manning & Menzies, 1980; Persson et al., 1997; Zitnick-Anderson & Pasche, 2016).

Many pea-infecting isolates of *A. euteiches* have been evaluated for their pathogenicity on various legume species and pea lines. Pathogenicity typically refers to the pathogen's virulence and aggressiveness (Quillevere-Hamard et al., 2018). Virulence is the ability to induce a susceptible host response (Parlevliet, 2002) while aggressiveness refers to the intensity of disease on susceptible hosts (Pariaud et al., 2009). Malvick and Percich (1998) reported field-dependent variation in pathogenicity across 114 isolates from pea fields in Minnesota, Oregon, and Wisconsin. In their earlier study (1998a), 96% of pea isolates were virulent on pea, 18% on alfalfa, and 14% on green bean. By contrast, Holub et al. (1991) found that 59% of their 37 pea

isolates were virulent on pea, 97% on alfalfa, and none on green bean. Pfender & Hagedorn (1982) also found pea isolates could infect beans, though with lower severity, while Malvick and Percich (1998a) reported similar symptom severity regardless of host origin. Random amplified polymorphic DNA (RAPD) analyses (Malvick et al., 1998) revealed genotypic variation linked to host origin and pathogenicity, although follow-up studies showed inconsistent associations. This suggests that host origin may influence pathogenic specialization across different hosts, and that there are variations in pathogen aggressiveness within hosts.

Wicker et al. (2001) grouped 89 French isolates of *A. euteiches* into four pathogenicity groups based on aggressiveness to five legumes. Typically, isolates were most aggressive to their host of origin but could infect multiple species. Pea isolates commonly infect other legumes, while isolates from alfalfa and green bean showed limited ability to infect pea. Common vetch (*Vicia sativa*) was broadly susceptible, suggesting it may sustain *A. euteiches* populations in the absence of pea. These findings supported previous groupings of pea isolates into 'pea' and 'pea/alfalfa' virulence groups (Holub et al., 1991; Malvick & Percich, 1998a, 1998b), challenging the idea of a pea-specific form such as *A. euteiches* f. sp. *pisi* (Pfender & Hagedorn, 1982).

In unpublished work by Chatterton and Banniza, two Canadian pea isolates did not infect most tested dry bean (*Phaseolus vulgaris* L.), faba bean (*Vicia faba*), chickpea (*Cicer arietinum*), or soybean (*Glycine max* L.) cultivars, reinforcing their non-host status. Historically, two pathotypes have been defined based on host specialization, alfalfa (pathotype I) and pea (pathotype II) (Pfender & Henshaw, 1981). Now there are multiple virulence groups which are also referred to as pathotypes, and are based on the degree of root rot severity that an isolate causes on a set of pea differential lines with various sources of partial resistance (Wicker and

Rouxel, 2001). Wicker and Rouxel (2001) showed that 88 French pea isolates belonged to a single genetic population (pathotype I) but exhibited wide variation in aggressiveness. American isolates formed separate virulence groups (pathotypes III and IV), which were generally less aggressive than the French isolates. Quillevere-Hamard et al. (2018) further identified three aggressiveness groups among 34 French isolates from pea, with some regions (e.g., Bourgogne) harboring more aggressive populations. French isolates are thus considered highly aggressive and valuable for resistance breeding reference panels. A recent Canadian study characterized the pathogenic variability of *A. euteiches*, using French pea differential lines to determine pathotypes of isolates collected from pea across Saskatchewan and Alberta, Canada, alongside several U.S. and reference isolates. A total of 33 Canadian isolates from pea were analyzed, and the majority (29/33) were classified as pathotype I, known to be the most virulent and dominant type in France (Sivachandra Kumar et al., 2021). Only three isolates matched pathotype III, and one U.S. isolate aligned with pathotype II. The identification of pathotype I as the predominant pathotype in Western Canada has significant implications for resistance breeding, as this pathotype exhibits virulence against all pea differentials used for classification. This study also highlighted the limited host range of alfalfa isolates, which were non-virulent on peas. In summary, most pea isolates of *A. euteiches* can infect legumes like alfalfa, lentil, and vetch with varying aggressiveness, while pea isolates are not virulent on crops ~~crops~~ such as dry bean, soybean, chickpea, and faba bean. Crop rotations involving susceptible alternative hosts could risk inoculum buildup or increased disease severity due to aggressive strains.

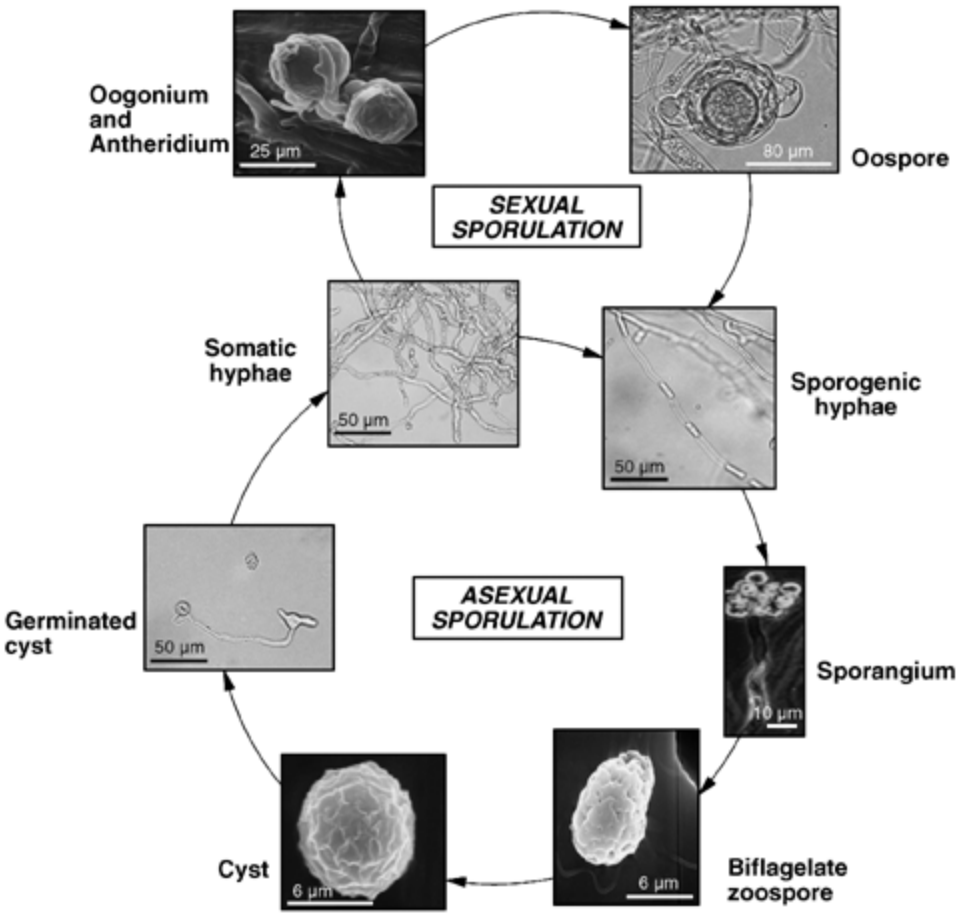

Figure 1.1. Diagrammatic representation of the life cycle of *A. euteiches.* (Gaulin et al., 2007). Reproduced with permission license (lic number 6152620116248) from John Wiley and Sons.

1.2.3 *FUSARIUM AVENACEUM*

Fusarium species are the most predominant among the pathogens in the PRRC. *F. avenaceum* is a fungal plant pathogen with a broad host range, including pulses, grain crops, potato, tomato, and asparagus (Foroud et al., 2014; Stępień et al., 2016). It has also been reported as an endophyte (Crous et al., 1995; Varvas et al., 2013) and an opportunistic pathogen of animals and fish (Batta, 2012; Makkonen et al., 2013). It is globally distributed and survives in the soil for several years as a saprophyte on dead organic matter, particularly crop residues. *F. avenaceum* is considered problematic in most part of the world including Canada, the USA,

Europe, Australia, and Asia due to its ability to infect over 80 plant genera (Chittem et al., 2015; Moslemi et al., 2017; Parihar et al., 2025; Sharma et al., 2022). In the Canadian prairies, *F. avenaceum* causes root rot in several rotational crops such as wheat, canola, and faba bean. *F. avenaceum* typically does not produce chlamydospores or microconidia (Yli-Mattila et al., 2018), and is known for producing toxic secondary metabolites such as moniliformin, beauvericin, enniatins, and fusarin C (Leslie & Summerell, 2008; Uhlig et al., 2007). However, mycotoxin contamination in peas is not considered a significant threat to human or animal health, as infections primarily affect the roots (Feng et al., 2010). Despite its genetic and morphological diversity, *F. avenaceum* does not segregate into host-specific pathotypes, complicating management through crop rotation (Satyaprasad et al., 2000; Yli-Mattila et al., 2018).

F. solani f. sp. *pisi* is also frequently associated with pea RRC and infects a wide range of hosts, including pea, vetch, alfalfa, clover, fenugreek, and numerous wild legumes (Kraft & Pfleger, 2001; Šišić et al., 2018; VanEtten, 1978). Although historically considered pea-specific, recent studies show that it infects up to 10 legume genera, with some acting as asymptomatic hosts (Šišić et al., 2018). On pea roots, *F. avenaceum* and *F. solani*, cause similar symptoms but *F. avenaceum* can be distinguished morphologically by its narrow and slender macroconidia (Leslie & Summerell, 2008). Symptoms on pea roots include brown to dark lesions on the root surface, often leading to cortical decay, root pruning, and overall reduction in root biomass, which contribute to stunted growth and yellowing of above-ground tissues (Safarieskandari et al., 2021).

Environmental conditions and cropping systems are known to influence pathogen aggressiveness (Karkanis et al., 2016; Laloi et al., 2016). For instance, variation in

aggressiveness among *F. avenaceum* isolates has been documented between pea samples collected from North Dakota (Chittem et al., 2015; Feng et al., 2010) and Alberta (Feng et al., 2010). Therefore, the varying levels of aggressiveness observed in these studies likely stem from the diverse sampling regions, though this requires further investigation. Genomic sequencing of *F. avenaceum* strains isolated from barley (Finland) and wheat (Canada) revealed 75–80 key biosynthetic enzymes involved in polyketide, non-ribosomal peptide, terpene, alkaloid, and indole-diterpene synthesis pathways (Lysoe et al., 2014). Additionally, novel metabolites such as fuscofusarin and JM-47 were identified. However, comprehensive studies focused solely on *F. avenaceum* are limited due to its frequent coexistence with other *Fusarium* species in disease complexes such as Fusarium head blight and RRC. Thus, its pathogenicity and genetic diversity require further exploration.

In pea, *F. avenaceum* first enters the seedling through the point of seed attachment. Typical symptoms of Fusarium root rot (FRR) include small brown to black lesions near the point of seed attachment or on the hypocotyl and epicotyl (Hwang et al., 1994; Kraft et al., 1993; Willsey et al., 2019). These lesions later coalesce into larger necrotic spots that can encircle the roots and extend 1–2 cm above the soil line on the stem. Yellowing of aerial parts and stem necrosis commonly occur in conjunction with co-infections. Early-season symptoms include poor emergence, stunted growth, and seedling collapse. Infection by *Fusarium* spp. impairs water and nutrient uptake, leading to poor plant development. Yield loss is often difficult to quantify due to the involvement of multiple pathogens and various indirect effects (Willsey et al., 2019). However, *Fusarium* spp. have been reported to cause yield losses of up to 60% in field pea (Chang et al., 2005; Gossen et al., 2016) and loss in lentil can be up to 100% based on environmental conditions. Additionally, exposure of pea plants to both *Fusarium* spp. and insects

such as the pea leaf weevil results in more severe root rot compared to *Fusarium* infection alone (Willsey et al., 2019).

F. avenaceum can survive as a saprophyte or infect weeds or volunteer crops to maintain its inoculum. *Fusarium* spp. persist in the soil primarily as chlamydospores and mycelia, enabling them to survive for several years in the absence of a host. Infected crop debris and contaminated seeds serve as major sources of inoculum for subsequent infection cycles. However, *F. avenaceum* do not produce chlamydospores but still survive effectively through infected plant residues, seeds, and soil. These pathogens also spread via water movement, farm machinery, and infected crop debris. Moderate to high soil moisture levels generally favor *Fusarium* infections. Poor drainage and soil compaction exacerbate disease severity. High humidity or precipitation sustained for at least 12 hours is typically required for infection. The epidemiology of *Fusarium* root rot is complex and influenced by a combination of environmental conditions, host susceptibility, and pathogen traits. Although classically *F. solani* f. sp. *pisi* is known to thrive in warmer soils (20–25°C) and *F. avenaceum* in cooler soils (15–20 °C) (Brennan et al., 2003), recent *in vitro* studies suggest a broader ecological adaptability. For example, Garcia et al. (2024) reported that many *F. avenaceum* isolates produced higher levels of mycotoxins under elevated temperature and moisture conditions, indicating that this species may also thrive under warmer, wetter environments. Broadly, temperatures ranging from 20 to 30 °C are conducive to infection by most *Fusarium* spp., supporting their role as major contributors to root rot in legumes under typical prairie field conditions (Osborne & Stein, 2007; Brennan et al., 2003; Xu, 2003).

Fusarium avenaceum is known to produce several secondary metabolites, commonly referred to as mycotoxins, that support its infection and proliferation in host tissues. These

include moniliformin, enniatins, beauvericin, aurofusarin, chlamydosporol, and fusarins (Uhlig *et al.*, 2007, Leslie & Summerell, 2008, Munkvold, 2017). Mycotoxins play critical roles in *Fusarium* pathogenesis. For example, the trichothecene deoxynivalenol (DON), produced by *F. graminearum*, acts as a potent inhibitor of protein synthesis and suppresses defense gene activation, leading to chlorosis and increased susceptibility (Rotter, 1996). Presence or absence of DON production has been shown to strongly influence pathogen aggressiveness and host resistance in wheat (Eudes *et al.*, 2001). However, the role of mycotoxins in *F. avenaceum*–pea interactions remains poorly understood. Evidence suggests that enniatins, although commonly produced by *F. avenaceum*, are not essential virulence factors in pea. In a functional analysis using ESYN1 mutants (disrupted and overexpressed), no significant difference in pea emergence or root rot symptoms was observed compared to the wild-type strain, suggesting enniatins are not required for disease development in pea (Eranthodi et al., 2020). While enniatins may not act as primary pathogenicity factors in pea, they could modulate host cell physiology altering ion flux, disrupting membranes, or enhancing the activity of other virulence effectors thus intensifying disease severity under certain conditions.

Recent metabolomic profiling by Witte et al. (2024) expanded our understanding of *F. avenaceum* pathogenicity by identifying several novel metabolites, including fungal decalin-containing diterpenoid pyrones (FDDPs), fusaoctaxins, sambutoxin, and fusahexin, some newly associated with this species. Pathogenicity assays on two pea cultivars demonstrated that certain compounds contributed to disease development, reinforcing the view that *F. avenaceum* utilizes a complex network of virulence factors rather than a single determinant.

1.3 MANAGEMENT OPTIONS FOR ROOT ROT

Given the complex and persistent nature of soilborne pathogens contributing to the pea RRC, particularly under favorable environmental conditions, the development of effective, integrated management strategies is critical for minimizing disease impact and sustaining pea production. At present, there are no fully effective chemical control options or resistant cultivars available for pea RRC; instead, only tolerant or partially resistant germplasm lines have been identified in multiple studies (Conner et al., 2013; Gossen et al., 2016; Hamon et al., 2013; C. Lavaud et al., 2015; Willsey et al., 2018). There have been extensive studies on managing ARR, but very few studies on management of Fusarium root rot, and thus this section will review relevant studies on ARR management.

The observations that the disease response to varying inoculum doses of *Aphanomyces euteiches* is influenced by soil type and pathogen co-occurrence, suggest that the disease requires site-specific disease management approaches, exacerbating the challenges in managing pea RRC (Chatterton et al., 2023). Heavy clay or silty soils, pH between 6.5-7.5, temperature from 20-25°C, and infected pea residues present an ideal environment for *A. euteiches* survival and inoculum maintenance in the form of oospore. Besides soil characteristics (Oyarzun et al., 1997), its saturation or waterlogged condition, pH and temperature greatly determine the disease severity. Also, the changes in soil nitrogen content through nitrogen fertilization affected both root rot severity and overall crop performance in peas and lentils (Hubbard, 2022).

The success of crop rotation in managing ARR largely depends on the duration of the rotation and the selection of non-host crops, as oospores of *A. euteiches* can persist in soil for up to 8-10 years (Hossain et al., 2012). Alternative hosts including lentil, alfalfa, and many wild species tend to reduce the effectiveness. Rotations with crops from family Poaceae such as maize

(*Zea mays* L.), oat (*Avena sativa*) and rye (*Secale cereal* L.) (Fritz et al., 1995; Tu & Findlay, 1986) and from family Brassicaceae such as mustard (*Brassica nigra* L.), turnip (*Brassica rapa* L.), cabbage (*Brassica oleracea*) and rapeseed (*Brassica napus*) (Chan & Close, 1987; Papavizas & Ayers, 1974; Tu & Findlay, 1986) are reported to reduce the severity of ARR. However, recent field trials in Alberta and Saskatchewan did not confirm these benefits (Chatterton, personal communication). Nonetheless, non-host cropping systems for 6-8 years are considered effective in the management of ARR. Such a lengthy regime of crop rotation is practically challenging and often discouraging for the growers.

Various treatments and agronomic practices have been explored to mitigate the effect of ARR. The use of undecomposed oat shoot residues as a soil cover has shown promise, increasing pea yield by 48% compared to residue-free treatments in *A. euteiches*-infested fields (Fritz et al., 1995). Similarly, green manures derived from non-host crops have been associated with reduced root rot incidence in peas (Williams-Woodward et al., 1997). However, it remains unclear whether the beneficial effects of non-host residues are due to improved soil nutrition or their inhibitory properties. Evidence suggests that certain non-hosts, such as brassicas, may suppress pathogen development through the release of bioactive compounds like isothiocyanates, which inhibited *A. euteiches* hyphal growth (Hossain et al., 2014). In some studies, higher free calcium concentration of soil or application of spent lime were linked to reduced ARR severity (Heyman et al., 2007; Hossain et al., 2012; Williams-Woodward et al., 1997). Nevertheless, field trials in Canada and the United States reported inconsistent outcomes, with lime or gypsum amendments failing to reliably improve yield or reduce disease severity (Hubbard, 2022). In contrast, another study demonstrated that precipitated calcium carbonate (PCC) significantly reduced conidial production, germination, and mycelial growth of six *Fusarium* species in vitro, and effectively

decreased root rot severity caused by *F. avenaceum* and *F. solani* in greenhouse and field trials, indicating its potential as a management strategy for Fusarium root rot in field pea (Chittem et al., 2016). However, a lack of association between soil calcium or pH and root rot severity levels observed in other studies (Ali et al., 2025; Hubbard, 2022; Karppinen et al., 2020), indicated that soil type and other geographic factors may influence the role of calcium in disease development. Despite the range of management strategies explored, none have consistently reduced *A. euteiches* infections below economically damaging thresholds under field conditions.

In Canada, a limited number of fungicides are registered for seed treatment targeting *A. euteiches*. INTEGO™ Solo (active ingredient: ethaboxam) offers early-stage protection against ARR, although its efficacy is largely restricted to the seedling phase (Wu et al., 2019). Ethaboxam-based formulations often include additional active ingredients such as fludioxonil and metalaxyl, broadening the target spectrum to include *Fusarium*, *Rhizoctonia*, *Ascochyta* spp., *Pythium*, *Phytophthora*, and *Aphanomyces* spp (Syngenta, Guelph ON, CA). However, field trials revealed no significant reduction in root rot severity following ethaboxam seed treatment (Willsey et al., 2021). Apron Maxx RTA (Fludioxonil, Metalaxyl-M and S-isomer, Syngenta, Guelph ON, CA) has shown effectiveness against seedborne *Fusarium* species and *R. solani* in faba bean (Chang et al., 2014) and pea (Franck et al., 2024), yet field applications did not reduce overall root rot severity, highlighting its limited effectiveness against later onset disease. Zeltera Pulse (Nufarm, AB, CA, metalaxyl, inpyrfluxam, mandestrobin, and ethaboxam) was recently registered for seed treatment targeted for all fungal and oomycete pathogen involved in the RRC, however disease and yield protection efficiency until the end of the season is not clearly known. Rancona Trio (UPL NA Ince, ON, CA, ipconazole, carbathiin, metalaxyl) is another seed treatment registered for pulses but is more effective at early-season disease suppression than

providing long-term efficacy over the growing season (Chatterton, personal communication). In green beans (*Phaseolus vulgaris*), hymexazol and captan seed treatment or hymexazol, propamocarb, and azoxystrobin soil drench reduced ARR (Watson et al., 2013). In the United States, sugar beet seed treatment by hymexazol is used to manage *Aphanomyces cochlioides* (Harveson et al., 2007) and although its effectiveness in reducing severity of ARR on pea was demonstrated in field trials in the Canadian prairies, it is currently not registered for use on pulse crops (Chatterton, personal communication). Pea seed treatment with Fosetyl-Al (aluminum tris) in a greenhouse trial effectively suppressed early stage symptoms of ARR but had less effect in later stages (Bruin, 1983; Oyarzun et al., 1990). Collectively, these results indicate there are no efficient chemical agents to fully control ARR and demand further research to identify effective management options.

Besides chemical control agents, research into biological control has identified several promising antagonistic microbes, including *Pseudomonas spp.*, *Bacillus mycoides*, *Streptomyces spp.*, *Rhizobium spp.*, *Pantoea agglomerans*, *Lysobacter capsica*, and *Burkholderia cepacia*, that had inhibitory effects on *A. euteiches* through mechanisms such as suppression of mycelial growth and zoospore germination (Heungens & Parke, 2000; Slezack et al., 2000). Spore-forming bacteria and arbuscular mycorrhizal (AM) fungi used as seed coating significantly suppressed ARR development in field conditions (Wakelin et al., 2002). Seed or granular treatment with spore-forming bacteria *Bacillus mycoides* significantly increased plot stand and yield. The AM fungi *Glomus intraradices* and *G. claroideum* induced tolerance of pea against ARR (Thygesen et al., 2004). Biocontrol agents, when used in combination with the fungicides, showed effective results compared to either of them. Fungal and bacterial species, such as *Clonostachys rosea*, *Pseudomonas fluorescens*, and *Burkholderia cepacia* (Palleroni and

Holmes), in combination with a fungicide increased seedling emergence compared to treatment with fungicide only (Parke et al., 1991; Wicker et al., 2003; A. G. Xue, 2003). Seed treatment using *C. rosea* strain ACM941 and fungicide Thiram 75 WP (thiram) or Apron FL (metalaxyl) improved the germination of pea in *A. euteiches* infested fields (A. G. Xue, 2003). More recently, a study demonstrated *Trichoderma longibrachiatum (TL)* treatment of seeds inhibited the growth of *F. solani* and *F. avenaceum* via mycoparasite activity and enhanced plant growth in snow pea (Boakye et al., 2022). Another study demonstrated that arbuscular mycorrhiza, *Trichoderma harzianum*, *Pseudomonas fluorescens* treatment provided protection against root rot caused by *Fusarium oxysporum* f. sp. *pisi* and increased plant growth and yield in pea plants (El-Sharkawy et al., 2021). However, the implementation of biological control in large-scale field operations remains limited. Despite these findings, no biological control products have yet proven fully effective under field conditions. Therefore, the most widely used management approaches for ARR include crop rotation and the evaluation of initial disease inoculum levels in the field prior to seeding, to inform crop selection.

Evaluation of other agronomic methods have also yielded limited responses. Isothiocyanate (a compound from Brassicaceae plants) showed a potential effect against ARR under controlled conditions (Hossain et al., 2014). In temperate regions, soil solarization in combination with green manure crops or biocontrol organisms was also reported to be effective in controlling ARR (Katan & DeVay, 1991; Ramirez-Villapudua & Munnecke, 1988). Despite many studies on agronomic methods to manage ARR, none has been effective in field conditions. Currently, an integrated approach including agronomic practices, such as early seeding date, crop rotation, use of seed treatment products that provide early-season suppression of *A. euteiches* and other active ingredients that target the other members of the RRC, and

cultivar selection combined with soil monitoring for pathogen load is the only approach to reduce yield losses caused by pea root rot. The development of cultivars with partial resistance will be an integral component of a robust disease management strategy, which will require a thorough understanding of defense responses to the predominant pathogens in the pea root rot.

1.4 PLANT DEFENSE RESPONSES

Plant defense to pathogens is a complex system including different passive and active defense mechanisms against a varied range of pathogens. Passive defense includes physical and chemical barriers such as cell wall and secondary metabolites that prevent pathogen entry and infection (Ding *et al.*, 2011). Active defense includes biochemical, molecular, and morphological changes such as pathogen induced cell wall thickening, oxidative burst, programmed cell death and expression of Pathogenesis related (PR) proteins (Ding et al., 2011, Wang *et al.*, 2019b). Plant defense is also categorized as basal, physical, biochemical, or molecular defense. For example, basal defense is a barrier created by root cell wall structural proteins, physical defense responses are agglutination and cross linking, biochemical defense response is pathogen genetic material degradation, and molecular defense response is an activation of pathogenesis related gene expression (Rashid, 2016). Roots can perceive a pathogen's presence and induce the immune system in a Pattern triggered immunity (PTI) response. PTI involves molecular events such as callose deposition (Millet *et al.*, 2010), transcriptional reprogramming (Boller & Felix, 2009), production of Reactive Oxygen Specites (ROS) and modified extensin distribution within the cell wall (Plancot *et al.*, 2013, Ribeiro *et al.*, 2006, Wojtasik *et al.*, 2011). These mechanisms in plant pathogen interaction are controlled by hormonal signaling pathways (Robert-Seilaniantz *et al.*, 2011, Shigenaga & Argueso, 2016).

Plants can recognize exogenous compounds from microorganisms that activate adapted immune responses in the hosts (Raaymakers & Van den Ackerveken, 2016, Boller & Felix, 2009, Latge, 2010). These compounds are called elicitors or microbe/pathogen-associated molecular patterns (MAMPs/PAMPS), which are recognized by pattern recognition receptors (PRRs) in the host (Kamoun, 2003, Boller & Felix, 2009). Cell wall polymers of pathogens, such as β-1,3;1,6-glucans in oomycetes, which are sources of oligoglucosides, act as PAMPs for plant surveillance mechanisms (Boller & Felix, 2009, Silipo *et al.*, 2010, Shibuya & Minami, 2001). PRR mediates recognition of PAMPs by triggering Ca^{2+} fluxes as signaling cascades (known as spiking) and mitogen-activated protein phosphorylation which further activates an array of plant defense responses (Attard *et al.*, 2008). Oligogalacturonides are produced by partial degradation of plant cell wall components, like pectin, by pathogen cell wall degrading enzymes. With a similar function to PAMPs, oligogalactutonides are termed damage-associated molecular patterns (DAMPS) as they also elicit defense responses such as the accumulation of ROS and PR proteins to protect plants from damage (Ferrari *et al.*, 2013). The production of ROS upon sensing of PAMPs or DAMPs is considered one of the earliest events of plant defense response and is implicated in initiating the hypersensitive response (HR) or programmed cell death (Nimchuk *et al.*, 2003). The hypersensitive response (HR) often involves programmed cell death, production of lytic enzymes, PR-proteins, and reinforcement of the cell wall to restrict pathogen spread.

1.4.1 DEFENSE RESPONSES TO *APHANOMYCES EUTEICHES*

A. euteiches infection triggers several defense responses in pea and related legumes, some resembling classical pathogen recognition processes and others deviating from typical models. Pathogen recognition (PRRs recognizing PAMPs/DAMPs) and early signaling events play a

central role in pea and related legume's responses to *Aphanomyces euteiches*. In ARR infection, oligogalacturonides act as endogenous elicitors that the plant interprets like PAMPs, amplifying immune responses against *A. euteiches*. Consistent with this, pea plants pretreated with oligogalacturonides in controlled conditions displayed significantly reduced ARR severity with high and stable protection efficiency of more than 45% compared to the control (Selim *et al.*, 2017). While there is currently no consistent or conclusive evidence of a classical HR in pea-*Aphanomyces euteiches* interactions, transcriptomic and biochemical studies have shown rapid accumulation of ROS and upregulation of ROS-associated defense pathways in pea roots upon *A. euteiches* detection (Kälin et al., 2024; Selim et al., 2017). In both pea and the model legume *Medicago truncatula*, early ROS bursts are considered key components of basal defense, often associated with the initiation of localized cell death to limit pathogen colonization.

In *A. euteiches*, chito-oligosaccharides, which are linked covalently with β-1,6-glucans and are essential for cell wall integrity, trigger nuclear-associated calcium fluxes independent of other signal transduction cascades in the host plant (Nars et al., 2013). Calcium influx into the cytoplasm and ROS generation are often coordinated, with ROS production being directly linked to calcium signaling pathways. Reactive oxygen species (ROS) are activated either directly after *Aphanomyces euteiches* infection through the perception of pathogen-derived elicitors such as cell wall associated glucan chitosaccharide fractions (Trapphoff *et al.*, 2009, Nars et al., 2013) or indirectly through infection-induced metabolic disturbances, including a decline in root nitrate content (Thalineau *et al.*, 2016).

The regulation of ROS production involves multiple host factors. In *M. truncatula*, the production of ROS is regulated by a small GTPase, MtROP9 (Kiirika et al., 2012). The knockdown of GTPase prevented the detection of nonexpressor of PR1 (NPR1) protein and

respiratory burst oxidase homologs (RBOH) resulting in reduced activity of primary antioxidative enzymes and defense compounds, which promoted colonization of *A. euteiches* (Kiirika *et al.*, 2012, Kiirika *et al.*, 2014).

In addition to signaling, structural reinforcement contributes to partial resistance. A defense response to *A. euteiches* infection in a partially resistant *M. truncatula* line A17 involved the accumulation of soluble phenolic compounds, enhanced pericycle cell divisions that formed additional cell layers, lignin deposition around the pericycle, and increased production of lateral roots, collectively reinforcing protection of the root stele (Djebali *et al.*, 2009). The strengthening of the cell wall was linked with reduced *A. euteiches* invasion in the vascular regions suggesting this may be a contributing factor to the partial resistance of the A17 line. The increased accumulation of lignin observed in resistant lines was associated with H_2O_2 scavenging mechanisms (Djébali *et al.*, 2011), allowing quicker polymerization of lignin around the central stele.

These structural defenses are genetically linked to major resistance loci. Two alleles of the major QTL AER1/prAe1 were associated with resistance in *M. truncatula*: a dominant allele conferred strong resistance in the line DZA045.5, while a recessive allele was linked to partial resistance in A17 (Djebali et al., 2009, Pilet-Nayel *et al.*, 2009b, Hamon *et al.*, 2010). Mapping studies further showed that AER1 conveys broad-spectrum resistance in both pea and alfalfa, either alone or in combination with other loci (Bonhomme et al., 2019). However, functional validation of AER1/prAe1 and its candidate genes in pea and *M. truncatula* remains outstanding.

Beyond structural defenses and major QTLs, additional mechanisms implicated in responses to *A. euteiches* include hormone-responsive genes, PR protein regulation, and genetic loci distributed across multiple chromosomes. Among the genes whose expression was altered

by *A. euteiches* infection in *M. truncatula*, genes encoding abscisic acid (ABA)-responsive proteins were over-represented suggesting there are similarities between drought and *A. euteiches* stress responses (Jacquet & Bonhomme, 2019). Although PR10 in the PR protein family showed strong induction after infection, its expression was not positively associated with resistance; instead, it appeared to correlate with *A. euteiches* growth within the plant, indicating a possible negative relationship with host resistance (Colditz *et al.*, 2005, Jacquet & Bonhomme, 2019). RNAi-mediated suppression of the PR10 gene in *M. truncatula* led to a compensatory induction of PR5 genes and was associated with reduced *A. euteiches* colonization. (Colditz et al., 2007). In addition to such gene-level responses, Hamon et al. (2010) identified 12 additive and 12 epistatic genetic factors for resistance distributed across eight chromosomes. Among these, Bonhomme et al. (2014) reported that resistance was linked to mutations in the interaction domain of an F-box protein (*MtAHL1*), a component of the ubiquitin–proteasome pathway. These mutations illustrate how regulatory proteins involved in protein turnover can contribute to the additive and epistatic genetic effects underlying *A. euteiches* resistance in *M. truncatula*.

Plant nutrition status, secondary metabolites, and hormones play a role in defense against *A. euteiches.* A recent study in *M. truncatula* resistance to *A. euteiches* revealed that nitrogen deficiency leads to reduced or enhanced susceptibility depending upon plant genotype. (Thalineau *et al.*, 2018). They further discovered the role of amino acid glutamine as an important nitrogen source for *A. euteiches* infection. Transcript profiling of *M. truncatula* seedlings and adult plant roots infected with *A. euteiches* identified upregulation of a gene encoding terpene synthase 10 (*MtTPS10*) (Yadav *et al.*, 2019). *MtTPS10* produces sesquiterpenes in the roots of *M. truncatula* which could be a part of plant defense against *A. euteiches*. The

heterologous expression of *MtTPS10* and production of sesquiterpenes in yeast inhibited zoospore germination and mycelial growth of *A. euteiches* (Yadav et al., 2019).

Secondary metabolites are another group of compounds playing an important role in defense against biotic stress in plants. A strong induction of phenolic compounds in root tissues during *A. euteiches* infection is likely part of resistance mechanisms (Jacquet & Bonhomme, 2019). The classic honey-brown coloration associated with *A. euteiches* infection is thought to result from the oxidation of phenolic compounds (Djebali et al., 2009, Djébali et al., 2011) and resistant *M. truncatula* lines often exhibit distinct root coloration patterns. Correspondingly, rapid production of phenolic compounds was detected in *A. euteiches* infected *M. truncatula* roots of lines A17 and DZA45.5 (Djebali et al., 2009). Oxidation of phenolics followed by root color changes has also been observed after infection with other oomycetes, such as *Bremia lactucae* in lettuce (*Lactuca sativa*) (Lebeda *et al.*, 2008). Strong induction of genes associated with the isoflavonoid biosynthesis pathway leading to the production of the phytoalexin, medicarpine, was revealed by transcriptome and proteome analyses (Colditz et al., 2005, Rey *et al.*, 2013). A large set of genes involved in flavonoid pathways were induced in *A. euteiches* infected *M. truncatula* plants indicating their role in response to the pathogen (Rey et al., 2013, Badis *et al.*, 2015). Studies comparing transcriptome data and biochemical analysis in resistant and susceptible lines revealed a higher accumulation of flavonoid compounds in resistant compared to susceptible lines of *M. truncatula* (Jacquet & Bonhomme, 2020). The proteome analysis of *A. euteiches* infected roots and cells of *M. truncatula* detected chalcone synthase or isomerase and isoflavone reductase (Colditz et al., 2005, Schenkluhn *et al.*, 2010, Trapphoff et al., 2009). Similarly, strong expression of many chalcone O-methyltransferases was detected in *A. euteiches* infected resistant lines of *M. truncatula* (Badis et al., 2015). Among them, a gene

encoding the enzyme, isoliquiritigenin 2′-O-methyltransferase, was highly induced and the enzyme was detected in proteome analysis of *M. truncatula* roots infected with *A. euteiches* (Colditz *et al.*, 2004, Jacquet & Bonhomme, 2019). The product 2′-O-methyl isoliquiritigenin, catalyzed by the enzyme, substantially impeded *A. euteiches* development and zoospore germination compared to pisatin, a major pea phytoalexin (Badis et al., 2015). The polyphenolic organic acids such as coumaric acid, vanillic acid, 4-aminosalicylic acid, 4-hydroxybenzoic acid, and 3,4-dihydroxybenzoic acid effectively suppressed the hyphal growth of *A. euteiches* in lentil (Bazghaleh *et al.*, 2018).

The *A. euteiches* and *M. truncatula* interaction has been well studied using genomic and bioinformatic approaches (Gaulin et al., 2008, Madoui *et al.*, 2007), including functional analysis of *A. euteiches* genes involved in metabolic pathways (Madoui *et al.*, 2009) and encoding effectors (Ramirez-Garcés *et al.*, 2016). The complete genome sequence of *A. euteiches* has been released which can help us understand more about the *A. euteiches*-pea pathosystem (Jacquet & Bonhomme, 2019, Gaulin *et al.*, 2018). Unlike other oomycetes, the *A. euteiches* genome contains a large repertoire of small secreted protein (SSP)-encoding genes that are highly expressed during plant-pathogen interaction (Gaulin, 2018). Gaulin (2018) have further identified an SSP which enters the plant nucleus to increase plant susceptibility to infection.

Little is known about the interaction that occurs within the *A. euteiches*-pea pathosystem. Pea resistance to *A. euteiches* is governed by polygenic inheritance and is expressed as a quantitative trait (Desgroux et al., 2016). Several QTL associated with partial resistance to ARR have been identified and validated (Desgroux et al., 2016, Pilet-Nayel at al., 2013). These QTL facilitate the development of pea varieties with improved resistance to ARR through breeding programs that incorporate marker-assisted selection (MAS) (Hamon *et al.*, 2011, Hamon et al.,

2013, Lavaud *et al.*, 2016, Lavaud et al., 2015b, Desgroux *et al.*, 2016, Pilet-Nayel *et al.*, 2002, Pilet-Nayel *et al.*, 2005). A recent study identified a minor QTL contributing to partial resistance in greenhouse and field experiments, and analyzed the gene content within this QTL in progeny of a cross between susceptible and resistant pea lines (Wu *et al.*, 2022, Wu *et al.*, 2021). Genes associated with ARR resistance were further analyzed using bulked segregant RNA-seq (BSR-seq) and cross-referenced with differentially expressed genes (Wu *et al.*, 2022). Over 2,300 genes were differentially expressed between resistant and susceptible lines, many of which were linked to plant defense pathways like jasmonate, ethylene, and salicylate signaling. Additionally, SNPs in 31 candidate genes were detected, offering valuable markers for breeding resistant pea cultivars. Kälin et al. (2024) conducted a transcriptomic analysis to explore the immune responses of two pea genotypes 'Linnea' (susceptible) and 'PI180693' (partially resistant) when challenged with two strains of *A. euteiches* differing in virulence at germinated seed stage (4 days after seeding). The research revealed that the plants' defense mechanisms were both time- and genotype-dependent, involving the activation of WRKY and MYB-like transcription factors, as well as genes associated with JA and ABA signaling pathways. Kälin et al. (2024) further identified a polymorphic gene, Psat7g091800.1, encoding a leucine-rich repeat receptor-like kinase similar to the Arabidopsis FLAGELLIN-SENSITIVE 2 receptor. Polymorphisms distinguishing resistant from susceptible pea cultivars suggest that allelic variation in this gene may influence pathogen recognition and thereby contribute to enhanced disease resistance. Goyal et al. (2024) explored the contribution of root exudates to pea resistance against *A. euteiches.* They observed that exudates from partially resistant lines (PI660736 and PI557500) significantly suppressed oospore germination compared to those from susceptible cultivars (CDC Meadow and AAC Chrome). Metabolomic profiling showed that resistant lines produced higher levels of

saponins, especially soyasaponin I and dehydrosoyasaponin I, which were found to strongly inhibit *A. euteiches* development, indicating a potential role of these compounds in mediating partial resistance. In another study, pea plants infected with *A. euteiches* along with other pea pathogens in field conditions had higher saponin content in grains of two pea cultivars Crécerelle and Firenza (Oliete et al., 2022).

1.4.2 PLANT DEFENSE RESPONSES TO *FUSARIUM AVENACEUM*

Fusarium spp. can invade plants by penetrating natural openings at the junction of primary and lateral roots, through mechanical wounds, and through actively growing tissues in the hypocotyl and epicotyl regions (Wang et al., 2024). *Fusarium* pathogen lifestyle plasticity complicates the characterization of host immune responses. Notably, *F. avenaceum* exhibits a biphasic infection strategy that includes both biotrophic and necrotrophic stages (Berrocal-Lobo & Molina, 2008; Kang et al., 2005). This dynamic transition between trophic phases enables the pathogen to subvert and evade host defenses, as plants typically activate distinct signaling pathways depending on whether the pathogen behaves as a biotroph or necrotroph.

While studies have begun to uncover the diversity of mycotoxins and virulence factors associated with *F. avenaceum*, the corresponding defense mechanisms in pea remain poorly understood. A recent phenotypic screening of 20 pea accessions with pigmented traits (varying flower and seed coat pigmentation) (e.g., CDC Acer, CDC Vienna, PBA Oura, Morgan, CDC Blazer, CDC Dakota, PI 280609) exhibited resistance or partial resistance, with minimal reductions in growth metrics under infection (Awodele et al., 2024). Most non-pigmented accessions were highly susceptible, though Cameor showed partial resistance at lower inoculum levels. Similar variation in susceptibility was observed in lentil accessions (Heineck et al., 2022) where root rot severity was negatively correlated with biomass. Further, genome-wide

association mapping study in lentil identified 11 QTLs across four chromosomes, including loci near orthologous genes linked to disease resistance, suggesting a genetic basis for variation in FRR resistance (Heineck et al., 2022) .

Root surface immune perception also plays a role in plant defense. Evidence from other species suggests that root surface cells are equipped with pattern recognition receptors (PRRs) capable of detecting PAMPs and DAMPs, thereby activating defense signaling. These receptors are often expressed at key pathogen entry points, such as the vascular cylinder of emerging lateral roots (Beck *et al.*, 2014, Chuberre *et al.*, 2018). In *Arabidopsis* and flax, border-like cells (BLCs) exposed to fungal elicitors, including chitin, fusaric acid, and *Fusarium oxysporum* mycelial extracts, exhibited a strong oxidative burst (Plancot et al., 2013), indicating early immune activation at the root-soil interface. In pea, border cells have also been implicated in pathogen defense, particularly through the formation of extracellular traps and secretion of antimicrobial compounds (Gunawardena *et al.*, 2005, Gunawardena & Hawes, 2002). However, the role of BLCs in *F. avenaceum* recognition and defense remains largely unexplored. Given that PAMP perception is known to vary with elicitor type (Millet et al., 2010, Plancot et al., 2013), it is essential to investigate how different components of *F. avenaceum*, such as fusaric acid or cell wall-derived signals, are perceived by pea roots, particularly at the border cell layer. Understanding this interface could provide key insights into early detection mechanisms that contribute to partial resistance in pea.

1.5 TOOLS TO DECODE RESISTANCE

Advances in next-generation sequencing (NGS) and omics technologies have transformed plant science, providing unprecedented insights into genome structure, gene

expression, and defense mechanisms, and enabling high-resolution genetic analysis in a wide range of crop species. The sequencing of the *Arabidopsis thaliana* genome using Sanger sequencing (The Arabidopsis Genome Initiative *et al.*, 2000) laid the foundation for modern genomics research. Among these, RNA sequencing (RNA-seq) has become a particularly powerful tool, offering greater accuracy and sensitivity than earlier methods such as microarrays (Lister et al., 2008, Alba *et al.*, 2004). RNA-seq enables transcriptome profiling without prior genome sequence knowledge, making it applicable to non-model crops. This technology has been widely adopted to dissect plant-pathogen interactions and identify defense-related pathways. For example, RNA-seq of wild and cultivated rice challenged with *Magnaporthe oryzae* revealed both common and genotype-specific defense responses, including activation of lipid metabolism, jasmonic acid, and phenylpropanoid pathways (Tian et al., 2018).

Whole-genome sequencing (WGS) complements transcriptomic data by providing reference frameworks for read alignment. In crops such as rice (Yuan et al., 2005; Yu et al., 2002; Goff et al., 2002), *Medicago truncatula* (Town, 2006), and pea (Boutet et al., 2016; Tayeh et al., 2015; Kreplak et al., 2019), genomic and transcriptomic resources have enabled the discovery of resistance mechanisms and genotype-specific responses to pathogens. Additionally, in pea, several QTL regions have been identified that contribute to partial resistance to *Aphanomyces euteiches* (Hamon et al., 2013; Desgroux et al., 2016; Leprevost et al., 2023). These QTLs provide valuable entry points for deeper investigation into the defense responses against major PRRC. Altogether, strategies such as integrating known QTLs with transcriptomic analysis may provide a powerful strategy to correlate genetic variation with defense gene expression, offering insights into the molecular basis of resistance.

1.6 THESIS OBJECTIVES

The root rot complex (RRC) of field pea, primarily caused by combination of oomycete and fungal pathogen complex, presents a significant and persistent challenge in temperate cropping systems. The persistent soilborne nature of these pathogens, coupled with overlapping host ranges, synergistic interactions, and a lack of fully resistant cultivars, complicates disease management. This chapter provided a comprehensive overview of the RRC in pea with particular focus on the key pathogens *Aphanomyces euteiches* and *Fusarium avenaceum*, which cause significant yield losses in Western Canada. Although several studies have dissected many aspects of host resistance and pathogen virulence, the molecular basis of partial resistance in pea to PRRC pathogens remains poorly characterized. This literature reviewed underscored the complexity of PRRC and the necessity of an integrated approach combining pathology, genomics, and molecular biology to resolve this challenge.

This thesis aimed to dissect the plant-pathogen interactions between field pea and the two major contributors to RRC, *A. euteiches* and *F. avenaceum,* using a multi-tiered approach combining phenotyping, temporal disease progression, and transcriptomic analyses.

Objectives:

1. Identification of partially resistant and susceptible pea lines to each of *A. euteiches* and *F. avenaceum* pathogens in greenhouse trials.
2. Developing *F. avenaceum* inoculation methods for consistent development of pea root rot under greenhouse conditions.
3. Temporal analysis of *Aphanomyces* root rot development after zoospore inoculation and *F. avenaceum* root rot development after conidia inoculation in field pea.

4. Assessment, through RNA-seq, of transcriptome response in partially resistant and susceptible pea lines to *A. euteiches* inoculation.
5. Assessment, through RNA-seq, of transcriptome response in partially resistant and susceptible pea lines to *F. avenaceum* inoculation.

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CHAPTER 2 : ASSESSMENT OF *FUSARIUM AVENACEUM* INOCULATION METHODS FOR CONSISTENT DEVELOPMENT OF PEA ROOT ROT UNDER GREENHOUSE CONDITIONS

This chapter has been published in the Canadian Journal of Plant Pathology. Inevitably, there might be some repetition of content between chapter 1 and introduction section of this chapter.

Pandit, S., Biscaglia-Horvath, K., Vucurevich, C., Amundsen, E., & Chatterton, S. (2025). Assessment of *Fusarium Avenaceum* Inoculation Methods for Consistent Development of Pea Root Rot under Greenhouse Conditions. *Canadian Journal of Plant Pathology*, 1-15. https://doi.org/10.1080/07060661.2025.2537831

ABSTRACT

Fusarium avenaceum (Fave) is the most dominant pathogen of the pea root rot complex in the Canadian prairies. Its increasing prevalence and broad host range present significant challenges, underscoring the urgent need for research to develop effective management strategies, including breeding for resistance. A critical aspect of efficient pathogen studies and screening pea lines for resistance under controlled conditions is selecting an appropriate inoculation method. In previous experiments, we observed that classical *Fave* inoculation methods such as seed inoculation either lacked uniform infection or killed plants before emergence. Therefore, we tested different *Fave* conidia inoculation methods to assess disease development in various pea lines after *Fave* exposure and refined these methods to develop an optimized indoor screening protocol for *Fave*. Among the tested methods, soil inoculation with *Fave* conidia resulted in the most consistent and uniform disease symptoms across repeated experiments. This method could be most effective for germplasm screening for partial resistance. A modified seed inoculation method also resulted in moderate disease levels making it the most effective for testing the virulence of different isolates of *Fave* on the same genotype. Root soaking resulted in uniform but low disease severity and can be used for studying host-pathogen interactions where observation during the infection process is important.

Keywords: Fusarium root rot, inoculation methods, soil inoculation, germplasm screening

2.1 INTRODUCTION

Field pea (*Pisum sativum* L.) is an important crop within the legume family species providing nutritional and soil health values. It is included in crop rotation to produce high-value seeds for whole food nutrition and plant-based protein ingredients, to manage disease/pests of non-legume crops, to enrich biodiversity within plant communities, and to supply a nitrogen benefit to subsequent crops (Lake et al. 2021). Canada is a leading field pea producer and exporter yielding 2.6 million tonnes (Mt) and exporting 2.4Mt in 2024 (Agriculture and Agri-food Canada, 2025). Pea root rot is one of the most devastating diseases of field peas worldwide caused by a complex of various fungal and oomycete pathogens including *Fusarium* spp., *Aphanomyces euteiches, Pythium* spp. and *Rhizoctonia solani*. The major root rot-causing agents in legumes such as pea and lentil are *Fusarium* species, of which *Fusarium avenaceum* has become increasingly important (Bodah et al. 2017). *Fusarium avenaceum* was the dominant pathogen in the pea root rot complex (PRRC) in the Canadian prairies, accounting for up to 80% of the pathogens isolated from diseased roots from commercial pea crops (Esmaeili Taheri et al. 2017; Chatterton et al. 2019; Safarieskandari et al. 2021). It was also among the most aggressive *Fusarium* species isolated from pea and the dominant pathogen in North Dakota and Montana pea fields (Willsey et al. 2018; Chatterton et al. 2019; Moparthi et al. 2021; Safarieskandari et al. 2021).

F. avenaceum is a generalist pathogen distributed worldwide with a wide host range including more than 80 genera of plant communities (Lysoe et al. 2014). This large host range includes many important crops and *F. avenaceum* is one of the major pathogens in cereal crops such as wheat and barley where it contributes to crown rot and is associated with Fusarium head

blight (Tyburski et al. 2014; Moparthi et al. 2021), oilseed crops such as canola where it causes root rot (Chen et al. 2014) and pulse crops including pea, lentil, and chickpea (Safarieskandari et al. 2021). Fusarium root rot (FRR) symptoms on pulse crops include initial reddish-brown streaks on primary and secondary roots that eventually coalesce to form dark/black discoloration of roots that extends to the epicotyl region. FRR is usually found in conjunction with other root rots, e.g. Aphanomyces root rot, that comprise the PRRC. FRR restricts the transport of nutrients and water, reducing plant foliar growth and uniformity during crop maturity. There are no efficient control measures nor any available resistant cultivars for FRR in field pea.

Efficient inoculation methods are essential for studies on epidemiology, etiology, disease resistance, host-pathogen interaction and disease control strategies (Das et al. 2015). In pea, seed soak inoculation before planting is one of the popular and standard methods for evaluating *F. avenaceum* infection (Willsey et al. 2019; Awodele et al. 2024). In other crop-pathogen systems, specialized inoculation techniques are employed to reflect the biology of infection. For example, point inoculation in wheat is a well-established method for delivering precise, localized inoculum in studies of Fusarium head blight (Eranthodi et al. 2020). Similarly, in root crops such as sugar beet, seed-soaking and root-dipping approaches are commonly used for soilborne pathogens (Lai et al. 2020). Since *F. avenaceum* is a relatively new pathogen described in pea, breeding efforts for disease resistance to this pathogen are also relatively new. Therefore, it is important to develop effective inoculation methods that result in consistent and uniform disease severity across repeated experiments, and provide a moderate level of disease severity to allow germplasm screening for breeding and study of plant-pathogen interaction.

Many inoculation techniques for screening *F. solani* on pea have been published (Porter et al. 2015), but it is unknown if these techniques are transferable to *F. avenaceum*.

Safarieskandari et al. (2021) reported excessive death of pea seeds before germination when the seed soak method commonly used for *F. solani* was implemented for *F. avenaceum*. Therefore, the objectives of this study were to test the efficacy of common *F. solani* inoculation methods with *F. avenaceum* inoculum, particularly to assess response of pea lines to *F. avenaceum* infection, and to refine these methods for an optimized indoor screening protocol. In addition, we conducted field screening in a disease nursery to assess disease severity under natural conditions and compared them to disease severity under greenhouse conditions.

2.2 MATERIALS AND METHODS

2.2.1 Fungal isolates and inoculum preparation

Fusarium avenaceum pea isolate Fa 1306.08 was used for all greenhouse experiments except for the inoculum concentration experiment in which two other wheat isolates (Fa DAOM 2420768 and Fa DAOM 242378) were used. Fa 1306.08 was obtained from field pea surveys in Alberta and was determined to be an aggressive isolate (Safarieskandari et al. 2021). The wheat isolates were obtained from AAFC's Canadian National Mycological Herbarium. All isolates were stored as a mixture of mycelia and conidia in 15% glycerol stock at -80ºC. To obtain fresh cultures for each experiment, a loopful of the glycerol stock suspension was transferred to potato dextrose agar (PDA) (amended with penicillin and streptomycin antibiotics at 150 mg/l concentrations), and grown at room temperature under ambient light. An actively growing mycelial plug of 5 mm diameter from the outer edge of the PDA plate (7 – 10-day-old cultures) was transferred to a 250 ml glass Erlenmeyer flask containing 100 ml of liquid carboxymethylcellulose (CMC) medium (Cappellini and Peterson 1965). The inoculated flask was then incubated on a shaker at 170-190 rpm at 25º C for 7-10 days until a cloudy suspension with visible chunks of mycelia was obtained. The culture was shaken aggressively to release

macroconidia followed by filtering through 4 layers of cheese cloth to remove mycelia and agar. Macroconidia were collected by centrifugation in a swinging bucket rotor at 4000 rpm for 10 min. The supernatant was poured off and the pellet was washed three times with sterile distilled water (SDW) using centrifugation as specified above. After three washes, the macroconidia were resuspended in SDW, and the concentration was determined using a hemocytometer. Further dilution was performed using SDW to bring the suspension to the required concentration.

2.2.2 Disease severity rating scale

In all the experiments, disease severity was rated on a scale of 1-7, where a rating score of 1 denotes a healthy plant; 2 denotes small reddish brown lesions at the seed attachment point; 3 denotes coalescing of localized root/epicotyl lesions approximately 180° around the tap root; 4 denotes lesions extending and completely encircling the tap root; 5 denotes increasingly discoloured and extended epicotyl lesions; 6 denotes epicotyl lesions encircling the root and extending up to the green stem; and 7 denotes a completely decayed root system (Figure 2.1, Schneider and Kelly 2000; Safarieskandari et al. 2021).

A. Inoculum concentration for seed soak method

Two pea genotypes, 'CDC Meadow' (susceptible) and 'Carman' (partially resistant, R. Conner, personal communication) were selected for this study. To identify the best combination of inoculum, isolate and concentration that cause moderate levels of disease severity, three *F. avenaceum* isolates; 1306.08, Fa DAOM 2420768, and Fa DAOM 242378 at four concentrations (1000, 5000, 10000, and 50000 macroconidia/ml) and a mock-inoculated control were tested. Seeds were surface-disinfested with 10% bleach (0.5% NaOCl) and a drop of Tween-20 by continually shaking at room temperature for 10 minutes followed by 3 rinses with SDW. For each concentration of the inoculum, genotype and growth media, 15 seeds per treatment

combination were soaked overnight (~16 hours) in conidia suspension (Porter et al. 2015). Seeds were then planted in to two growth media, Cornell mix A (1:1 peat moss:vermiculite with fertilizer additives, Boodley and Sheldrake, 1972) or vermiculite, in 2"x 2" SVD pots (32 pots per tray, A.M.A Horticulture, Canada) contained in 0.7 L clear plastic bags to prevent cross-contamination. The vermiculite was sterilized by 2 rounds of autoclaving, 24 h apart, 48 h prior to planting, but the soil mix was not sterilized due to undesired changes in peat moss characteristics after autoclaving. There were three replicate pots per treatment combination, with 5 seeds planted per pot. Plants were grown in the greenhouse with 22/18^{o}C (day/night) and 16 h photoperiod. Pots were watered as needed to maintain moist conditions, and all pots were watered with equal amounts of water. Plants were grown for 28 days, and then roots were washed to remove growth media and assigned a disease severity rating (DSR). Seeds that did not emerge were checked for germination. Seeds that were germinated (viable) but unable to emerge out of the soil/ vermiculite surface due to high FRR severity were rated 7.

B. Screening of pea germplasm lines using two conventional methods

Two methods of inoculation, overnight seed soak and cornmeal layering, were used to screen 14 pea genotypes. The overnight seed soak method in *Fave* conidia suspension and cornmeal layering have been described as standard screening protocols for FRR caused by *F. solani* f. sp. *pisi* (Porter et al. 2015). Pea genotypes were selected that had been reported to have partial resistance to FRR caused by *F. solani* (Bodah et al. 2016). For both inoculation methods, seeds of each genotype were first surface-disinfested as described above. For the seed soak method, seeds were then soaked overnight in 10,000 conidia/ml of *Fave* isolate 1306.08, followed by planting in rootrainers containing autoclaved vermiculite with 2 seeds/cell (32 cell tray, Rootrainer International, Canada) at a depth of 2.5 cm. For the cornmeal layering method of

inoculation, sand meal was prepared using sand, cornmeal and water in a ratio of 9:1:2 and was autoclaved for 45 minutes. After cooling to room temperature, eight – 5 mm agar plugs from actively growing cultures of *Fave* were mixed thoroughly with 60 grams of the sterilized sand meal, and incubated at room temperature for 7 days. The mixture was mixed thoroughly daily with a sterile spatula and immediately prior to layering. Layering was performed in rootrainer cells (32 cells per tray) with a ratio of 2:2:1 of vermiculite, sand meal and vermiculite. For planting, seeds (2 per cell) were placed on top of the sand meal then covered with vermiculite. There were 5 replicates per genotype arranged in a split-plot design, with the inoculation method as the main plot (rootrainer tray), and then genotypes randomized within the cells of the tray. The non-inoculated 'CDC Meadow' control cells were contained in their own tray to prevent cross-contamination. Plants were grown in a standard greenhouse with 22/16^{o}C (day/night) and 16 h photoperiod. Plants were watered as needed to maintain moist conditions. The experiment was performed twice.

C. Evaluation of revised inoculation methods

After determining the optimum inoculation concentration and comparing two inoculation methods in the greenhouse, we further compared four revised inoculation methods in pea genotypes 'CDC Meadow' and 'CDC Dakota'. The inoculation approaches included a revised seed soak method, inoculation at planting time, post-emergence root inoculation and a root soak method. **1) Revised seed soak method:** Surface sterilized pea seeds were soaked in 10,000 conidia/ml for 4 hours followed by planting in rootrainers containing autoclaved vermiculite with 2 seeds/cell. **2) Inoculation at planting time:** Rootrainer cells were filled to three-quarters with vermiculite and 2 seeds per cell were placed. This was followed by pipetting 1 ml of 10,000 *Fave* conidia/ ml suspension onto seeds and covering the seeds with autoclaved vermiculite. After

covering the seeds with vermiculite, another 1 ml of the 10,000 conidia/ml suspension was pipetted on the surface of the vermiculite in each cell. **3) Stem base inoculation:** Surface sterilized pea seedlings were planted in rootrainers with 2 seeds/cell and were allowed to grow for 2 weeks followed by pipetting 5 ml of 10^6 *Fave* conidia/ml at the stem base of each seedling (10 ml total per cell). **4) Root soaking:** Surface sterilized pea seedlings were planted in rootrainers with 2 seeds/cell. Peas were grown for 2 weeks and then the rootrainer books were soaked in 10^6 *Fave* conidia/ml suspension for 1 hr. The *Fave* suspension was contained in a plastic tub deep enough to completely submerge the cells. All four inoculation methods were tested at once in the same experiment, with seeds planted on the same day, and plants harvested and rated at the same time. Plants were grown in the greenhouse under the conditions described above. Disease rating was performed 4 weeks after seeding for all four methods. There were 4 replicate blocks per treatment per genotype, and methods (1-3) applied to the two cultivars were randomized within blocks assigned to rows within the rootrainers. Method #4 (root soaking) was contained within one rootrainer tray since the whole tray was soaked. Non-inoculated controls were included for each treatment method but were also contained in a separate rootrainer to avoid cross-contamination. Non-inoculated controls were excluded from statistical analysis since no disease was observed. The experiment was performed twice.

D. Soil inoculation experiment

After trying various methods, we then tested Cornell soil mix inoculation of *Fave,* as we were not satisfied with the level of disease severity and ability to distinguish between genotypes. Seeds of 22 genotypes were surface disinfested as described above. Genotypes were selected from previous descriptions of their reaction to Fusarium or Aphanomyces root rot (McGee et al. 2012, Table 2.2). A conidia suspension (100,000 conidia/ml) of Fa 1306.08 was prepared and

Cornell soil mix was inoculated with 10,000 conidia/g of dry soil. A disease-free check of the pea cultivars 'CDC Meadow' and 'K2' was included by only adding SDW to the potting mix prior to planting. Inoculated soil was then used to fill 5 cm x 5 cm SVD pots (32 pots per tray, A.M.A Horticulture, Canada) at 250 g per pot. Four seeds were planted per pot at a depth of 2.5 cm with four replications per genotype. Pots were placed in 0.7 L plastic bags to maintain moisture and to avoid cross-contaminating the disease-free checks. The position of the treatment pots (22 genotypes + 2 disease-free checks) from each replicate were randomized within a tray, and one tray was considered a block. Plants were grown in standard greenhouse conditions with 22/16^{o}C (day/night) and 16 h photoperiod and watered moderately. Roots were rated for disease severity using the 1-7 scale, 28 days after planting. A summary describing all four of the inoculation experiments (A-D) is provided in Table 2.1.

E. Germplasm screening in a field disease nursery

Twenty-six pea genotypes in 2020 and nineteen in 2021 (Table 2.2) were evaluated under field conditions to evaluate the resistance level of pea genotypes against a mixed infection. Seven genotypes were not included in the second year due to the unavailability of seeds. The field trial was performed in microplots in established pea root rot field disease nurseries with four replications per genotype. Microplots were used due to the small number of seeds available for each genotype. Eighty-four microplots were constructed by excavating a 26 cm deep by 23 cm diameter size hole at the disease nursery site at the Lethbridge Research and Development Centre using an auger drill attached to a BobCat. PVC irrigation pipe (22.9 cm diameter) cut to 25.4 cm depth was fitted into each hole, and back-filled with the excavated soil. The disease nursery was inoculated with *F. avenaceum* in 2014 (Willsey et al. 2018), and also contained natural populations of *F. solani* which were maintained by growing peas at this site every other

year since 2014. In each microplot, twenty seeds per genotype were hand-seeded at 3.8 cm depth. The site was irrigated with hand-move pipes with a target of 2.5 inches of water applied weekly for 6 weeks from mid-June to late July. In both years, root sampling was done at 6 and 8 weeks post-seeding and each plant was rated for disease severity on a scale of 1-7. An average disease severity rating for each replicate was calculated before performing statistical analysis. In 2020, because many plants were damaged by ground squirrels and replicates were lost, all plants from all replicate microplots were calculated together to determine an average DS for each genotype. Therefore, statistics could not be performed on the 2020 data set.

2.2.3 Data analysis

All the greenhouse and 2021 field disease severity ratings (DSR) were analyzed using the Kruskal-Wallis test (non-parametric one-way ANOVA) in R to examine whether a statistically significant difference occurred between genotypes and treatments that is reflected in median disease severity levels. The average rating of all plants in one pot or one microplot was first calculated and the average value used in the statistical analysis. A significance in Kruskal-Wallis test was followed by Dunnet test using the R package 'FSA'. Because the Kruskal-Wallis test cannot be performed on multi-factorial data, where applicable, analysis was performed separately for each treatment factor (e.g. cultivars, soil type and isolates in experiment A, etc.). Data were combined across repeated trials for analysis if Levene's test for homogeneity of variances was not significant.

2.3 RESULTS

Disease symptoms from select treatments and experiments are depicted in Figure 2.2. Symptoms and the sites of infection in host roots varied between inoculation methods. For example, peas from the modified seed inoculation method and the soil inoculation method

showed localized symptoms mainly on the point of seed attachment and taproot (Figures 2.2A, 2.2D) whereas root soak (Figures 2.2C, 2.2G) and stem base inoculation (Figure 2.2F) caused symptoms on the tap and lateral roots. Pea plants from the field microplots showed blackening of the tap root, along with browning and loss of lateral roots (Figure 2.2E).

A. Inoculum concentration for seed soak method

Two pea genotypes, 'CDC Meadow' (susceptible) and 'Carman' (partially resistant) were tested with three *F. avenaceum* isolates; 1306.08, Fa DAOM 2420768, and Fa DAOM 242378 at four concentrations (1000, 5000, 10000, and 50000 macroconidia/ml) and a mock-inoculated control. All three isolates (Fa 1306.08, Fa DAOM 2420768 and Fa DAOM 242378) for all concentrations in both cultivars, 'CDC Meadow' and 'Carman', resulted in similar levels of disease severity. Plants with control groups (0 conidia/ml) had minimal to no discoloration for all the isolates in both genotypes (Figures 2.3 and 2.4). For the cultivar 'Carman' grown in soil, plants treated with concentrations ≥ 10,000 conidia/ml had higher DSR than in control groups except for isolate Fa DAOM 242378 in which DSR was significantly higher only at 50,000/ml (Figure 2.2). When grown in vermiculite, concentrations ≥ 5000 conidia/ml resulted in significantly higher DSR as compared to the control group (Figure 2.3) for all isolates.
For 'CDC Meadow' grown in soil, concentrations ≥ 5000 conidia/ml resulted in significantly higher DSR as compared to control groups for all isolates (Figure 2.4). When grown in vermiculite, all the concentrations resulted in higher disease severity compared to the control with 10,000 and 50,000 conidia/ml causing the highest and most consistent level of disease (Figure 2.4).

In the case of susceptible cultivar 'CDC Meadow' grown in both soil and vermiculite, an increase in the conidia inoculum concentration resulted in significantly decreased emergence.

However, in ‘Carman’, emergence was higher and was not significantly affected by an increase in conidia concentration (Supplementary Figures 1 and 2). Based on the results from this experiment, we selected 10,000 conidia/ml for the next round of experiments and vermiculite as a growth media, as this seemed to provide more discriminatory power for partial resistant lines. All other greenhouse experiments used this level of inoculum concentration.

B. Screening of pea germplasm lines using two conventional methods

Fourteen pea genotypes were screened for disease symptom severity under two different methods (seed soaking, cornmeal layering) of inoculation. Variances were not equal between the repeated trials, and thus each trial was analyzed separately. In the genotype screening experiment, the non-inoculated ‘CDC Meadow’ control did not show root discoloration symptoms for either soak or cornmeal layering methods (Figure 2.5). For the seed soak method, ‘K2’ was the only genotype that was significantly different from the inoculated ‘CDC Meadow’ control (Figure 2.5A). ‘Carman’ had a mean lower root rot rating than ‘CDC Meadow’ but it was not significantly different, although it was significantly lower than some other lines (5001, 5007, ‘Carneval’, PI 660736). In the cornmeal layering method, none of the genotypes performed differently from the inoculated susceptible control ‘CDC Meadow’ (Figure 2.5B). Disease levels were generally lower in the plants inoculated using the cornmeal layering method (Figure 2.5) as compared to the seed soak method. Similar trends in results were found in trial 2 (Supplementary Figure 3).

C. Evaluation of revised inoculation methods

Four revised inoculation methods, the revised seed soak method, at-planting inoculation, stem base inoculation and root soak, were tested for disease severity induction in pea genotypes ‘CDC Meadow’ and ‘CDC Dakota. All revised inoculation methods resulted in no discoloration

of roots in the mock-inoculated control groups (data not shown). For ‘CDC Meadow’, all methods of inoculation resulted in significantly different DSRs from each other. The revised seed soak method resulted in the highest level of disease followed by root soak, then stem base inoculation and then at-planting inoculation (Figure 2.6). For ‘CDC Dakota’, disease levels in plants inoculated with the root soak method was highest followed by revised seed soak and then the other two methods. Disease levels were generally lower in ‘CDC Dakota’ compared to ‘CDC Meadow’, except for the root soak method where mean DSR was numerically similar in both genotypes, although the two cultivars were not directly compared to each other using statistical analysis. A similar trend was observed in trial 2, except inoculation at planting had the highest disease severity rating for ‘CDC Dakota’ (Supplementary Figure 4).

D. Soil inoculation experiment

In the soil inoculation experiment, data from both the experiments were combined for analysis because Levene’s test indicated variances were equal. Of the twenty genotypes tested, seventeen genotypes resulted in significantly higher DSR compared to the mock-inoculated susceptible ‘CDC Meadow’ (Figure 2.7), and were not significantly different from inoculated ‘CDC Meadow’. Only ‘CDC Dakota’ and ‘Carman’ had significantly lower DSR compared to inoculated ‘CDC Meadow’, and were not significantly different than the non-inoculated controls.

E. Germplasm screening in a field nursery

Despite high disease levels (DSR ≥5) in all the genotypes in 2020, statistical analysis could not be performed due to a high number of missing replicates caused by ground squirrel damage. Out of 26 germplasm screened in 2020 in the field, 9 genotypes resulted in numerically lower disease severity compared to CDC Meadow at 6 weeks post seeding (wps). Some genotypes also had numerically lower DSR compared to inoculated ‘CDC Meadow’ at 8 wps

(Supplementary Figure 5). Of nineteen genotypes screened in 2021 in the field, only genotype ‘K2’ had significantly lower DSR at 6 wps; however, the severity increased rapidly in the next two weeks making it no different from the susceptible cultivar ‘CDC Meadow’ at 8 wps (Figure 2.8). Five genotypes, ‘CDC Dakota’, AphTol2, ‘Carman’, PI660731 and PI 557501 had significantly lower DSR compared to ‘CDC Meadow’ at 8 wps (Figure 2.8).

2.4 DISCUSSION

Inoculum concentration and inoculation approaches are the essential to controlled and reproducible experiments in plant-pathogen studies. We tested several inoculation methods to determine which was optimal for conducting greenhouse experiments for purposes such as germplasm screening and assessing plant pathogen interaction between with *F. avenaceum* and pea.

The classical overnight seed soak in conidia suspension method resulted in the highest level of disease in the susceptible cultivar ‘CDC Meadow’. However, seeds were often heavily colonized by *F. avenaceum* as evidenced by mycelia growing over the germinated seed and most plants failed to emerge due to high disease pressure. Similar observtions in previous studies (Eranthodi et al. 2020; Safarieskandari et al. 2021) prompted this current study on inoculation methods. The seed soak method did work well for the partially resistant cultivar ‘Carman’ where emergence was higher and disease severity lower than ‘CDC Meadow’. This is similar to what was observed for ‘CDC Dakota’, a dun-colored cultivar, by Eranthodi et al (2020), where disease severity was much lower than ‘CDC Meadow’ after using seed soak inoculation. However, ‘Carman’ is a variety of unknown origin with purple flowers and a coloured seed coat. These traits have been linked to early seedling resistance (Bodah et al. 2016; Awodele et al. 2024) but

are agronomically undesirable and almost all commercial cultivars have white flowers, and colorless seed coats with yellow or green cotyledons

A modified seed soak method with a lower inoculum concentration and shorter incubation period improved emergence in 'CDC Meadow' but disease levels reached maximum levels in 9 of 13 genotypes that were tested. Furthermore, observations in the field indicate that FRR usually starts later in the growing season, during early flowering around 8 weeks after seeding, and not in the early seedling stage (Willsey et al. 2021). Thus, a seed inoculation method may not be the most appropriate if the objective of the study is to understand disease development under biologically-relevant conditions. Despite its limitations, the revised seed soak method may be useful in testing for seed-coat mediated resistance, testing the virulence of *Fave* isolates or refreshing virulence of stored isolates.

Cornmeal layering and other grain meal layering has been a common method of inoculation with various *Fusarium* species such as *F. oxysporum* and *F. solani* (Porter et al. 2015; Güler Güney and Güldür 2018; Lai et al. 2020). In comparison to the modified seed soak method, the cornmeal layering method did not perform at a satisfactory level to differentiate the genotypes with different levels of resistance and/or tolerance to *Fave.* We observed that disease symptoms stayed in the root zone that was in direct contact with the inoculum in the cornmeal layer (Figure 2.2B) and did not spread past this point, and thus disease severity remained low. Similarly, the colonized grain inoculation method did not allow *F. culmorum* and *F. pseudograminearum* to successfully induce crown rot of wheat (Erginbas-Orakci et al., 2016).

The root soak method was also an effective method of inoculation as it resulted in uniform and moderate disease levels in both 'CDC Meadow' and 'CDC Dakota'. This method allows direct contact of roots with a spore suspension for a prolonged time and allows pathogens

to enter the vascular system. Studies suggest that in the root soaking process, pathogen entry is unaffected by the resistance level of the host genotype at the root epidermal level (Eynck et al. 2009; Michielse and Rep 2009; Lai et al. 2020) and therefore this method may not be an appropriate method for screening germplasm resistance or for assessing seed-coat mediated resistance. This method requires producing a large amount of inoculum and is demanding for large-scale experiments. Furthermore, FRR resulting from the root soak method did not resemble those resulting from infection in the field; following the root soak method, root browning was generally confined to the lateral roots and the tap root was unaffected (Figure 2.2C, 2.2G). However, this method can be useful when a single, defined inoculation time point is needed to study multiple discrete time points post-inoculation -, such as for transcriptomic or metabolomic studies.

The stem base and direct seed inoculation approaches were unsuitable methods for germplasm screening for resistance based on our observations in pea, as they resulted in low disease severity and therefore differentiating the resistant nature of pea genotypes can be difficult. In evaluating Fusarium crown rot inoculation methods in wheat, stem base inoculation also resulted in the lowest level of disease severity (Erginbas-Orakci et al. 2016). Although these methods result in low disease severity, infection was centered on the epicotyl and stem base (Figure 2.2F). Therefore, this approach may be appropriate for time-sensitive and combination studies such as plant defense, and exogenous hormone application studies where pathogen inoculum might be affected by hormones/chemicals. For example, to prevent *Fave* from using hormones/chemicals and modifying its counter-defense pathways, chemicals/hormones can be applied to prime the plant defense pathways and washed away via heavy watering before inoculating pathogen at the stem base.

Soil inoculation was the most effective method that resulted in moderate levels of disease and was also the only method where the variances were homogeneous over two repeated trials. Seeds germinated and emerged uniformly (85- 100% emergence rate, data not shown), and moderate levels of disease prevented plants from dying early. Symptoms were also the most similar to what is observed in the field (Figure 2.2E), with a blackened tap root and some lateral root browning (Figure 2.2D). Therefore, this method was the most appropriate for screening pea lines for partial resistance. Using this method, 'Carman' and 'CDC Dakota' showed high partial resistance (DSR < 3) to *Fave*, confirming the importance of seed-coat mediated resistance. The non-pigmented lines 5001, 'Prodigy', 'K2', and an Aphanomyces-tolerant line (confidential) showed intermediate levels (DSR = 3 – 5) of partial resistance compared to 'CDC Meadow'. The soil inoculation method worked more effectively compared to other methods such as stem base and seed inoculation for many other *Fusarium* species in other crops as well (Olivain et al. 2006; Maitlo et al. 2016).

There were some discrepancies between the results of the soil inoculation method and field screening, which was expected due to the nature of the root rot complex in field soil. In the field, at 8 weeks post-seeding, 'Carman' showed intermediate partial resistance (DSR = 4), whereas 'CDC Dakota' (DSR = 5.2) and the Aphanomyces-tolerant line (DSR = 5.2) showed a reduction in disease severity compared to 'CDC Meadow' (DSR = 6.5) but disease severity was still high. Other pathogens like *F. solani* and various *Pythium* spp. (data not shown) were present in the field soil used in the microplots. Multiple pathogens can act synergistically to increase disease (Willsey et al. 2018). Furthermore, since rating was performed at 4 weeks in the greenhouse but later in the field (6 and 8 weeks), this could account for the higher severity in the field, as disease had more time to develop. Since greenhouse screening can be performed quickly

and is less labour- and space-intensive, the soil inoculation method can be used to screen lines and narrow down the number for follow-up field screening.

In conclusion, since FRR symptoms appear later in the pea lifecycle (early flowering) in the Canadian prairies, inoculation methods that allow slower root rot development at a later seedling stage of plant growth is recommended. Thus, in the greenhouse experiments, the soil inoculation method with *Fave* conidia was the most effective method for germplasm screening for partial resistance. The modified seed soak inoculation method may be effective for testing the virulence of different isolates of *Fave* on the same genotype, although disease severity levels may be too high, or for testing for seed-coat mediated resistance. The root soak method is an effective method of inoculation for experiments such as time course trials where one may want to measure disease development and study related defense responses at various time points after inoculation for a longer period. Thus, care should be taken in selecting the most appropriate inoculation method depending on the purpose of the experiment.

Acknowledgements

The authors acknowledge all present and past members of the pulse pathology lab, AAFC Lethbridge.

Declaration of interest statement

The authors state that they have no competing interests to disclose.

Funding

The work was supported by the AgriScience Pulse Cluster project ASC-06-A9 with funding provided by Agriculture and Agri-food Canada, Alberta Pulse Growers, Saskatchewan Pulse Growers, and Manitoba Pulse and Soybean Growers Associations.

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TABLES AND FIGURES

Table 2.1. Summary of greenhouse experiments performed to assess optimal inoculation methods for *F. avenaceum* and pea root rot development.

Experiments	Inoculation Method	Dose (conidia / ml or g soil)	Fa isolate(s)	Cultivars	Growth media	Growth containers
A	Seed soak overnight	0 – 500,000	1306.08, DAOM 2420768, DAOM 242378	CDC Meadow and Carman	Vermiculite and Cornell Mix	SVD pots
B	1) Seed soak overnight; 2) Cornmeal layering	1) 10,000 2) Unknown	1306.08	14 pea genotypes, including CDC Meadow and Carman	Vermiculite	Rootrainers
C	4 methods: 1) seed soak 4 hours; 2) Inoculum on seed at planting; 3) stem base inoculation; 4) Root soaking	1) 10,000 2) 10,000+ 10,000 3) 5 ml of 10^6 4) 10^6 soak	1306.08	CDC Meadow and CDC Dakota	Vermiculite	Rootrainers
D	Soil inoculation	10,000 conidia/ g dry soil	1306.08	22 genotypes; including CDC Meadow, CDC Dakota and Carman	Cornell Mix	SVD pots

Table 2.2. List of pea cultivars and genotypes used in greenhouse experiments and field screening in 2020 and 2021. All pea lines have a non-pigmented seed coat colour, except 'Carman' and 'CDC Dakota' (in bold).

Genotype	Source and reference	Experiments
PI 660736	USDA GRIN database, McGee et al. 2012	B, D, Field nursery 2020, 2021
PI 660735	USDA GRIN database, McGee et al. 2012	Field nursery 2020
PI 660734	USDA GRIN database, McGee et al. 2012	Field nursery 2020
PI 660733	USDA GRIN database, McGee et al. 2012	Field nursery 2020
PI 660732	USDA GRIN database, McGee et al. 2012	Field nursery 2020, 2021
PI 660731	USDA GRIN database, McGee et al. 2012	Field nursery 2020, 2021
PI 660730	USDA GRIN database, McGee et al. 2012	Field nursery 2020, 2021
PI 660729	USDA GRIN database, McGee et al. 2012	Field nursery 2020, 2021
PI 557502	USDA GRIN database, Kraft et al. 1992	D, Field nursery 2020
PI 557501	USDA GRIN database, Kraft et al. 1992	D, Field nursery 2020
PI 557500	USDA GRIN database, Kraft et al. 1992	Field nursery 2020, 2021
PI 557501	USDA GRIN database	B
PI 606701	USDA GRIN database	D
PI 606700	USDA GRIN database	Field nursery 2020
PI 606698	USDA GRIN database	Field nursery 2020
PI 606699	USDA GRIN database	D
PI 606696	USDA GRIN database	D, Field nursery 2020, 2021
Carneval	R. Conner; unknown	B, Field nursery 2020, 2021
SW Prodigy	R. Conner; unknown	B, Field nursery 2020, 2021
K2	L. Porter; Bodah et al. 2016	B, D, Field nursery 2020, 2021
5007	L. Porter; Bodah et al. 2016	B, D, Field nursery 2020
5006	L. Porter; Bodah et al. 2016	B, Field nursery 2020
5005	L. Porter; Bodah et al. 2016	B, Field nursery 2020, 2021
5004	L. Porter; Bodah et al. 2016	B, Field nursery 2020, 2021
5003	L. Porter; Bodah et al. 2016	B, Field nursery 2020
5001	L. Porter; Bodah et al. 2016	B, D, Field nursery 2020, 2021
Carman	R. Conner; Feng et al. 2011	A, B, D, Field nursery 2020, 2021
CDC Meadow	Purchased commercial seed, Warkentin et al. 2007	A, B, C, D, Field nursery 2020, 2021
CDC Dakota	Purchased commercial seed, Warkentin 2010 (Unpublished)	C, D, Field nursery 2020, 2021
AAC Comfort	Bing et al. 2018	D
AAC Chrome	Bing et al. 2019	D
Aphano tolerant 1(AphTol1)	KWS seeds	D, Field nursery 2021
Aphano tolerant 2(AphTol2)	KWS seeds	D, Field nursery 2021

Figure 2.1. Visual representation of the root rot rating scale applied to roots in the various experiments. 1 = healthy plant, 2 = small reddish brown lesions at the seed attachment point, 3 = coalescing of localized root/epicotyl lesions approximately 180 degrees around the tap root, 4 = lesions extending and completely encircling the tap root, 5 = increasingly discoloured and extended epicotyl lesions, 6 = epicotyl lesions encircling the stem extending up to the green stem, and 7 = completely decayed root system. Scale was modified from Schneider and Kelly, 2000 and Safarieskandari et al. 2021.

Figure 2.2. Visual representation of plant disease symptoms resulting from various inoculation methods and field conditions. The images illustrate symptom severity and variation in plant response, highlighting differences in effectiveness among different conditions for pathogen infection studies. A: CDC Meadow, modified seed soak; B. CDC Meadow, cornmeal layering; C. CDC Meadow, root soak; D. CDC Meadow, soil inoculation; E. CDC Meadow, field microplot; F. CDC Dakota, stem base inoculation; G. CDC Dakota, root soak

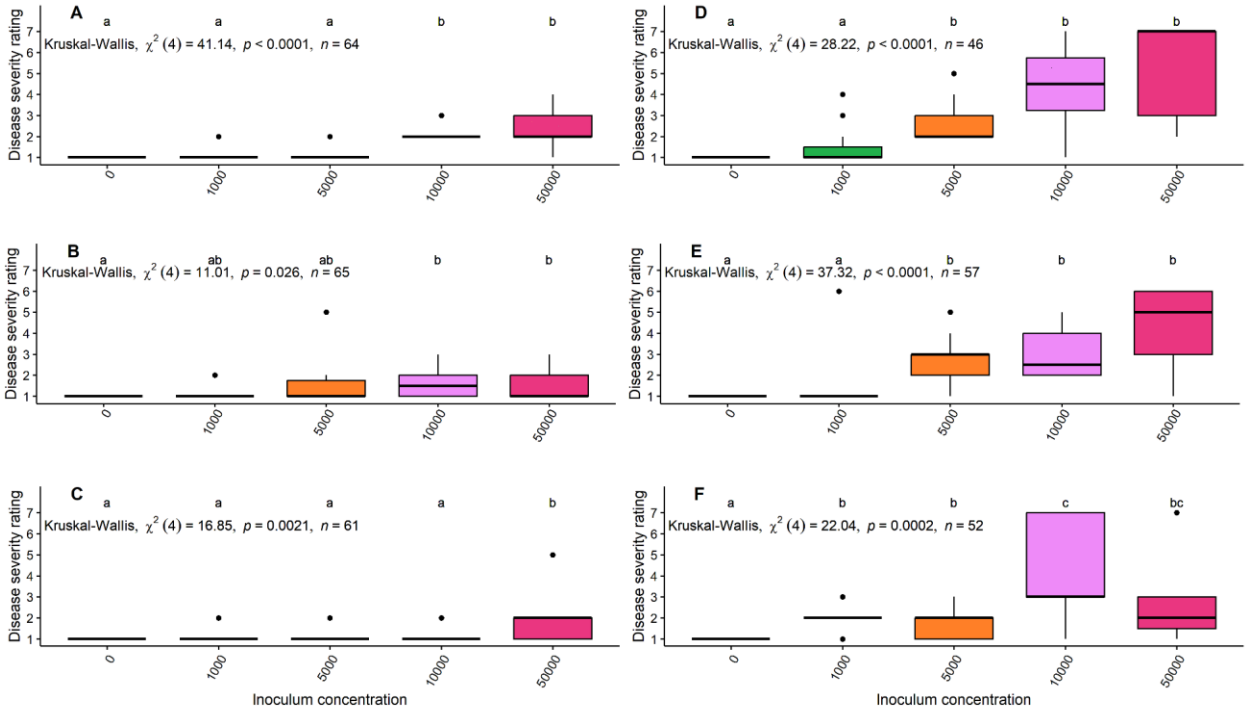

Figure 2.3. Box plot showing effect of different *Fave* inoculum levels on disease severity rating of genotype 'Carman' (pigmented seed coat) grown in soil (left) or vermiculite (right). (A,D): Isolate pea 1306.08, (B, E): Fa DAOM 2420768 and (C, F): FA DAOM 242378. Boxes with different letter groupings are significantly different. Each box represents the interquartile range of disease severity scores for a specific genotype, with the median indicated by the horizontal line within the box. Whiskers denote variability outside the upper and lower quartiles, and outliers are represented by individual points.

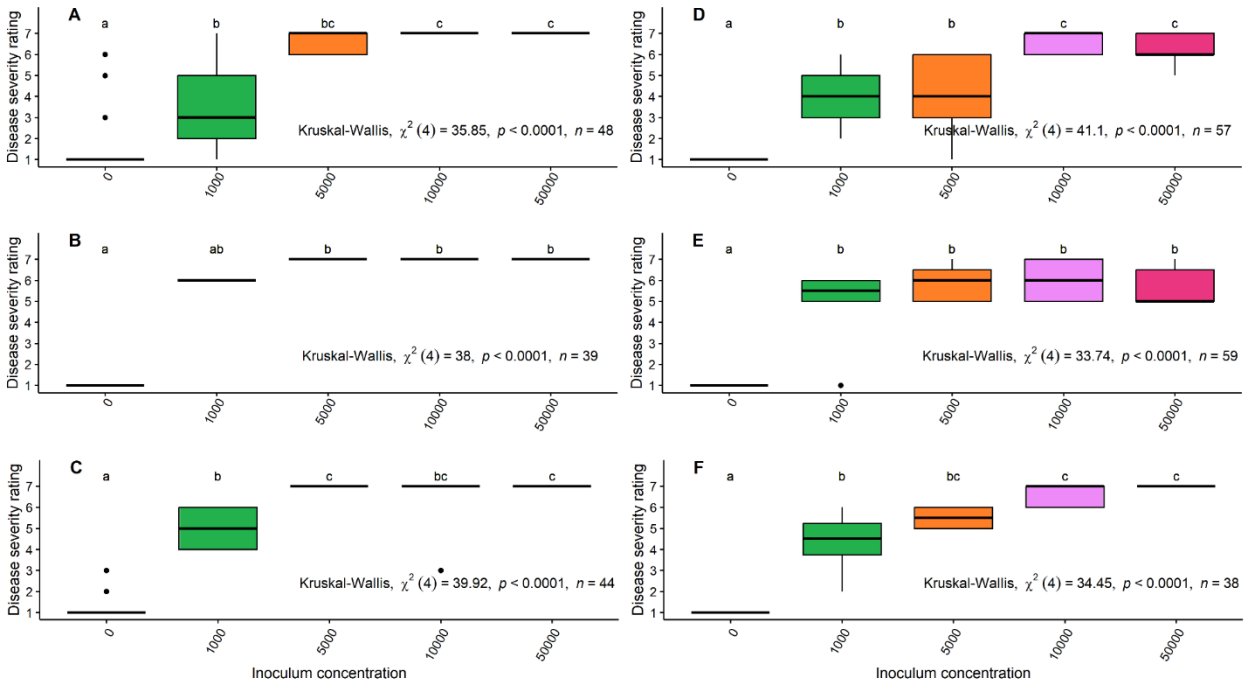

Figure 2.4. Effect of different *Fave* inoculum levels on disease severity rating of genotype 'CDC Meadow' (non-pigmented seed coat) grown in soil (left) or vermiculite (right). (A,D): Isolate pea 1306.08, (B, E): Fa DAOM 2420768 and (C, F): FA DAOM 242378. Boxes with different letter groupings are significantly different. Each box represents the interquartile range of disease severity scores for a specific genotype, with the median indicated by the horizontal line within the box. Whiskers denote variability outside the upper and lower quartiles, and outliers are represented by individual points.

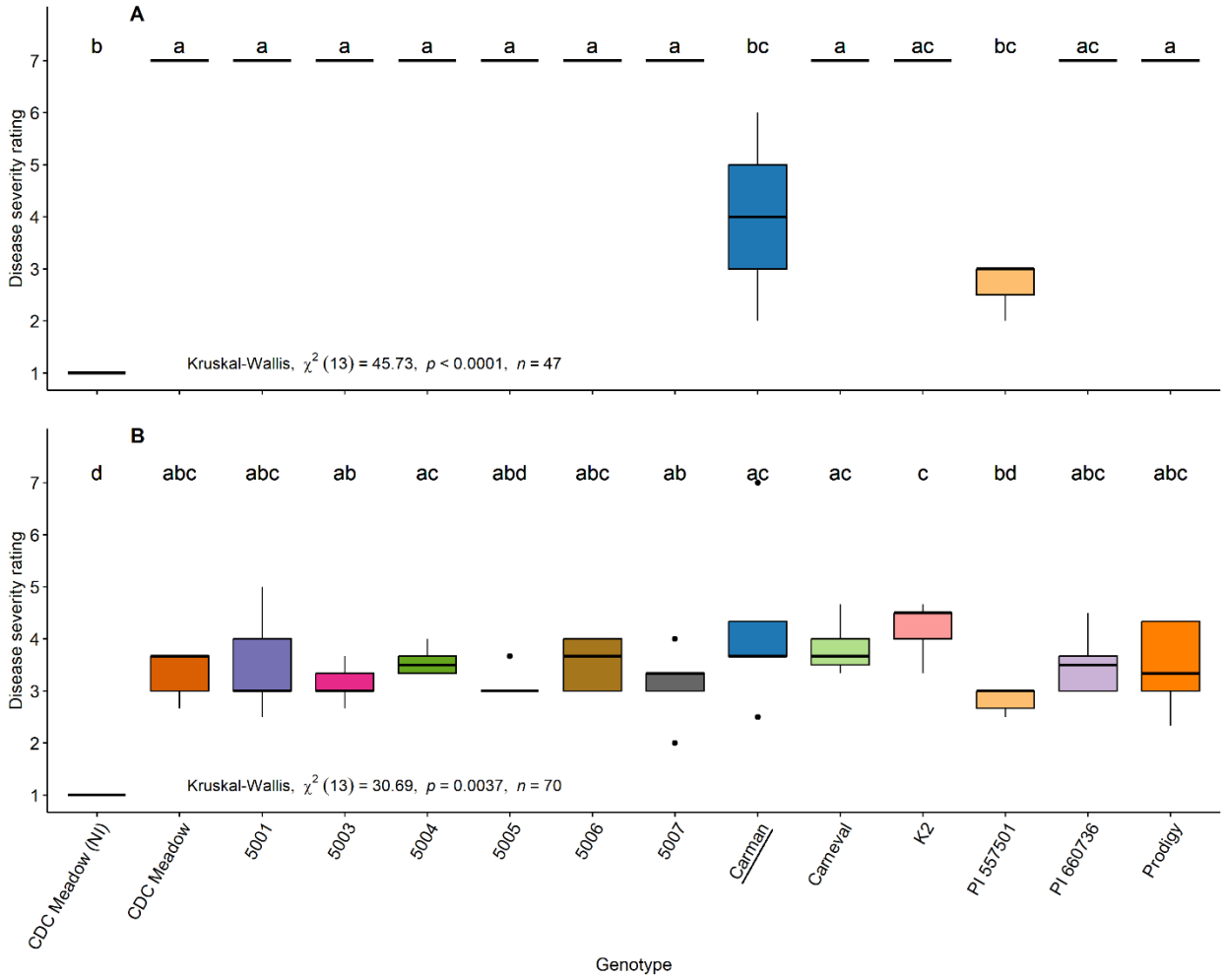

Figure 2.5. Box plots showing disease severity rating of 14 genotypes, of which only 'Carman' has a pigmented seed coat, following *Fave* inoculation using seed soaking method (A) or corn meal layering method (B) within trial number 1. Boxes with different letter groupings are significantly different. Each box represents the interquartile range of disease severity scores for a specific genotype, with the median indicated by the horizontal line within the box. Whiskers denote variability outside the upper and lower quartiles, and outliers are represented by individual points.

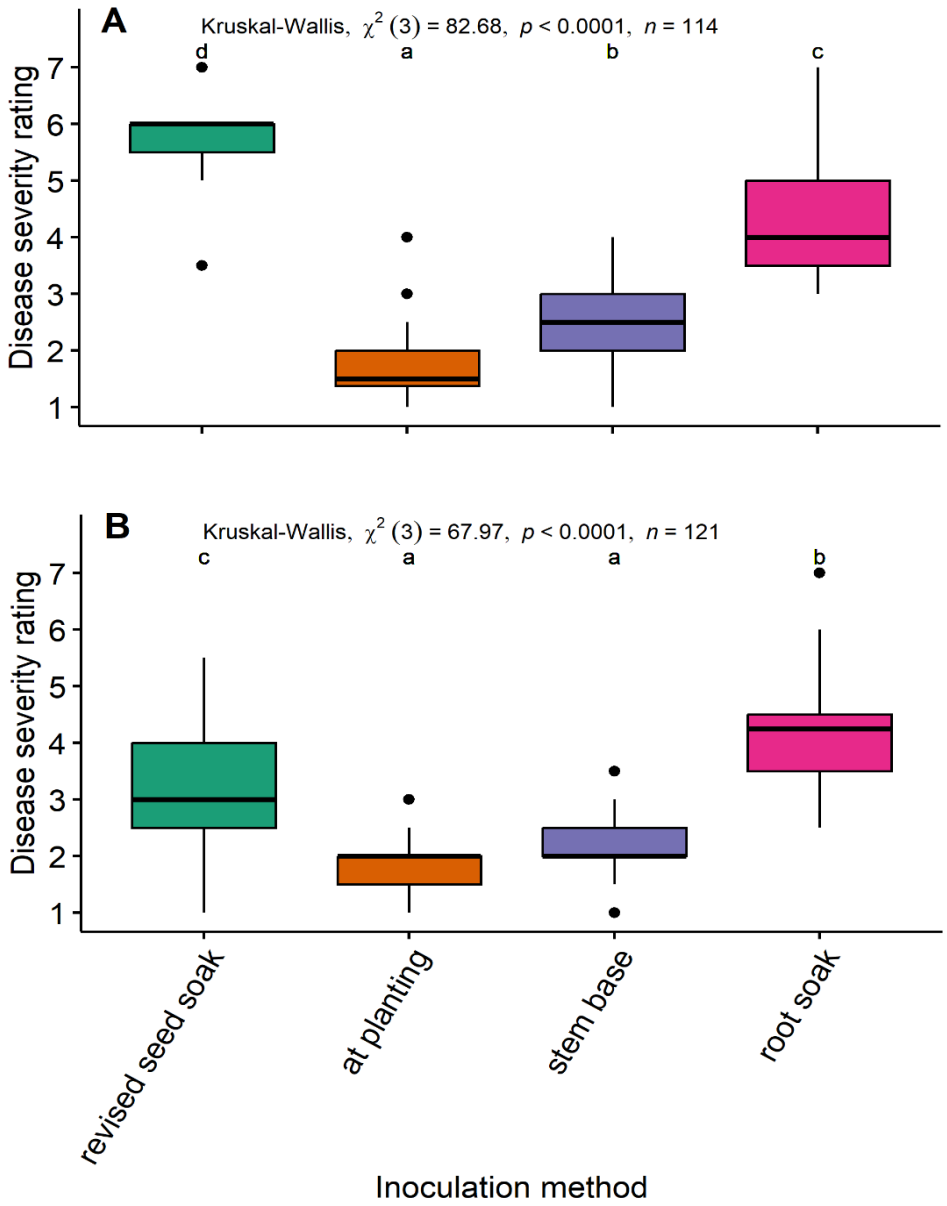

Figure 2.6. Boxplot showing disease severity rating of genotype 'CDC Meadow' (A, non-pigmented seed coat) and 'CDC Dakota' (B, pigmented seed coat) following *Fave* inoculation using 4 inoculation methods. Results shown are from trial #1. Boxes with different letter groupings are significantly different. Each box represents the interquartile range of disease severity scores for a specific genotype, with the median indicated by the horizontal line within the box. Whiskers denote variability outside the upper and lower quartiles, and outliers are represented by individual points.

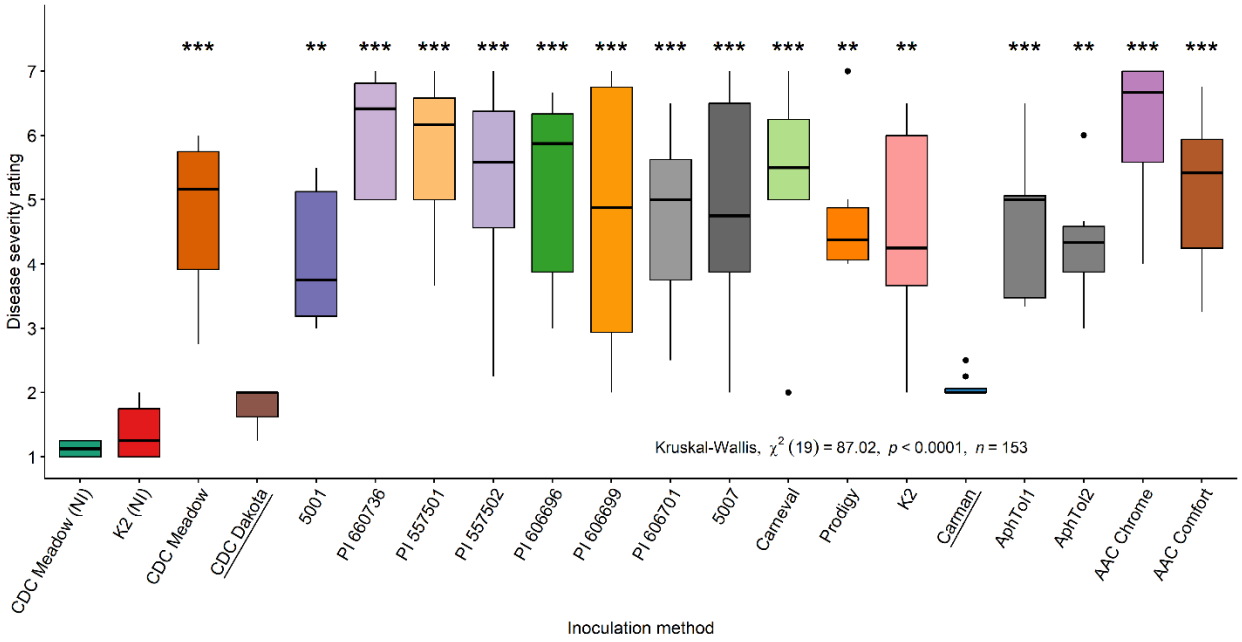

Figure 2.7. Box plots showing disease severity of 22 genotypes, of which 'CDC Dakota' and 'Carman' have pigmented seed coats, using soil inoculation method from two combined trials. Each box represents the interquartile range of disease severity scores for a specific genotype, with the median indicated by the horizontal line within the box. Whiskers denote variability outside the upper and lower quartiles, and outliers are represented by individual points. * represents $0.01 \leq p < 0.05$, ** represents $0.001 \leq p < 0.01$, *** represents $p < 0.001$ compared to non inoculated CDC Meadow.

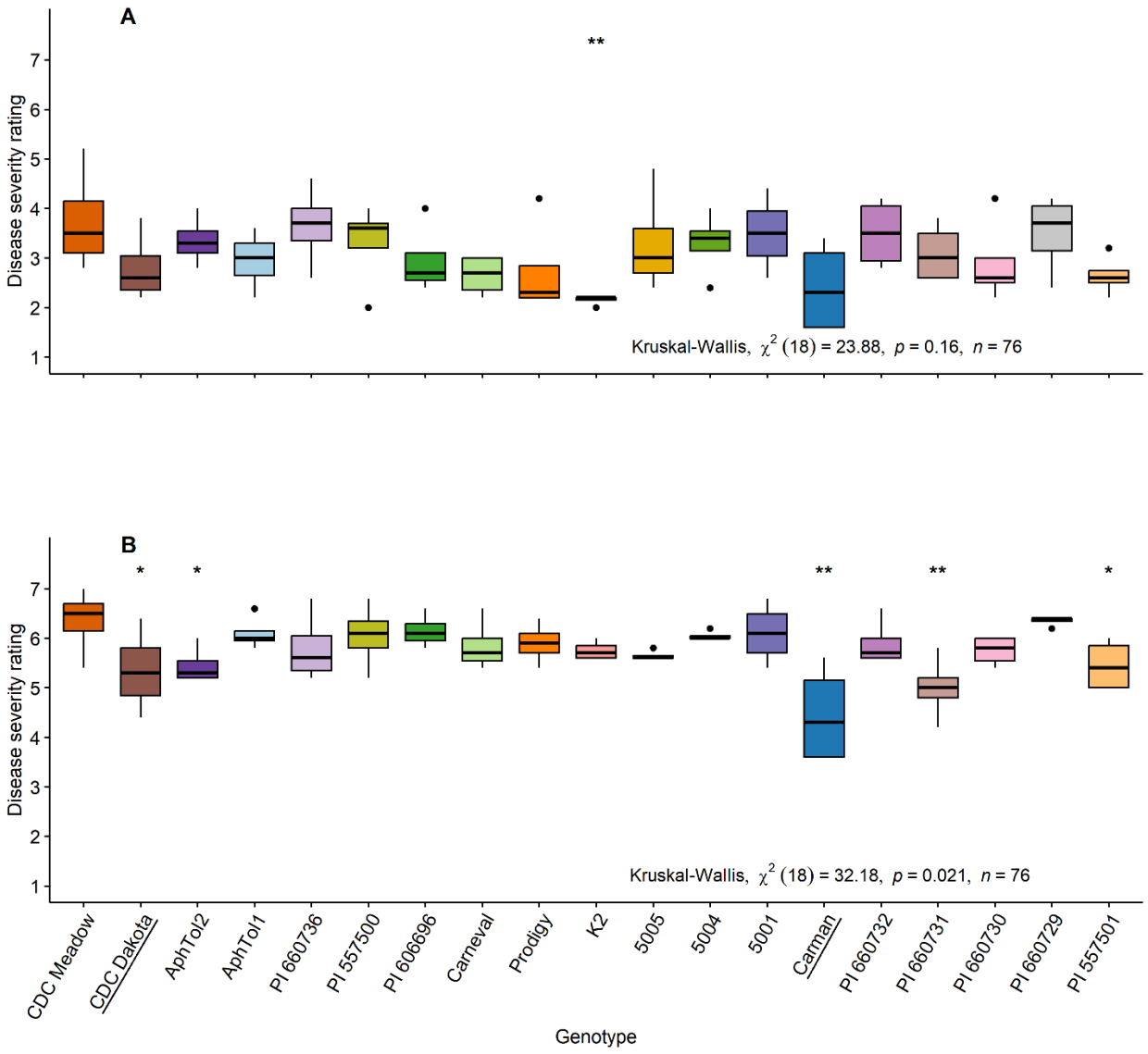

Figure 2.8. Box plots showing disease severity rating of 19 genotypes, of which 'CDC Dakota' and 'Carman' have pigmented seed coats, screened in the field in the year 2021, and rated at 6 wps (weeks post seeding, A) and at 8 wps (B). Each box represents the interquartile range of disease severity scores for a specific genotype, with the median indicated by the horizontal line within the box. Whiskers denote variability outside the upper and lower quartiles, and outliers are represented by individual points. * represents $0.01 \leq p < 0.05$, ** represents $0.001 \leq p < 0.01$, *** represents $p < 0.001$.

CHAPTER 3 : TEMPORAL ANALYSIS OF APHANOMYCES ROOT ROT AND FUSARIUM ROOT ROT DEVELOPMENT AFTER SPORE INOCULATION OF FIELD PEA

ABSTRACT

Pea root rot, caused by a complex of pathogens such as *Aphanomyces euteiches* and *Fusarium avenaceum*, is a significant threat to pea production, leading to severe yield losses. This study investigates the temporal dynamics of pathogen colonization, disease progression, and symptom development in two susceptible pea cultivars, CDC Meadow and CDC Dakota, following inoculation with *A. euteiches* zoospores and *F. avenaceum* macroconidia. We evaluated pathogen detection, disease severity, and root biomass over multiple time points using hydroponic and greenhouse experiments. Quantitative PCR (qPCR) analysis revealed that *A. euteiches* detection occurred as early as 12 hours post-inoculation (hpi) in hydroponic conditions, with qPCR detection 4 days post-inoculation (dpi) and visible symptoms appearing by 4 dpi in greenhouse conditions in vermiculite. In contrast, *F. avenaceum* was detected by qPCR at 4 dpi, manifesting symptoms at 6 dpi. Both pathogens exhibited cultivar-specific variations in disease progression, with CDC Meadow showing earlier symptom development compared to CDC Dakota. The study highlights the critical latent period between pathogen introduction and symptom onset, emphasizing the importance of early time points (2–24 hpi for *A. euteiches* and up to 6 days for *F. avenaceum*) for studying host-pathogen interactions and defense mechanisms. These findings provide a standardized framework for understanding PRRC dynamics and inform strategies for disease management and resistance breeding in pea crops.

3.1 INTRODUCTION

Pea root rot (PRR) is a devastating disease caused by a complex of pathogens from oomycete and fungal groups, including the major contributors *Aphanomyces euteiches*, *Fusarium* spp., *Pythium* spp., and *Rhizoctonia solani* (Willsey et al., 2018), collectively known as the pea root rot complex (PRRC). *Aphanomyces euteiches* and *Fusarium avenaceum* are the primary pathogens responsible for PRR and the highest yield losses in the Canadian prairies (Chatterton *et al.*, 2019).

PRR is characterized by brown-black or reddish lesions that begin on lateral roots and progressively spread to the taproot, hypocotyl and epicotyl regions, leading to root softening and significant loss of root mass. Under conditions of high inoculum density and favorable environmental factors during the early growth stages, the disease can result in complete yield loss (Gossen *et al.*, 2016, Chatterton et al., 2019, Esmaeili Taheri *et al.*, 2017). Studying PRRC is particularly challenging due to the following factors: a) interactions among the pathogens themselves and with the host, b) diverse taxonomic classifications of the pathogens, c) synergistic effects that enhance pathogen aggressiveness, d) overlapping disease symptoms, and e) variations in environmental preferences. To better understand host-pathogen interactions, it is essential to study each pathogen both individually and as part of the complex. Research indicates that pathogens involved in the PRRC caused greater disease severity when present together as a complex compared to individual infections (Willsey et al., 2018). Notably, symptoms of PRR may not appear immediately after pathogen colonization. Infection, colonization and growth in roots are highly influenced by plant growth conditions and the initial inoculum load.

Defining the time period between pathogen introduction and symptom manifestation is critical for scientific studies focusing on plant defense mechanisms and plant-pathogen

interactions. This phase, often referred to as the *latent period*, provides valuable insights into the dynamic processes occurring during infection, including pathogen colonization, host response activation, and the progression of disease. Understanding events during the latent period is essential to plant defense studies, because many host defense mechanisms, such as reactive oxygen species (ROS) bursts, nitric oxide (NO) signaling, and early transcriptional changes, occur rapidly after pathogen introduction. These early responses often dictate the plant's ability to resist or tolerate infection. Defining events that occur during this time allows researchers to monitor key molecular and biochemical changes, such as the activation of defense-related genes, accumulation of secondary metabolites, and synthesis of pathogenesis-related (PR) proteins. Missing these critical early time points can lead to incomplete data, obscuring the temporal dynamics of defense activation.

This research aimed to develop a standardized inoculation system to achieve uniform disease establishment in host roots for downstream transcriptomic studies of both Aphanomyces and Fusarium root rots. It also sought to determine key time points for pathogen colonization, symptom development, disease progression, and pathogen biomass accumulation in roots following inoculation with *Aphanomyces euteiches* zoospores (asexual spores) and *Fusarium avenaceum*.

3.2 MATERIALS AND METHODS

3.2.1: Time course trial for *A. euteiches* infection and disease progression

3.2.1.1 Inoculum production

Twenty mycelial plugs from the outer edges of an actively growing *A. euteiches* isolate *Ae1* culture on PDA were added to 200 mL Erlenmeyer flasks containing 40 mL of freshly prepared peptone glucose broth and incubated in the dark for 2-3 days (Windels, 2000). Peptone

glucose broth was prepared by dispensing 20g/L peptone and 5g/L glucose in deionized water followed by autoclaving. Following 2-3 days of incubation, the inoculated broth was decanted, and the mycelial plugs were washed using 50% mineral salt solution (MSS) by swirling the suspension vigorously to dispense salt solution throughout all mycelial networks. After mixing, the solution was left to sit for 1.5 hours at room temperature. After 1.5 hours the suspension was discarded and washed again with 50% MSS in a similar manner. After the second wash and incubation, 30 mL of full-strength MSS was added to the flasks and incubated overnight at room temperature. Subsequently, the solution was decanted and aliquoted to count zoospores using a hemocytometer and brought to the required concentration by adding full-strength MSS.
Refer to chapter 2 for Fusarium macroconidia inoculum preparation approach.

3.2.1.2 Planting, inoculation and sampling

A mini-experiment was conducted using the *Aphanomyces euteiches*-susceptible pea cultivar CDC Meadow in a disinfected hydroponic system with vermiculite as seeding media in sterile water. Seeds were sown in autoclaved vermiculite and grown with 22/16ºC (day/night) and 16 h photoperiod. After 14 days of growth, plants were inoculated with *A. euteiches* zoospores at a concentration of 10^3 spores/mL, applying 5 mL of the inoculum per hydroponic pot. Root samples were collected at 2, 6, 12, and 24 hours post-inoculation (hpi) for qPCR analysis to determine the timing of pathogen colonization. Plants were rated for disease symptoms (honey brown discoloration of roots) at 2, 24 hpi and every alternate day until 5dpi. DNA extraction was done using DNAeasy plant mini kit (Qiagen, Montreal QC Canada). The qPCR was performed using a QuantStudio Flex 6 system with a 96-well plate with cycling conditions 95 °C for 3 min; 40 cycles of 95 °C for 15 s and at 60 °C for 60 s and with 3 technical replicates. The reaction included PrimeTime MM 2x reagent (Integrated DNA Technologies,

Canada), which contains DNA polymerase, dNTPs, $MgCl_2$, and other enhancers and stabilizers. The primers and probes used for qPCR analysis are detailed in Table 3.1.

Further, a comprehensive experiment was conducted to study the timing of infection and disease progression in greenhouse conditions using solid rooting media. Seeds of two *A. euteiches*-susceptible pea cultivars, CDC Meadow and CDC Dakota, were treated with fungicide Apron Maxx RTA before seeding. The seeds were sown at a depth of 2.5 cm in rootrainers filled with autoclaved vermiculite and grown with 22/16ºC (day/night) and 16 h photoperiod. After 14 days of growth, plants were inoculated with *A. euteiches* zoospores at a concentration of 10^3 spores/mL, using 2-liter suspension per rootrainer containing 32 plants. Roots were soaked in the inoculum for 1 hour and then the inoculum was removed from the trays. Mock-inoculated control plants were treated with a mineral salt solution ($CaCl_2$:KCl: $MgSO_4$ in a 3.7:1:7 ratio per liter of water) in a similar way.

Plants were sampled in four replicates per treatment at multiple time points: 2, 6, 12, and 24 hpi; 2 days post-inoculation (dpi); and every alternate day until 20 dpi. Sampled plants were assessed using a 0–5 disease severity scale where 0 indicates plant is healthy and 5 indicates plant is dead due to disease; all other values indicate different order of disease severity with increase in severity in increasing order. Samples were also weighed to determine root biomass. Samples from 2hpi, 12hpi, 4dpi, 6dpi and 14dpi were stored at -20°C for DNA extraction and qPCR in a similar way as aforementioned in mini hydroponic experiment. The qPCR protocol was conducted in the same manner as in the hydroponic experiment. To assess reproducibility two independent experiments were carried out.

3.2.2 Time course trial for *Fusarium avenaceum* (*Fave)* infection and disease progression

Seeds of two *Fusarium avenaceum*-susceptible pea cultivars, CDC Meadow and CDC Dakota, were prepared for seeding by surface sterilization with 10% bleach for 10 minutes. The seeds were sown at a depth of 2.5 cm in rootrainers filled with autoclaved. Plants were grown under controlled conditions with 22/16ºC (day/night) and 16 h photoperiod. After 14 days of growth, plants were treated with a suspension of *F. avenaceum* macroconidia (5 x 10^4 spores/mL) at a rate of 2 litres per rootrainer containing 32 plants. The roots were soaked in the inoculum for 1 hour and the inoculum was then removed from the trays. Mock-inoculated control plants underwent the same procedure using sterile double-distilled water (ddH_2O). Samples were collected in four replicates per treatment at multiple time points: 2, 6, 12, and 24 hours post-inoculation (hpi); 2 days post-inoculation (dpi); and every alternate day up to 21 dpi. Plant roots were evaluated on a 1–7 disease rating scale where a rating score of 1 denotes a healthy plant, 2 denotes small reddish brown lesions at the seed attachment point, 3 denotes coalescing of localized root/epicotyl lesions approximately 180° around the tap root, 4 denotes lesions extending and completely encircling the tap root, 5 denotes increasingly discoloured and extended epicotyl lesions, 6 denotes epicotyl lesions extending up to the green stem, and 7 denotes a completely decayed root system (Safarieskandari et al., 2021; Schneider & Kelly, 2000). The samples were also weighed for their fresh biomass. Samples from 2hpi, 12hpi, 4dpi, 6dpi and 14dpi were stored at -20°C for DNA extraction and qPCR analysis. Two independent experiments were conducted to ensure reliability.

DNA extraction and qPCR were done using same protocol as in *A. euteiches* time course experiment. Primers and probes used in qPCR are listed in Table 3.1.

3.3 RESULTS

3.3.1 *A. euteiches* infection and disease progression

In the mini hydroponic trial conducted using susceptible cultivar CDC Meadow for *A. euteiches* disease progression, qPCR analysis first detected the pathogen in the inoculated group at 12 hours post-inoculation (hpi) after which levels remained consistent through 24 hpi (Figure 3.1C). In contrast, mock-inoculated plants showed no amplification for the pathogen, with Ct values remaining above 35, indicating absence of pathogen DNA (Figure 3.1B). Amplification of pea housekeeping genes (PHG) remained consistent across both treatments, with Ct values around 23–24, confirming the integrity of plant DNA and normalization of qPCR data. qPCR, which detects pathogen DNA, is most useful during early infection before visible symptoms appear; therefore, assays were conducted up to 24 hpi to quantify initial pathogen establishment in the hydroponic system. Disease severity ratings increased rapidly after 2 days post-inoculation (dpi), reaching a score of ~3 by 3 dpi and remaining stable through 5 dpi (Figure 3.1A). No symptoms were observed at 2 hours post-inoculation (hpi) or 24 hpi, indicating that visible symptoms manifested between 1 and 3 dpi.

In contrast, the greenhouse experiment using vermiculite as the growth medium exhibited a slower progression of *Ae1* infection load increase and disease symptoms. The qPCR results showed that for inoculated CDC Meadow, Ct values for *A. euteiches* quantification decreased from ~35 at 2 hpi to ~20 by 14 dpi with detection threshold crossed at 4dpi. A similar pattern was observed in CDC Dakota, with detection at 4dpi and a sharp decline in Ct values at 6 dpi and remaining low thereafter (Figure 3.3). In contrast, the control groups (Panels B and D) showed consistently high Ct values (~35-36) throughout the experiment, indicating no detectable pathogen presence. Meanwhile, Ct values for the pea gene (Psat tubulin) remained relatively stable across all treatments and time points, validating sample quality and serving as an internal control. In the *Ae* inoculated groups, the DSR remained at 0 until 6 dpi, after which it steadily

increased, reaching approximately 3 by 14 dpi and peaking between 4.5 and 5 by 18 dpi (Figure 3.2). The DSR trajectory aligns closely with the decrease in Ct values observed in the *Ae1* inoculated groups (Figure 3.3 Panels A and C), reflecting a direct correlation between pathogen biomass accumulation and symptom expression. Notably, qPCR analysis revealed that pathogen colonization occurred 2-3 days before visual symptoms became apparent. The Ct value for *Aphanomyces euteiches* was just below the detection threshold Ct value in root tissues at 4 dpi in CDC Meadow whereas the Ct value was right at the detection threshold value at 4 dpi in CDC Dakota (Figure 3.3). A marked increase in pathogen biomass was evident between 12 hpi and 4 dpi, further indicating early and active root colonization preceding symptom development. The earlier detection of the pathogen at 24 hpi in the hydroponic system compared to 4 dpi in the vermiculite setup underscores the influence of growth medium on disease progression and highlights the importance of early time points for studying plant defense responses to *Ae*, provided favorable growing conditions. There was no difference in fresh biomass of roots in the period of 3 weeks after inoculation.

3.3.2 *Fave* infection and disease progression

In the greenhouse experiment investigating the temporal dynamics of disease development caused by *Fave*, visible symptoms first appeared around 4 dpi in the lateral roots of both CDC Meadow and CDC Dakota. These symptoms, characterized by reddish lesions, emerged slightly earlier in CDC Meadow than in CDC Dakota under identical conditions. These symptoms progressed steadily over time, with disease severity rating (DSR) values reaching approximately 4.5 to 5.0 in *Fave*-inoculated CDC Meadow by 20 dpi (Figure 3.4, top panel). In contrast, disease development was slightly delayed and less severe in CDC Dakota in early days, where DSR values peaked near 4.5 at 20 dpi (Figure 3.4, bottom panel). Control treatments in

both cultivars exhibited consistently low DSR values (~1-2), indicating minimal background symptom expression unrelated to *Fave* infection. Although both cultivars eventually exhibited substantial symptom development, the rate of disease progression differed. In CDC Meadow, a noticeable rise in DSR was observed between 6 and 10 dpi, followed by a steady increase through 20 dpi. CDC Dakota showed a similar pattern but with a lag of approximately 2 days and slightly lower DSR values during the early stages (6-12 dpi). These findings suggest that while both cultivars are susceptible to *Fave* under greenhouse conditions, *F. avenaceum* was slightly more aggressive and caused faster disease development on CDC Meadow compared to CDC Dakota.

qPCR analyses confirmed the molecular presence of *F. avenaceum* in root tissues by 4 dpi, approximately two days prior to the onset of visual symptoms in both cultivars (Figure 3.5). This early detection window reinforces the interpretation that pathogen colonization precedes external symptom expression, highlighting the 4-6 dpi interval as a critical period for early host-pathogen interaction in this system.

Despite the progression of disease symptoms, no significant differences were observed in fresh root biomass across treatments during the three weeks following inoculation.

3.4 DISCUSSION

In the hydroponic system, where infection progressed rapidly, assays conducted within 12–24 hpi were sufficient to confirm pathogen establishment and quantify initial pathogen load. By 24 hpi, pathogen DNA levels clearly indicated successful colonization since pathogen levels increased from 6 hpi indicating active growth of the pathogen on the roots, and highlighting the value of qPCR in capturing early infection events. These results establish a baseline for

understanding pathogen entry and proliferation, providing a framework for comparing resistant and susceptible host responses in subsequent analyses.

It is important to consider the sequential stages that occur between pathogen introduction and visible symptom development in plants, which reflect both pathogen virulence strategies and host immune responses. The transition from pathogen introduction to visible symptom development involves multiple sequential stages, reflecting both pathogen virulence strategies and host immune responses. Upon encountering a suitable host and favorable environmental conditions, primary inoculum such as zoospores or conidia germinates. Zoospores, in particular, exhibit rapid chemotactic movement toward roots, attaching in a short time and initiating infection (Doehlemann et al., 2017). Conidia, on the other hand, are non-motile and require passive contact with the root surface for activation.

Following attachment, the pathogen initiates host penetration through enzymatic degradation of physical barriers using cell wall degrading enzymes such as cellulases and pectinases (H. Wang et al., 2019). Concurrently, plants detect conserved microbial signatures known as pathogen-associated molecular patterns (PAMPs), triggering PAMP-triggered immunity (PTI) (Doughari, 2015; Y. Wang et al., 2019). However, successful pathogens often suppress this defense via effector-mediated interference, leading to colonization and, eventually, disease symptom expression (Sephton-Clark & Voelz, 2018). Our experiments demonstrated that a temporal gap exists between initial pathogen colonization and the appearance of visible symptoms, which is both pathogen-and environment dependent.

Based on our findings, the critical lag period between initial infection and symptom appearance typically spanned 2-4 days. This period is essential for dissecting the molecular interplay between pathogen virulence and host defense. For both *A. euteiches* and *F. avenaceum*,

the early stages post-inoculation are decisive: pathogen biomass increases rapidly, while host responses are either mounting or being suppressed. Studying this window is therefore key for identifying resistance mechanisms and developing targeted interventions.

For *A. euteiches*, the mini hydroponic trial provided ideal conditions for rapid zoospore activity and early infection. Zoospores, being motile, attached and initiated infection on pea roots within 30 minutes. Supporting this, previous observations from our lab (Chatterton lab, data not shown) showed a marked decline in free zoospore numbers within 30 minutes of exposure to pea roots, indicating swift colonization. Under these conditions, symptoms began to appear by 24 hpi, and DSR reached ~3 by 5 dpi much earlier than in greenhouse trials with solid substrate, where symptom onset was delayed. These observations underscore that the period before 24 hpi is a critical window for host-pathogen interaction studies under ideal (hydroponic) conditions, where both early immune recognition and pathogen suppression of defenses are actively occurring. Thus, to capture these dynamic interactions, we selected 2, 6, 12, and 24 hpi as molecular sampling time points. In greenhouse conditions and solid growth media while qPCR detection confirmed high pathogen biomass at later stages (e.g. 4 dpi), such stages likely reflect a breakdown of host defense and offer limited insight into resistance mechanisms. Focusing on the pre-symptomatic phase allows us to better resolve the kinetics of defense signaling and pathogen establishment.

In contrast, *F. avenaceum* follows a slower infection trajectory. Unlike zoospores, *F. avenaceum* macroconidia are non-motile and require a medium for transfer to the host surface. Our observations were consistent with previous reports that FRR symptoms typically emerge around 5–6 dpi, progressing from initial pathogen contact to visible lesion development and ultimately to full colonization by 9 dpi. Therefore, we selected 2 hpi (initial exposure to host), 3

dpi (during infection preprocess), 6 dpi (symptom manifestation/development), and 9 dpi (fully colonized stage) as representative time points for your next experimental design. Early molecular detection by qPCR at 2 hpi and 3 dpi captured the onset of pathogen establishment, while later time points (6 and 9 dpi) reflected the expected transition to symptom manifestation and extensive tissue colonization. Together, these results align with the established trajectory of FRR development, confirming the reliability of our system for monitoring both early and late stages of infection.

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TABLES AND FIGURES

Table 3.1. Primers and probes used in *A. euteiches* detection from pea root samples.

Organism	Target	Direction	Primer name	Sequence information
A. euteiches	ITS	Forward Primer	Ae1.2_ITS1_F	CCT GCG GAA GGA TCA TTA CC
		Reverse Primer	Ae1.2_ITS_R	AAA ATT ACA TCG GTT CCT TGC G
		Probe	Ae1.2-ITS_Pr	/56-FAM/TTC TTT ATG/ZEN/AGG CTT GTG CTC TT/3IABkFQ/
F. avenaceum	*EF-1α*	Forward Primer	AveF	GCTTATCTGCACTCGGAACC
		Reverse Primer	AveR	CGCGTAATCGAAGGGATATT
		Probe	AvePr	HEX-CGACAAGCGAACCATCGAGA-BHQ-1
Pea (*Pisum sativum*)	*β TUB1*	Forward Primer	Psat-Tub_F	TGGTTCTTGACAATGAAGCCTTG
		Reverse Primer	Psat-Tub_R	AGGGTCAACATAGGAGAGTGAA
		Probe	Psat-Tub_Pr(hex)	/5HEX/CT GAA GCT T/ZEN/A GTA ACC CAA GCT GTA AGC T/3IABkFQ

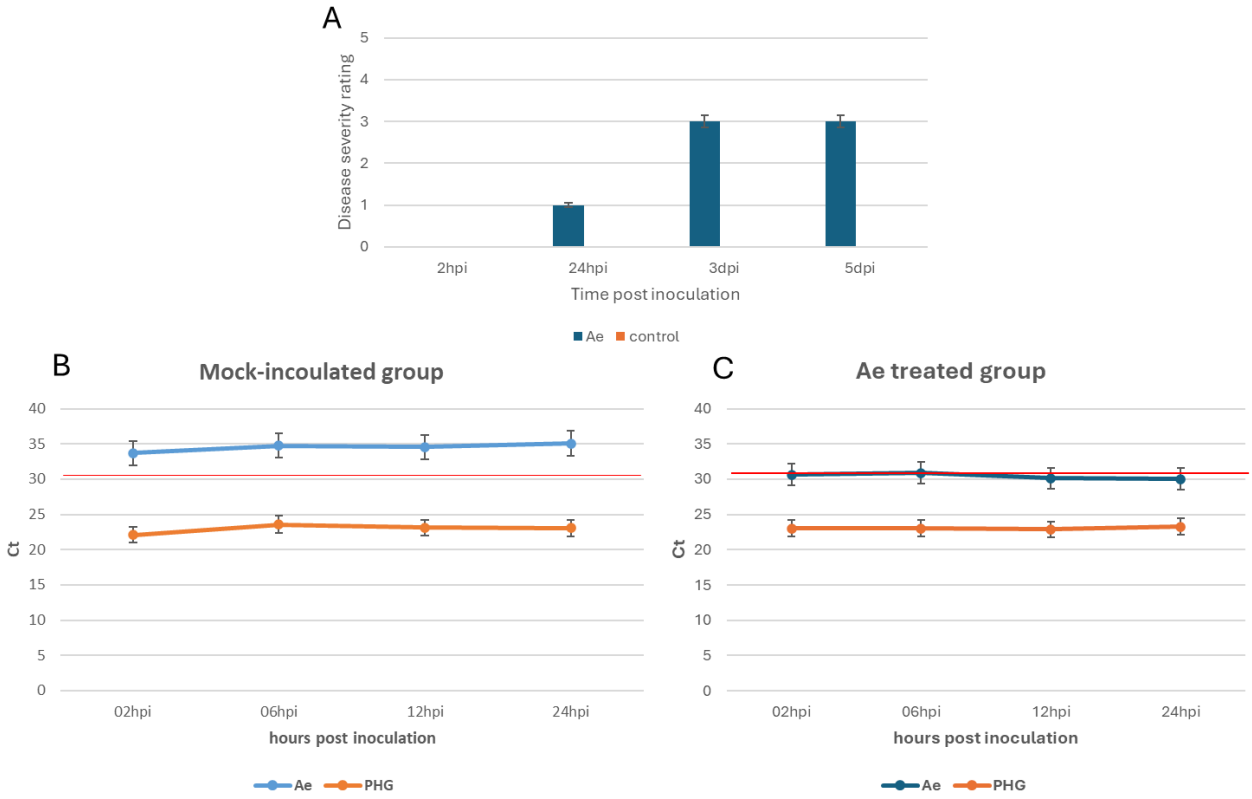

Figure 3.1. Disease severity ratings and *A. euteiches* biomass assessment via qPCR. Disease severity ratings of *A. euteiches*-inoculated or mock-inoculated plants grown in a hydroponic system at various time points up to 5 dpi (A). qPCR cycle-threshold (Ct) values for pathogen detection are shown for mock-inoculated plants (B) and *A. euteiches*-treated plants (C) in the hydroponic system. *Ae* indicates qPCR detection of *A. euteiches* ITS gene and PHG detection of pea housekeeping gene (β-Tubulin). The red line indicates the limit of detection for *Ae*, and values above this line are considered negative

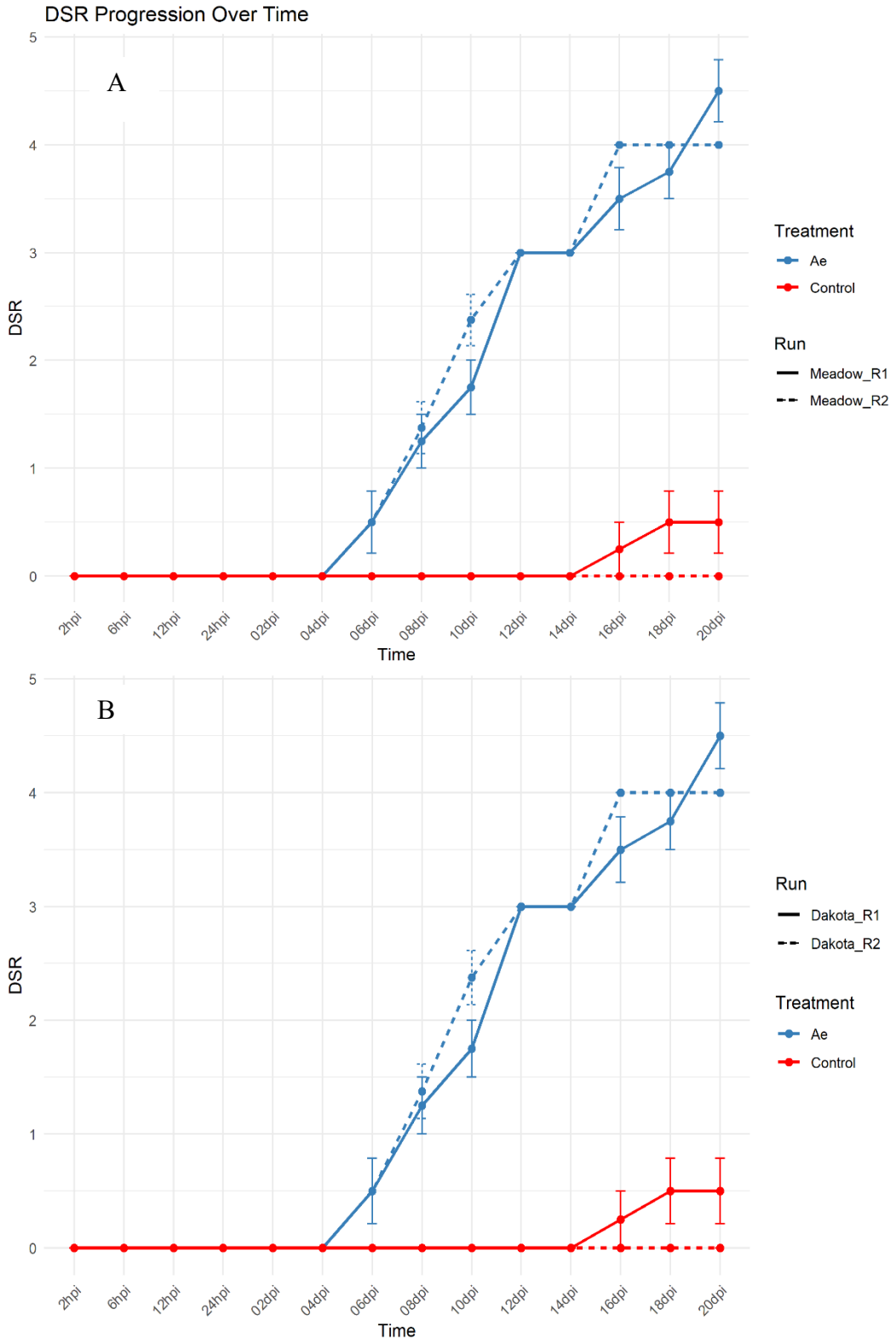

Figure 3.2. Disease severity ratings (DSR) over time for *A. euteiches* inoculated plants (red) or mock-inoculated plants (blue) grown under greenhouse conditions, shown for CDC Meadow (A) and CDC Dakota (B). _R1 and _R2 represent replicated experimental runs for each cultivar.

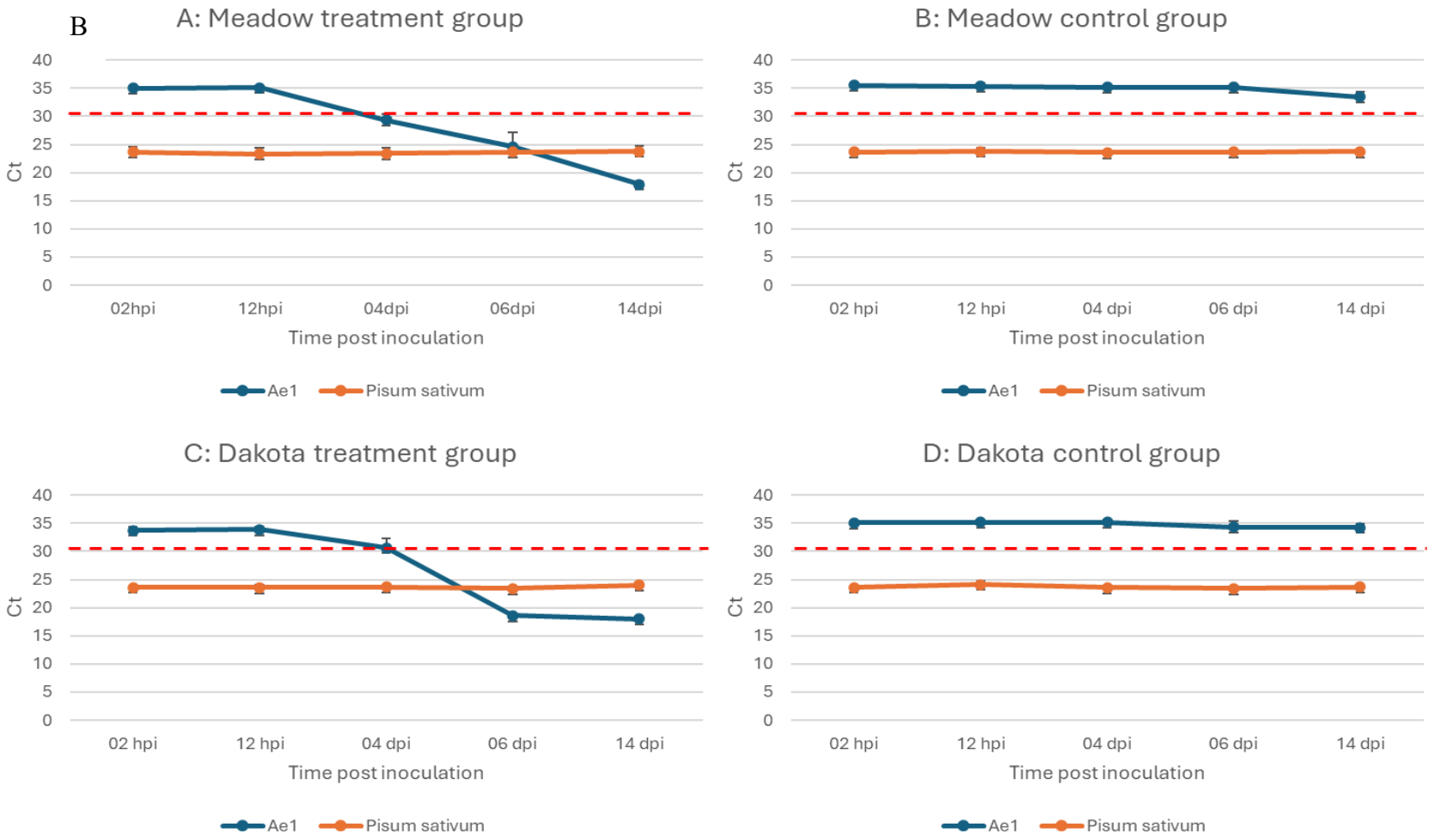

Figure 3.3. Quantitative PCR results showing pathogen detection and change in DNA quantity over time for *A. euteiches*-inoculated (A, C) and control (B, D) groups in CDC Meadow (A, B) and CDC Dakota (C, D). The red dashed line indicates the cycle-threshold (Ct) limit for pathogen detection. Mean values are combined from 2 experiments. Blue line represents the Ct value for *Ae* (ITS) gene and orange line represent Ct value progression for pea house keeping genes (β-Tubulin).

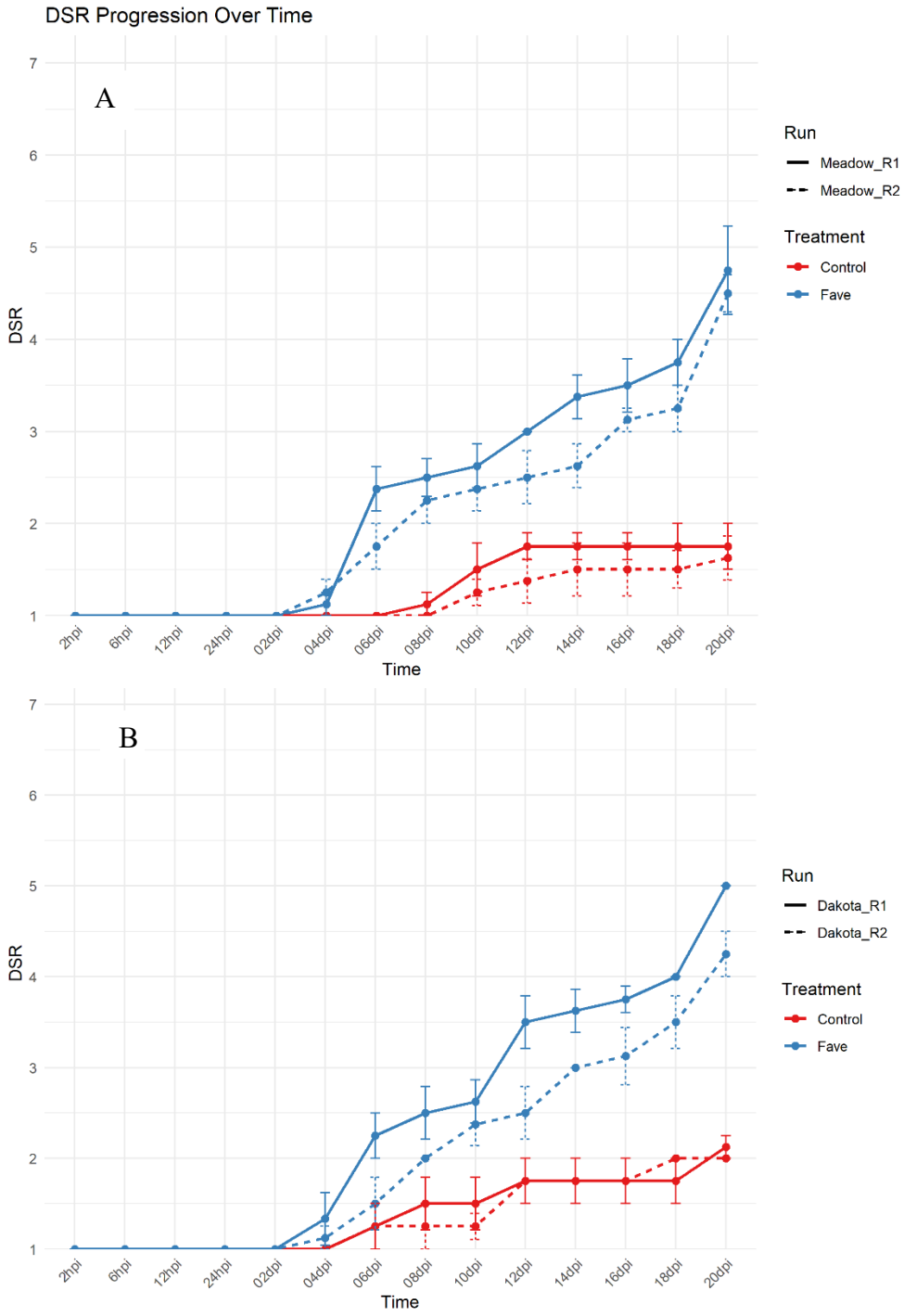

Figure 3.4. Disease severity ratings (DSR) over time for *F. avenaceum*-inoculated (blue; *Fave*) and mock inoculated (red; control) plants grown under greenhouse conditions, shown for CDC Meadow (A) and CDC Dakota (B). _R1 and _R2 represent replicated experimental runs for each cultivar.

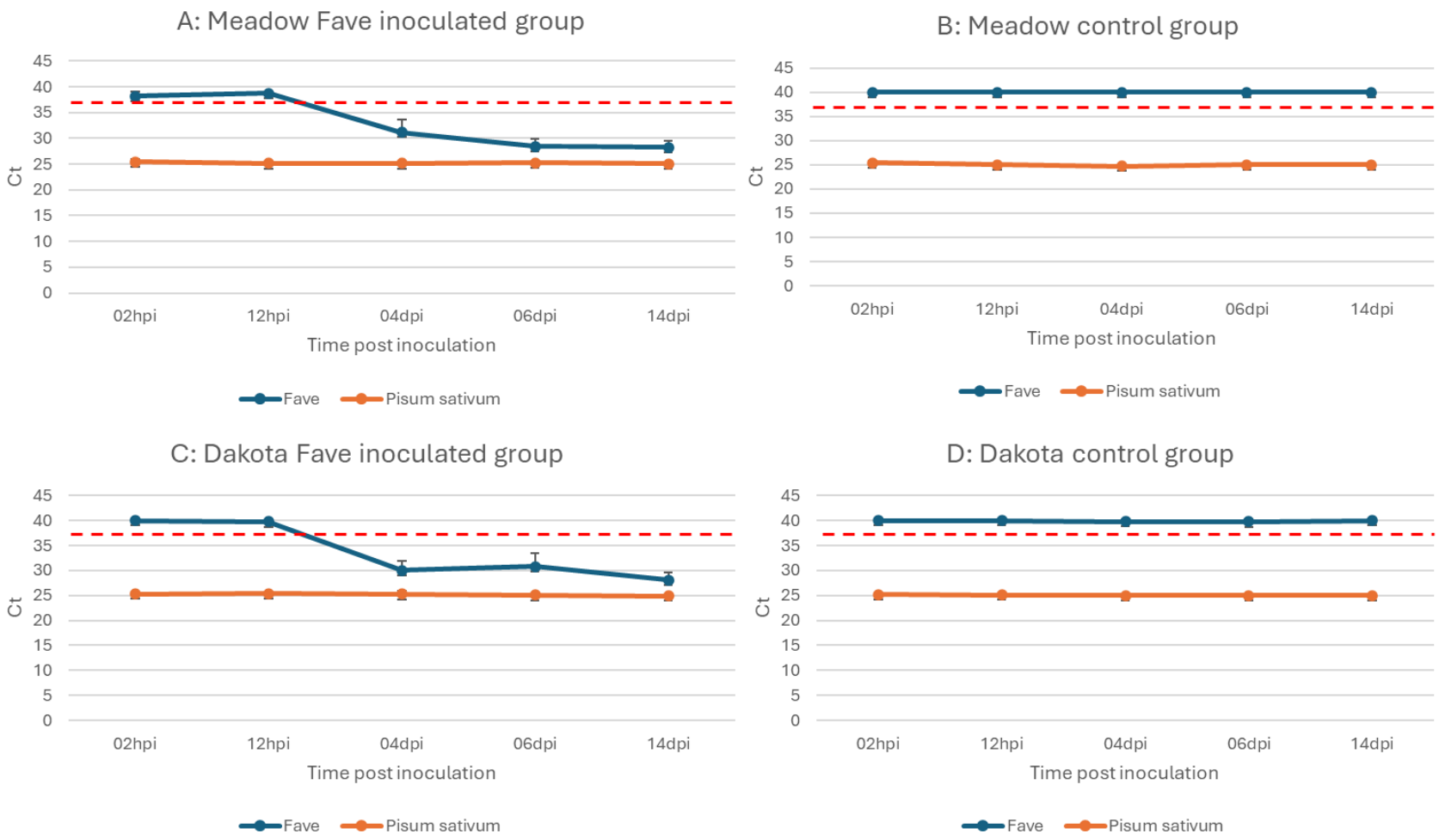

Figure 3.5. Quantitative PCR results showing pathogen detection and disease progression over time for *F. avenaceum* inoculated (A, C) and control (B, D) groups in CDC Meadow (A, B) and CDC Dakota (C, D). The red dashed line indicates the cycle threshold (Ct) for pathogen detection. Blue line represents the Ct value for *Fave* (EF-1α) gene and orange line represent Ct value progression for pea house keeping genes(β-Tubulin).

CHAPTER 4 : TRANSCRIPTOMIC AND TARGETED METABOLOMIC ANALYSES OF PARTIALLY RESISTANT AND SUSCEPTIBLE PEA GENOTYPES REVEAL DIFFERENTIAL DEFENSE RESPONSES DURING THEIR INTERACTION WITH *APHANOMYCES EUTEICHES*

This chapter is accepted for publication in Legume Science. Inevitably, there might be some repetition of content between chapter 1 and introduction section of this chapter.

Pandit, S., Hodan Halane, H., Lim, N. Z-L., Singer, S. D., Dhaubhadel, S., Goyal, R., Zhang, W., Foroud, N. A., Schultz, E., & Chatterton, S. (2025). Transcriptomic and targeted metabolomic analyses of partially resistant and susceptible pea genotypes reveal differential defense responses during their interaction with *Aphanomyces euteiches*. Legume Science (Manuscript accepted).

ABSTRACT

Aphanomyces root rot (ARR) incidence and prevalence are increasing throughout pea-growing regions worldwide, highlighting the need for effective management strategies to safeguard yields. Understanding pea defense against ARR is essential for developing resistant cultivars. To decipher defense mechanisms, a comprehensive transcriptional study was conducted using RNA-seq from roots of partially resistant (PR, PI 660736, PI 660729, PI 557550 and 5001) and a susceptible (S, CDC Meadow) pea genotype at various time points (2, 6, 12, and 24) hours post pathogen inoculation (hpi). We found significant differences in metabolic pathway enrichment between the PR and S genotypes, with notable distinctions at 6 and 24 hpi. At 6 hpi, *ethylene-activated signaling* and *phosphorylation pathways* were enriched across all PR genotypes, whereas *response to abscisic acid* and *response to stress* were enriched only in the S genotype. By 24 hpi, PR genotypes demonstrated upregulation of processes related to *regulation of DNA-templated transcription*, and *RNA biosynthetic and metabolic process*, while the processes linked to *shift in photosynthesis and energy reallocation* were associated with S genotype. The PR genotypes also exhibited upregulation of critical defense signaling genes, including WRKY transcription factors, ethylene response factors, MAPKs, and JAZ proteins, which remained unchanged in the S genotype. RNA-seq and targeted metabolomics revealed the upregulation of

pisatin (a phytoalexin) biosynthesis genes and the accumulation of pisatin in the PR genotypes. Our results suggest that partial resistance is controlled by the ethylene signaling pathway, which modulates transcription factor activity and initiates wholescale changes in transcription, leading to activated phenylpropanoid and ultimately pisatin biosynthesis. These findings provide novel insights into pea partial resistance to *Aphanomyces euteiches*, paving groundwork for resistance breeding and alternative strategies to mitigate the impact of ARR.

Keywords: ARR, Partial resistance, RNA-seq, Defense.

4.1 INTRODUCTION

Field pea (*Pisum sativum* (L.), 2*n*=14) is among the most important legume crops in the world in terms of production, consumption and nutritional value alongside dry bean (*Phaseolus vulgaris)*, chickpea (*Cicer arietinum*) and lentil (*Lens culinaris)* (Joshi & Rao, 2017; Singh, 2017). Field pea covers about 8% percent of the global pulse growing area and accounts for 14.6% of global pulse production (Bhat et al., 2022; DPD-GOI, 2023; FAO, 2024; Joshi & Rao, 2017). It is an important source of protein for humans and livestock (Hossain et al., 2012), and provides essential amino acids, such as lysine and tryptophan, that are deficient in cereal grains (Kumari & Deka, 2021). It is also important for soil health as it helps improve soil fertility due to its association with *Rhizobium* bacteria, which fix atmospheric nitrogen in root nodules (Meena et al., 2018). The major field pea growing areas include North America, Europe, Australia, and Asia (Gossen et al., 2016). Between 2014 and 2022, Canada was the largest pea exporter in the world and also among the top producers, accounting for 25% of global production in 2022, of which more than 80% came from the Canadian prairies (AAFC, 2020). However, in 2022, Russia overtook Canada in both pea production and export (Boersch, 2024). This decline in

production in Canada may be due, at least in part, to root rot pressure which has led to acreage reduction of this crop.

Aphanomyces root rot (ARR), which is caused by the oomycete, *Aphanomyces euteiches*, is a major threat to pea production worldwide and it is now the biggest constraint to total annual pea production in Canada (Wu et al., 2018). It is the most destructive soil-borne pathogen of field pea in temperate and humid environments, with a host range that is limited to the *Fabaceae* family. Indeed, several crops within this family, such as lentil, dry bean, clover, faba bean, alfalfa, are also susceptible. *A. euteiches* has a broad geographical range, affecting crops in North America, Australia, New Zealand, Europe, and Japan (Gangneux et al., 2014). Major pathotypes of *A. euteiches* infecting pea in Canada are pathotype I (is virulent on all six lines of the French pea differential set, the standard used to define pea pathotypes) with a few isolates belonging to pathotype III (avirulent on MN313 but virulent on the other five) (Moussart et al., 2024; Sivachandra Kumar et al., 2021; Wicker & Rouxel, 2001). The lifestyle of *A. euteiches* is characterized by filamentous growth habit, nutrition by absorption of nutrients after enzymatic degradation of host cell, and reproduction via spores. It starts as biotroph but later kills host cells to continue its lifecycle classifying it as a hemibiotrophic oomycete (Gaulin et al., 2007; Thalineau et al., 2018). ARR symptoms include honey-brown lesions on cortical tissues of the root that progress to the destruction of the cortex, resulting in stunting and chlorosis of the shoots and premature death (Willsey et al., 2018). Yield reduction can be as high as 100% when ARR is severe (McGee et al., 2012; Wu et al., 2018).

There are no effective management options for root rot diseases in pea and disease-resistant genotypes are not commercially available. The biggest challenge in ARR management results from the ability of oospores (resting spores) to remain viable in the soil for up to a decade

as they are resilient to temperature fluctuations and desiccation (Kraft et al., 1990; Zitnick-Anderson et al., 2021). Oospores germinate in the presence of root exudates to form sporangia and release motile zoospores that encyst on root tissue. Infection can occur at any plant growth stage (Chatterton et al., 2019; Gaulin et al., 2007). The only option to reduce inoculum levels in the soil is crop rotation of up to 10 years, which not only limits the availability of land for cultivation of pulses but importantly has an overall negative impact on cropping systems due to the loss of pulses in a rotation. While producers do have access to seed treatments such as Rancona Trio (ipoconazole, carbathiin and metalaxyl; UPL Agrosolutions, Ontario Canada) and Zeltera Pulse (ethaboxam, inpyrfluxam, mandestrobin and metalaxyl; Nufarm, Alberta Canada) that suppress *A. euteiches* infection during the early seedling stage, these seed treatments options do not reduce disease severity when infection occurs at later plant growth stages (Willsey et al., 2021). Biocontrol agents have also been tested and shown promising results *in vitro,* however they are less consistent under field conditions and their efficacy often depends on environmental conditions, formulation and application timing (Godebo et al., 2020; Wakelin et al., 2002). Other cultural practices such as avoiding waterlogging, improving drainage, and delayed planting in wet springs can be some options to minimize yield losses due to ARR but are difficult to carry out in field operations (Allmaras et al., 2003; Karppinen et al., 2020). An ideal disease management strategy or component thereof would be to grow resistant genotypes. Unfortunately, field pea varieties with high levels of ARR resistance are not available. Breeding efforts have led to the development of some genotypes with polygenic partial resistance (PR), such as PI 660736, PI 660729 (McGee et al., 2012), and PI 557500 (USDA-GRIN). This germplasm can be studied to facilitate the further development of resistant genotypes for commercial purposes. Previous research has identified major and minor effect quantitative trait loci (QTL) associated with PR to

ARR. Major-effect QTL include *Ae-Ps 4.5* and *Ae-Ps7.6* on chromosomes (chr) 4 and 7, respectively, while minor-effect QTL are identified on chr 1, 2, 3, 4, 5, and 6 (Desgroux et al., 2016; Hamon et al., 2011; Clément Lavaud et al., 2015; Leprévost et al., 2023; Pilet-Nayel et al., 2002; Pilet-Nayel et al., 2005).

Plants can recognize external stimuli from pathogens/microbes or abiotic sources. Once triggered by pathogens, plants activate immune responses that result in complex morphological, physiological, and biochemical mechanisms of defense in the early infection process (Boller & Felix, 2009; Latge, 2010; Raaymakers & Van den Ackerveken, 2016). This capacity of plants to recognize pathogens is not only limited to conserved molecules of pathogens but also variable elicitor molecules (Dodds & Rathjen, 2010). These conserved molecules are termed pathogen-associated molecular patterns (PAMPs), and the corresponding host plant molecules that recognize them are termed pattern recognition receptors (PRRs), which are mainly present in plasma membranes (Boller & Felix, 2009; Kamoun, 2003; Y. Wang et al., 2019). For example, cell wall polymers of pathogens such as the oligoglucosides β-1,3;1,6-glucans, act as PAMPs for plant surveillance mechanisms. PRRs mediate the recognition of PAMPs by triggering Ca^{2+} fluxes as signaling cascades (known as spiking) and protein kinases cascades that signal and activate a series of host responses (Attard et al., 2008). Plant immunity activated by these PAMPs is termed PAMP-triggered immunity (PTI) (Dodds & Rathjen, 2010; Jones & Dangl, 2006). PAMP recognition initiates multiple orders of signaling events resulting in responses such as a burst of reactive oxygen species (ROS), cell wall strengthening by callose deposition, and the activation of defense gene expression (Zipfel, 2014). PTI does not always successfully defend against pathogens since pathogens also deploy secreted proteins known as effectors that act as a counter-defense to PTI and promote pathogen proliferation in the host. This condition of

suppressed PTI in plants due to pathogen effectors is termed effector-triggered susceptibility (ETS). Effectors can act in the cell or the apoplast and are recognized by R proteins directly or indirectly. Recognition activates effector-triggered immunity (ETI), another layer of host plant defense that is stronger than PTI Growing evidence indicates that PTI and ETI share a large number of components.

In field pea, studies to understand defense responses to *A. euteiches* are limited (Goyal et al., 2024; Hosseini et al., 2015; Kälin et al., 2024; Lavaud et al., 2016; Clément Lavaud et al., 2015; Pilet-Nayel et al., 2002; Pilet-Nayel et al., 2005; Wu et al., 2021; Wu et al., 2022). As such, the development of enhanced genetic and genomic resources will aid in understanding disease resistance responses. The development of computational resources for handling next-generation sequencing (NGS) tools such as RNA sequencing (RNA-seq) provides a powerful approach for transcript analysis. RNA-seq allows for the high-throughput quantification of RNA transcript levels with high sensitivity and broad genome coverage. RNA-seq can be utilized to study the root transcriptional response of partially resistant pea genotypes and molecular pathways associated with partial resistance. To improve the level of resistance in breeding lines, the identified defense-related genes can be incorporated through marker-assisted selection or gene editing approaches. The availability of a reference *P. sativum* genome with most of the genes annotated provides a valuable opportunity to investigate the transcriptome for plant response after inoculation with *A. euteiches* (Kreplak et al., 2019; Yang et al., 2022). A comprehensive metabolomics study further helps to understand biochemical changes that occur after pathogen inoculation and thus complements and validates the gene expression data provided by RNA-seq. In this study we first screened a larger set of pea genotypes in field microplots and greenhouse to confirm their differential resistance level and selected contrasted

genotypes for RNA-seq study. We then identified differentially expressed genes in roots between a susceptible (S) genotype (CDC Meadow), and PR genotypes (PI 660736, PI 660729, PI 557500, and 5001) infected with *A. euteiches* and further validated major results with metabolomics.

4.2 MATERIALS AND METHODS

4.2.1 Inoculum preparation

4.2.1.1 Oospore production

Moderately aggressive isolates of *A. euteiches Ae1, Ae6* and *Ae7* (all pathotype I, (Sivachandra Kumar et al., 2021) isolated from pea roots in Saskatchewan, Canada, were stored on cornmeal agar plugs in sterile distilled water in glass tubes at room temperature. An agar plug was transferred to a potato dextrose agar (PDA, Sigma) plate and maintained at room temperature for up to a month. To prepare oospores, 5-day-old mycelial plugs on PDA were transferred to sterile homogenized and filtered oatmeal broth in 250 mL Erlenmeyer flasks with 5 plugs/30 mL broth (Windels, 2000). The flasks were incubated in the dark for 30 days at room temperature. After incubation, the *A. euteiches* mycelial mats with oospores were homogenized in a Waring blender for 5 mins followed by filtering using four layers of cheesecloth to separate the oospores (Gangneux et al., 2014). The filtrate was then centrifuge filtered through 100-µm cell strainers (VWR, Edmonton AB) at 4,000 rpm. The concentration of oospores in the suspension of each isolate was counted using a hemocytometer and diluted to appropriate concentrations.

4.2.1.2 Zoospore production

Twenty mycelial plugs from the outer edges of an actively growing *A. euteiches* culture on PDA were added to 200 mL Erlenmeyer flasks containing 40 mL of freshly prepared peptone glucose broth and incubated in the dark for 2-3 days (Windels, 2000). Peptone glucose broth was prepared by dispensing 20g/L peptone and 5g/L glucose in deionized water followed by autoclaving. Following 2-3 days of incubation, the inoculated broth was decanted, and the mycelial plugs were washed using 50% mineral salt solution (MSS) and swirling the suspension vigorously to dispense salt solution throughout all mycelial networks. After mixing, the solution was left to sit for 1.5 hours at room temperature. After 1.5 hours the suspension was discarded and washed again with 50% MSS in a similar manner. After the second wash and incubation, 30 mL of full-strength MSS was added to the flasks and incubated overnight at room temperature. Subsequently, the solution was decanted and aliquoted to count zoospores using a hemocytometer and brought to the required concentration by adding full-strength MSS.

4.2.2 Germplasm screening under greenhouse conditions

To confirm partial resistance and choose genotypes for downstream analyses, 31 pea genotypes (Table S1) (Bodah et al., 2016; Feng et al., 2010; Kraft, 1992; Porter et al., 2014) including one known S genotype (CDC Meadow), were screened for their response to *A. euteiches* (*Ae1*) inoculation. To minimize infection by seed-borne pathogens such as *Fusarium* and *Pythium* spp., seeds were surface sterilized in 10% commercial bleach for 3 minutes followed by treatment with the fungicide Apron Maxx RTA (metalaxyl and fludioxonil, Syngenta, Canada) at 325 mL/100 kg. Vermiculite was autoclaved and then inoculated with oospores at 200 oospores/g of vermiculite. The inoculated vermiculite was used to fill the 32 cells of a rootrainer block (Rootrainer International, Canada) and 2 pea seeds of each genotype

were planted into each cell in a randomized complete block design with 10 replicated blocks. Plants were grown under standard greenhouse conditions at 22°C/ 16°C (day/night) with a 16 h photoperiod. After 3 weeks of growth, plants were also inoculated with 5 mL/cell of 1000 zoospore/mL suspension. Two weeks after zoospore inoculation, all plants were rated for disease severity on a scale of 0-5, where 0 indicates a healthy plant and 5 indicates a plant that is dead due to ARR (Pilet-Nayel et al., 2009). The experiment was repeated twice. Disease severity rating (DSR) score data on individual plants were analyzed using the non-parametric Kruskal Wallis test and adjusted for multiple comparisons using the Dunnet test with R software version 4.4.0 (RStudio, 2020).

4.2.3 Field experiment

All but six genotypes that were initially screened in the greenhouse (Table S1) were further evaluated under field conditions in 2020 to confirm the level of resistance; the excluded genotypes lacked sufficient seed for field evaluation. The 23 tested genotypes, along with 1 inoculated and 1 non-inoculated S genotype (CDC Meadow), were evaluated in microplots with three replications. Seventy-five microplots were constructed at the Lethbridge Research and Development Centre to ensure soil is retained within the plots, each with a 26 cm deep x 23 cm diameter hole fitted with a PVC irrigation pipe (25.4 cm length, 22.86 cm diameter). Each microplot pipe was back-filled with (i) the excavated field soil mixed with 2.3 kg of *A. euteiches* infested soil obtained from a nearby pea field in Lethbridge, Alberta (which have unknown isolates), that was confirmed to cause high disease severity levels and (ii) 500 mL of *A. euteiches* oospores (*Ae6* and *Ae7* isolates from Alberta fields, pathotype I) (Sivachandra Kumar et al., 2021) inoculated vermiculite to ensure uniform and virulent inoculum was present. In each

microplot, twenty seeds per genotype were hand-sown at a depth of 3.8 cm. Roots were sampled 2 months post-seeding and rated for disease severity on a scale of 0-5. The disease severity rating for each replicate was calculated for all genotypes as follows: DSR = $[\sum(R_i \times N_i)/(N_t)]$, where Ri = disease severity rating for category i, Ni = number of plants in category i with rating R_i, N_t = total number of plants evaluated). In 2021, seven genotypes selected from the 2020 field test were subjected to re-evaluation under field conditions as described for 2020.

4.2.4 Planting, inoculation, and root sampling for RNA-seq

Five pea genotypes were considered for the RNA-seq experiment based on greenhouse screening and field tests, as well as previous studies (McGee et al., 2012). Four of the genotypes were designated as partially resistant (PR genotypes) to *A. euteiches* and one was an elite S-genotype (Table 4.1). Experimental design consisted of five pea genotypes, three replicates, four time points, and two treatments for RNA-seq, with an extra set of three replicates maintained for 14 days post-inoculation (dpi) for disease severity rating.

Briefly, seeds were surface sterilized with 10% bleach followed by treatment with Apron Maxx RTA at 325 mL/100 kg. Two seeds per cell were then sown in sterilized vermiculite in rootrainers and grown under the same greenhouse conditions as in the germplasm screening. After 14 days of growth, the rootrainers were soaked (ensuring roots were immersed) in 2 L of *A. euteiches*-isolate *Ae1* zoospore suspension containing 1000 zoospores per/mL for one hour to ensure that there was a discrete time point of infection. Mock-inoculated control plants for each genotype were soaked in full-strength MSS for one hour. The treatments were arranged in a randomized split-plot design with three replications with germplasm as the main factor and time of sampling as the sub-plot factor. Roots (2 root systems/cell/sample) were sampled for RNA

extraction at 2, 6, 12, and 24 hours post-inoculation (hpi; from when the trays were removed from the inoculum) in the three replicates since a preliminary experiment indicated that *A. euteiches* infection could be detected in roots via qPCR as early as 24 hpi (data not shown). Time points before 24 hpi were considered with the aim of capturing early defense responses before the roots are colonized by the pathogen. Following sampling, roots were carefully washed, immediately flash-frozen in liquid N_2, and stored at -80°C until RNA extraction.

The remaining plants were rated for their disease severity on a scale of 0-5 at fourteen dpi,. The DSR data at 14dpi were analyzed in the same way as in the germplasm screening.

4.2.5 RNA extraction, library preparation, and sequencing

Total RNA was extracted from root samples using the RNeasy Plant Mini kit (Qiagen Inc., Toronto, ON, Canada) according to the manufacturer's instructions. Residual DNA was removed using RNase-free DNaseI (Thermofisher Scientific, Ottawa, ON). DNA removal was further confirmed by PCR Test using Invitrogen Platinum PCR SuperMix High Fidelity (Thermofisher Scientific, Ottawa, ON) followed by gel electrophoresis. RNA quality was verified using a Bioanalyzer and Agilent RNA 6000 Nano kit (Agilent Technologies, Mississauga, ON, Canada), while quantity and integrity were again assessed before library preparation using a 5K / RNA / Charge Variant Assay LabChip GX and RNA Assay Reagent Kit (Perkin Elmer, Woodbridge, ON, Canada).

Library preparation and sequencing were performed by a service provider (Genome Québec Centre D'Expertise et de Services, Montreal, QC, Canada). In brief, 250 ng of total RNA was used for mRNA enrichment using the NEBNext Poly (A) Magnetic Isolation Module (New England BioLabs), and cDNA synthesis was achieved with the NEBNext RNA First Strand

Synthesis and NEBNext Ultra Directional RNA Second Strand Synthesis Modules (New England BioLabs, Whitby, ON, Canada). The remaining steps of library preparation were carried out using the NEBNext Ultra II DNA Library Prep Kit for Illumina (New England BioLabs, Whitby, ON, Canada). Libraries were quantified using the Kapa Illumina GA with Revised Primers-SYBR Fast Universal kit (Kapa Biosystems, Toronto, ON, Canada), and average fragment size was determined using a LabChip GX (PerkinElmer, Woodbridge, ON, Canada) instrument. Sequencing was performed using a NovaSeq 6000 S4 platform (Illumina Inc., San Diego, CA, USA) with 100-bp paired-end reads.

4.2.6 Processing of RNA-sequencing data

Illumina RNA-seq FASTQ files were processed using the nf-core/RNA-seq pipeline (Ewels et al., 2020), version 3.11.1. Briefly, raw reads were initially trimmed using TrimGalore version 0.6.7, (https://doi.org/10.5281/zenodo.5127899), and trimmed reads were then aligned to the *Pisum sativum* reference genome using the STAR aligner version 2.7.10a (Dobin et al., 2013). Reference genome (FNA/FA files) and annotations (GTF files) of Caméor (*Pisum_sativum*_v1a) genotype from Ensembl (release 56) was used to align the FASTQ files (Kreplak et al., 2019). Read quality was also assessed using various tools (e.g., Picard, QualiMap, FastQC, etc.) included in the nf-core/RNA-seq pipeline. Aligned reads were then quantified using RSEM version 1.3.1 (Li & Dewey, 2011) producing gene count matrices.

All downstream analyses were conducted using R (version 4.3.1). Principle component analysis (PCA) was carried out to confirm that biological replicates were grouped by genotype and treatment Read counts were TMM-normalized using the edgeR package (Robinson et al., 2009). Differential expression analyses were performed between inoculated and mock-inoculated

groups at each time point for each genotype, respectively. Genes that met both a false discovery rate (FDR) threshold of 0.05 and a $\log_2$-fold-change threshold of ≤ 1 and ≥ 1 were considered to be differentially expressed genes (DEGs). The fold-change threshold as 1 was determined using the RNASeqPower package in R (Hart et al., 2013). Upset plots were created using the R package 'UpsetR'(Conway et al., 2017), while heatmaps and the mapping of DEGs onto chromosomes were created using the R package ggplot2 (Wickham, 2011). GO term enrichment analysis was performed using g:profiler (Raudvere et al., 2019) at a threshold of $P<0.05$. Pathway analysis was performed using the iDEP web application (Ge et al., 2018). The sequence data generated in this study are available at the National Center for Biotechnology Information (NCBI) Sequence Read Archive (BioProject ID PRJNA1208110).

4.2.7 Metabolomic study

Root samples (100 mg) from inoculated and mock-inoculated plants of S genotype CDC Meadow and PR genotype PI 660736 harvested at 2, 6, 12 and 24 hpi were subjected to root metabolite extraction with some modifications (Anguraj Vadivel et al., 2021). Briefly, frozen root samples were ground in liquid N2 and extracted in methanol:water (4:1, v/v). Samples were then sonicated for 15 min followed by centrifugation at 11,000 × *g* for 10 min at 4 °C. Subsequently, supernatant (700 µL) was evaporated under N_2 gas before being re-suspended in 380 µL of methanol:water (1:1, v/v). The samples were then filtered using a 0.2 µm PTFE syringe filter (Millipore, USA).

4.2.7.1 Liquid Chromatography-Mass Spectrometry

For high-resolution mass spectrometry analysis, samples (5 µL) were injected onto an Agilent 1290 HPLC coupled to a Q-Exactive Quadrupole Orbitrap mass spectrometer (Thermo Fisher Scientific, USA) with a Zorbax Eclipse Plus RRHD C18 column (2.1 x 50 mm, 1.8 µm) maintained at 35°C. The samples were run at a flow rate of 0.3 mL/min. The mobile phases were water with 0.1% formic acid (A) and acetonitrile with 0.1% formic acid (B). Phase B was held at 0% for 0.75 min, increased to 22% over 0.5 min, to 35% in 2.75 min, to 100% over 3.5 min, held for 2.5 min, and returned to 0% over 30 seconds. Heated electrospray ionization (HESI) conditions included spray voltages of 3.9 kV (ESI+) and 3.7 kV (ESI-), capillary temperature at 400 °C, probe heater at 450 °C, sheath gas at 17 arbitrary units, auxiliary gas at 8 arbitrary units, and S-Lens RF level at 45%. Detection was conducted using a top 5 data-dependent acquisition (DDA) in positive ion mode with full MS scans between *m/z* 100-1200 at 35,000 resolution, automatic gain control (AGC) target of $3x10^6$, maximum injection time (IT) of 128 ms, and intensity threshold of $8x10^5$. MS/MS spectra were collected at 17,500 resolution, AGC target of $1x10^6$, max IT of 60 ms, and isolation window of 1.2 *m/z*, using a normalized collision energy (NCE) of 30.

Raw files from LC-MS were converted to mzml format with ProteoWizard (Kessner et al., 2008) and imported to R v4.3.2 using the XCMS package (Smith et al., 2006). Features were detected using the centWave method with the following prefilters: part per million (ppm) of 5, LC trace retained with 5 scans and minimum 5000 intensity, signal: noise threshold of 5, and a noise of $1.0x10^6$ (Tautenhahn et al., 2008). Feature grouping criteria were established for analytes found in a minimum of 25% of all samples, considering a retention time deviation of 10 seconds and a *m/z* width of 0.015. The 'fillPeaks' function was applied with default settings.

Targeted compounds were identified by comparing retention times and *m/z* to authentic standards. When necessary, all features were normalized using Z-score transformation. Significant

differences in metabolite levels in *P. sativum* roots subjected to two treatments across various time-points were determined using a two-way analysis of variance (ANOVA) followed by a post-hoc Tukey's for multiple comparisons. A p-value of <0.05 was considered statistically significant. All data analysis and visualization were performed and generated using R v4.3.2 through RStudio v2023.6.2.0 (RStudio, 2023).

4.3 RESULTS

4.3.1 Identification of pea genotypes that are partially resistant to *A. euteiches* infection

First, genotypes partially resistant to *A. euteiches* infection were identified through a combination of greenhouse and field trials. Under greenhouse conditions, of 30 genotypes tested against susceptible CDC Meadow, a significantly lower DSR than the S genotype CDC Meadow was found in 13 genotypes (results from experiment 1 shown, Supplementary Figure 1). Under field conditions, 9 of 23 genotypes tested had lower DSR than non-inoculated CDC Meadow, and 20 of 23 genotypes had lower DSR than inoculated CDC Meadow in 2020 (Supplementary Figure 2). Plants across all genotypes, including the non-inoculated plants of the S genotype CDC Meadow, showed symptoms of Fusarium root rot and the presence of *F. avenaceum*, *F. solani* and *F. redolens* was confirmed by PCR as described previously (Chatterton *et al*. 2019; data not shown). From the greenhouse and field trials, three genotypes, PI 660729, PI 557500 and 5001 (Table 4.1), were identified as having increased resistance. The fourth genotype, PI 660736 was also considered in our study as it performed well in our greenhouse trial and has been characterized as a major resistance source in pea in previous studies (McGee et al., 2012; Sivachandra Kumar et al., 2021). Among these, genotype PI 557500 was the least affected by ARR in the field and also had fewer disease symptoms in the greenhouse-based screening.

4.3.2 RNA-seq analysis of PR and S pea genotypes

To confirm PR status of the four selected genotypes prior to performing RNA extraction, a subset of each genotype from the RNA-seq inoculation experiment were grown for additional 14 dpi for disease severity assessment. All PR genotypes (PI 660736, PI 660729, PI 557500 and 5001) exhibited significantly lower disease severity compared to S genotype CDC Meadow (Figure 4.1). These results validated our previous screening results and identified that these genotypes and the experiment was appropriate for continuing downstream RNA-seq analysis.

Subsequently, one hundred twenty samples, comprising inoculated and mock-inoculated groups from the five selected genotypes at four different time points (2, 6, 12 and 24 hpi) with three biological replicates of each, were sequenced. An average of over 75 million mapped reads per sample was obtained and over 95% of the raw reads were mapped to the pea reference genome (Supplementary file 1). PCA revealed clustering of the three replicates from each treatment for each sampling time within the host genotypes. In all genotypes, there was a distinct differentiation between treatments at 2hpi which became less distinct with increasing time indicating that the effect of *A. euteiches* treatment significantly impacted the gene expression patterns as the disease progressed with time (Supplementary Figures 3 and 4). PCA also revealed that mock-inoculated samples of S genotype CDC Meadow grouped separately, while all PR genotypes grouped together (Supplementary Figure 5). However, there was no clear differentiation among the inoculated genotypes at any time point (Supplementary Figure 6).

To focus analysis, the timing of substantive gene expression changes following *A. euteiches* infection was assessed. Of 23,583 DEGs with an FDR below 0.05 and a $\log_2$ fold-change greater or lower than 1 that were obtained through comparisons between *A. euteiches* inoculated and mock-treated samples for each genotype at each time point, 19,315 (82%) were

annotated using Gprofiler (Raudvere et al., 2019). In general, the highest number of genes was differentially expressed between inoculated and mock-treated S genotype at early time points, with a subsequent decrease over time. In PR-genotypes PI 660736, PI 660729 and PI 557500, the highest number of upregulated genes tended to be observed at 6 and 24 hpi (Supplementary file 2). Common and exclusive genes for both upregulated and downregulated gene categories for each genotype and time point are represented in upset plots (Figure 4.2).

At 6 hpi, the largest number of upregulated DEGs was shared among three PR genotypes, PI 660736, PI 660729, and PI 557500 (intersection size = 328, Figure 4.2A). PI 660729 exhibited the highest overall number of upregulated genes (set size > 1200), while 189 genes were shared among all four PR genotypes (PI 660736, PI 660729, PI 557500, and 5001). The S genotype, CDC Meadow, had 317 upregulated genes unique to its profile. In the downregulated gene set at 6 hpi, PI 660729 again had the largest overall set (> 400) and the largest unique genes (intersection size = 273), whereas only 20 downregulated genes were common to all four PR genotypes (Figure 4.2B). By 24 hpi, the PR genotype PI 557500 had the largest total number of upregulated genes (set size > 1200) and a unique subset of 651 genes, with 63 upregulated genes shared among all four PR lines (Figure 4.2C). Notably, PI 557500 also had the highest number of downregulated genes at this time point (> 1500) and the largest unique downregulated genes (intersection size=844), while 219 downregulated genes were shared among all four PR genotypes (Figure 4.2D). Since our RNA-seq downstream analysis revealed that the 6 hpi and 24 hpi time points appear to be the most interesting in terms of host defense response, subsequent analyses focused on these specific points.

4.3.3 GO term enrichment analysis

Up and downregulated genes in the four PR genotypes (PI 660736, PI 660729, PI 557500, and 5001) and S genotype CDC Meadow at 6 hpi and 24 hpi compared to mock-inoculated controls were subjected to GO term analysis (Supplementary Figures 7, 8, 9, 10). At 6hpi, in the molecular function (MF) category, the 'oxidoreductase activity' term was enriched in upregulated genes at 6 hpi across all genotypes, while transcription related terms such as 'DNA binding transcription factor were enriched only in PR genotypes (Supplementary Figure 7). In the biological process (BP) category, PR genotypes showed strong enrichment for terms such as 'ethylene-activated signaling pathway', 'phosphorylation', and 'intercellular signal transduction', while in the susceptible CDC Meadow, terms such as 'response to abscisic acid', and 'response to stress' were exclusively enriched. In the cellular component (CC) category, terms such as 'extracellular region' and 'membrane' were the most enriched among upregulated genes from the PR-genotypes and terms such as 'photosystem', 'thylakoid' and 'membrane' were enriched in CDC Meadow. Among downregulated genes from CDC Meadow at 6 hpi, GO terms related to metabolic processes such as 'organic substance metabolic processes', were highly enriched in the BP category (Supplementary Figure 8). In PR genotypes PI 660729 and 5001, CC 'membrane' was consistently enriched among downregulated genes at 6hpi.

At 24 hpi, GO terms in the MF category, including 'protein kinase activity', 'ribonucleotide binding', and 'ion binding' were consistently enriched among upregulated genes in PR genotypes PI 660736, PI 660729, and PI 557500 (Supplementary Figure 9). In the BP category, terms such as 'regulation of DNA-templated transcription', 'regulation of RNA biosynthetic and metabolic process', and 'regulation of nucleobase compound metabolic process' were enriched among upregulated genes of all genotypes. GO terms such as 'regulation of gene expression', and 'regulation of macromolecule biosynthetic and metabolic process', were

enriched in upregulated genes of PR-genotypes PI 660736, PI 660729, PI 557500. In contrast, GO terms of BP such as ‘circadian rhythm’ and ‘rhythmic process’ were exclusively enriched among upregulated genes in the susceptible CDC Meadow. Within the CC category, the most enriched term among upregulated genes was ‘membrane’ and ‘cell periphery’ in PR genotypes PI 660736, PI 660729, and PI 557500, whereas in PR genotype 5001 no such enrichment was noticed. In CDC Meadow in the same category, terms such as ‘photosystem’ were enriched.

Among downregulated genes at 24 hpi, GO terms in the MF category such as ‘enzyme inhibitor activity’, ‘monooxygenase activity’, ‘oxidoreductase activity’, and ‘heme binding activity’ were exclusively enriched in PR genotypes except 5001 whereas in PR genotype 5001 terms such as ‘zinc ion binding’, were enriched (Supplementary Figure 10). In the BP category, the ‘lipid metabolic process’ term was enriched among all four PR genotypes, whereas in CDC Meadow, terms related to ‘regulation of circadian rhythm’, were exclusively enriched. Finally, in the CC category, ‘extracellular region’ was the most enriched term among downregulated genes from PR genotypes except 5001 whereas in CDC Meadow the ‘membrane’ term was enriched.

4.3.4 Pathway analysis

DEGs were further analyzed using iDEP for enriched pathway networks (Ge et al., 2018). At 6 hpi, gene sets that were the most significantly upregulated across all PR (PI 660736, PI 660729, PI 557500, and 5001) and S (CDC Meadow) genotypes in inoculated compared to mock treated samples were related to the biosynthesis of secondary metabolites, metabolic pathways, phenylpropanoid biosynthesis, MAPK metabolic pathways, and flavonoid biosynthesis (Supplementary Figure 11). However, fold-changes observed in the susceptible CDC Meadow were lower than in the four PR genotypes. Conversely, gene sets related to glycolysis-

gluconeogenesis, pyruvate metabolism and metabolic pathways were downregulated in PR genotype PI 660736, and in 5001 gene sets related to plant-pathogen interaction were downregulated, but not the remaining PR genotypes or CDC Meadow (Supplementary Figure 12). At 24 hpi, gene sets related to plant-pathogen interaction and MAPK signaling pathways were consistently upregulated in all four PR genotypes (Supplementary Figure 11), whereas gene sets related to the biosynthesis of secondary metabolites and plant hormone signal transduction were consistently downregulated (Supplementary Figure 12).

4.3.5 DEGs related to defense against pathogens

Among the DEGs identified, 58 key defense-related genes, filtered by fold-change and orthologous function based on model crops, are listed in Table 4.2 and displayed in a heatmap (Figure 4.3). The highest number of defense-related genes were differentially expressed at 6 hpi, followed by 24 hpi, mostly in the four PR genotypes (PI 660736, PI 660729, PI 557500 and 5001; Figure 4.3).

Various genes encoding putative resistance (R) proteins were upregulated at 6 or 24 hpi in all PR genotypes (Figure 4.3). For example, genes encoding disease resistance-responsive dirigent domain proteins (Psat7g199960 and Psat4g104200) and nucleotide-binding site-leucine-rich repeat (NBS-LRR) protein (Psat4g088560) were upregulated in all PR genotypes at 6 hpi, but downregulated or unresponsive in CDC Meadow (Figure 4.3). At 24 hpi, Psat2g140480, Psat3g107040 and Psat5g213640 encoding putative R proteins and an NB-ARC domain disease resistance protein, respectively, were upregulated in all PR genotypes, but were unresponsive in the S genotype.

Genes involved in redox balance (e.g. peroxidases and mutases) were mainly upregulated at 6 hpi and occasionally at 24 hpi in PR genotypes. For example, genes encoding lignin peroxidase (Psat5g250400, Psat5g086720) and thylakoid-bound ascorbate peroxidase (Psat3g008320) were upregulated at 6 hpi in all PR genotypes (Figure 4.3). On the contrary, both of these genes were transcriptionally unresponsive or downregulated in CDC Meadow and in some PR genotypes at 24hpi. Similarly, genes such as Psat6g148680, encoding bisphosphoglycerate mutase family protein, were upregulated in some PR lines, but not the susceptible CDC Meadow, at 6 hpi or 24hpi.

Protein phosphorylation-related genes, such as those encoding receptor kinases, MAP kinases, stress-induced kinases, and calcium-dependent kinases were also upregulated in PR genotypes (particularly PI 660736) at 6 hpi, but were transcriptionally unresponsive at 24 hpi and in CDC Meadow at both time points (Supplementary Figure 13, Table S2). The gene encoding MAP kinase I (Psat7g203360) was upregulated at 6 hpi in the PR genotypes PI 660736, PI 660729, and PI 557500, but were downregulated in all PR genotypes by 24 hpi (Figure 4.3). A similar transcriptional response was also observed for MAPKK and MAPKKK at 6 hpi and 24 hpi in the aforementioned three PR genotypes (Figure 4.3).

Genes encoding defense-related transcription factors (TFs) were predominantly upregulated at 6 hpi or 24 hpi. Numerous WRKY TF family genes associated with biotic and abiotic stress responses, hormone signaling, and development, were upregulated at 6 hpi in the PR genotypes. For example, WRKY TFs-encoding Psat6g026720 and Psat6g026680 were upregulated in PI 660736 and PI 660729 at 6 hpi, and downregulated in all PR genotypes at 24 hpi. In contrast, WRKY-encoding Psat5g068680 and Psat4g160360 were upregulated at 24 hpi in all PR genotypes but remained at a basal level in CDC Meadow. Similarly, ET related TFs

(e.g., Psat4g073120, and Psat0s415g0080) were upregulated in all four PR genotypes at 6 hpi, while the majority were downregulated at 24 hpi, and were unresponsive in CDC Meadow. Genes encoding NAC-like TFs (e.g., Psat2g173520 and Psat1g202400) were upregulated in PR genotypes PI 660736, PI 660729, and PI 557500 but not in CDC Meadow at 6 hpi. Likewise, genes encoding Myb TFs (e.g., Psat5g161000, Psat5g121120, and Psat4g080720) were upregulated in all PR genotypes at 6hpi, but not the susceptible CDC Meadow (Figure 4.3).

Genes involved in various plant growth hormone pathways were differentially regulated in PR genotype. Multiple JA biosynthesis-related genes were also upregulated in all four PR genotypes at 6 hpi (e.g. Psat3g190720, and Psat3g190560 encoding allene oxide cyclases (AOC)). Furthermore, Psat1g156720, encoding a pathogenesis-related bet V1 family protein/PR10 like protein that acts in plant defense in association with the JA/ET signaling pathway in many crops, was also consistently upregulated at 6 hpi in all four PR genotypes (Figure 4.3). Many genes encoding transcriptional regulators involved in JA signaling such as jasmonate zim domain (JAZ) proteins (e.g. Psat1g195000, Psat1g194920, Psat1g13608, and Psat0s766g0040), were upregulated in PR genotypes PI 660736, PI 660729, PI 557500 at 6 hpi, but were unresponsive or downregulated in PR genotypes at 24 hpi and in the susceptible CDC Meadow at both time points (Figure 4.3). Similarly, multiple ethylene response factors (ERF) encoding genes (e.g., Psat4g073120, Psat6g049880, Psat0s415g0080, and Psat3g149240) were consistently upregulated in aforementioned three PR genotypes but not in the CDC Meadow at 6 hpi. In addition, genes that encode ABA-inducible bHLH-type TFs (e.g., Psat0s1094g0040) were upregulated in all PR genotypes at 6 hpi, but showed basal or downregulated expression at 24hpi and in the S genotype at both time points (Figure 4.3). Major genes involved in JA and ET signaling pathways and their putative roles are illustrated in Figure 4.4.

Protein degradation, controlled by F-box mediated protein ubiquitination, is important for regulating cellular responses. Various F-box proteins were differentially expressed at 6 and 24 hpi in PR genotypes. For example, Psat5g260120 and Psat1g179200, encoding F-box and F-box-like protein respectively, were upregulated in PR genotypes PI 660736, PI 660729, and PI 557500 at 6 hpi, remained at a basal level 24 hpi while Psat4g103120, which encodes F-box protein, was upregulated in all four PR genotypes at 24 hpi. In contrast, the majority of genes encoding F-box-related genes were downregulated or unresponsive in CDC Meadow at both time points. Besides F-box proteins, no other ubiquitination-related genes were differentially expressed across genotypes or time points. (Figure 4.3).

Many genes in the phenylpropanoid pathway leading to the biosynthesis of the phytoalexin, pisatin, were upregulated at 6 hpi in all PR genotypes, particularly in PI 660736, while the majority of these genes were either downregulated or transcriptionally unresponsive in the susceptible CDC Meadow (Figure 4.6A). For example, Psat2g069120, encoding flavonone synthase for pisatin biosynthesis was significantly upregulated in PR genotypes and downregulated in CDC Meadow. Similarly, Psat6g000240, the gene that encodes CoA ligase-like protein, which, among different metabolic reactions, catalyzes steps of the phenylpropanoid biosynthetic pathway, was upregulated in all PR genotypes but was downregulated in the S genotype CDC Meadow at 6 hpi (Figure 4.3). As well, transcripts of genes encoding putative AMP-dependent synthetase/ligase (e.g., Psat7g263280, Psat6g000320, Psat4g011560, and Psat2g188920) phenylalanine lyase (PAL), and PAL-like proteins (Psat3g072160, Psat3g040960, and Psat6g072360) accumulated 2-8 times more in PR genotype PI 660736 at 6 hpi as a result of *A. euteiches* infection (Figure 4.6A). Similarly, Psat0s2956g0120, which encodes isoflavone 4'-O-methyltransferase responsible for phytoalexin synthesis, was also

upregulated in PI 660736. In contrast, similar upregulation of genes involved in phytoalexin biosynthesis was not visible in the susceptible CDC Meadow. As the infection reached 24 hpi, a sharp decline in the transcripts level was noticeable.

4.3.6 Location of ARR defense-related DEGs in the pea genome

When mapped to linkage groups/chromosomes, ARR defense-related genes were distributed among all 7 chromosomes of the pea genome. Most of the defense-related genes mapped to chr 3 (Linkage group 5), chr 4 (LG 4) and chr 5 (LG 3). In terms of upregulated defense-related genes in PR lines, genes encoding JAZ proteins mapped to chr 1 (LG 6) and chr 4 (LG 4), while genes encoding ERF TFs mapped to chr 3 (LG 5), chr 4 (LG 4) and chr 6 (LG 2). Genes encoding Myb TFs mapped to chr 2 (LG 1), chr 4 (LG 4) and chr 5 (LG 3), and those encoding WRKY TFs mapped to chr 4 (LG 4), chr 5 (LG 3) and chr 7 (LG 7). All upregulated genes encoding F-box proteins were mapped to chr 2 (LG 1, Figure 4.5). Upregulated gene Psat4g160360 encoding WRKY family transcription factor was located in confidence interval of major QTL *Ae-Ps.* Genes Psat7g199960, encoding disease resistance-responsive, dirigent domain protein, Psat7g203360, encoding MAPKKK, and gene Psat7g2211800, encoding AP2-like ethylene-responsive transcription factor, were all located within the confidence interval of QTL *Ae-PS7.6.*

4.3.7 Metabolic profiling

The RNA-seq data showed the upregulation of phytoalexin biosynthesis genes upon *A. euteiches* infection, and we investigated whether this altered the pisatin and its precursor metabolite levels. A targeted metabolomic analysis was performed on *P. sativum* roots at various

time points post-inoculation with *A. euteiches*. Because the contrast was highest between the S genotype CDC Meadow and PR genotype PI 660736, we focused the analysis on these two lines. PI 660736 roots accumulated higher levels of several metabolites, including *trans*-cinnamate, 4-coumaroyl-CoA, and daidzein, compared to CDC Meadow under mock-inoculated conditions (Figure 4.6B). Upon *A. euteiches* infection, the accumulation of early pisatin pathway metabolites rapidly increased within 2h in PI 660736 roots, while no such changes were observed in the case of CDC Meadow. Consistent with this, pisatin levels and other metabolites involved in its biosynthesis were found at much higher levels 24 hpi in PI 660736 compared to CDC Meadow roots. The results clearly showed an enhanced metabolic response in the resistant PI 660736 line compared to the susceptible CDC Meadow. Some metabolites also showed dynamic changes over the infection period. For instance, prunetin and isoliquiritigenin levels were initially reduced, followed by some recovery at later time points in PI 660736, but a similar pattern was not observed in CDC Meadow.

To evaluate relative pisatin levels in *A. euteiches*-inoculated susceptible and partially resistant genotypes, we subsequently quantified pisatin levels in CDC Meadow and PI 660736 roots at 2 hpi and 24 hpi. Pisatin levels were significantly higher in infected samples compared to mock-inoculated controls in PR genotype PI 660736 at 24hpi ($p < 0.05$, Figure 4.6C, Table S3 and S4). At 2 hpi, infected PI 660736 accumulated substantially more pisatin than mock controls and two-way ANOVA showed significance difference in cultivars, but this contrast was not significant after Tukey's multiple-comparison correction. While PI 660736 exhibited a substantial increase in pisatin levels at both 2 hpi and 24 hpi, with the highest levels observed at 24 hpi ($p < 0.001$), CDC Meadow did not display a noticeable increase in pisatin levels following infection with time. A significant interaction between genotypes and treatment groups was observed at both 2 hpi ($p <$

0.01) and 24 hpi ($p < 0.001$). Taken together, our metabolomic analyses suggest that the resistant pea genotype, PI 660736, possesses stronger innate immunity compared to the susceptible CDC Meadow. Additionally, its rapid synthesis of phytoalexin pisatin possibly contributes to its stronger partial resistance against *A. euteiches* infection.

4.4 DISCUSSION

The PR genotypes (PI 660736, PI 660729, and PI 557500) were confirmed through ARR resistance screening under greenhouse and field conditions. However, disease severity in the PR lines was reduced by less than 1 DSR category compared to S genotype and was inconsistent between field and greenhouse trials. The inconsistency could result from different external conditions such as temperature and moisture or from variation in the *A. euteiches* isolate used for inoculation. In a comparative aggressiveness assay, the isolates used for our field inoculation (*Ae6* and *Ae7*) were classified as highly aggressive, whereas the greenhouse inoculum isolate (*Ae1*) exhibited moderate aggressiveness (Sivachandra Kumar et al., 2021). Pea genotypes PI 660736, PI 660729, and PI 557500 were previously registered for their partial resistance to ARR (Kraft, 1992; McGee et al., 2012), while 5001 was registered for its partial resistance to Fusarium root rot (Bodah et al., 2016; Porter et al., 2014) and PI 557500 (90-2079) had a high level of partial resistance to pathotype III of *A. euteiches* (Goyal et al., 2024; Clément Lavaud et al., 2015) , suggesting PR genotypes may be active against multiple pathogens.

4.4.1 GO enrichment distinguishes PR and S responses over time

We examined time-based transcriptomic responses in PR and S genotypes to define the defense programs. Compared to the S genotype at multiple time points, transcriptomic data from the roots of all four partially resistant genotypes revealed a higher cumulative number of *A.*

euteiches-responsive DEGs. Enrichment of 'DNA binding transcription factor activities' oxidoreductase activities, and 'ethylene-activated signaling pathway' indicate that PR genotypes initiate *A. euteiches* defense responses through ROS, ethylene signaling and transcriptional changes. Enrichment of GO term 'extracellular region' in PR genotypes indicates that active defense often takes place in the apoplast.

Transcriptomic analysis indicated that, in contrast to the PR genotypes, the S genotype mounted a relatively weak early biotic stress response to *A. euteiches*, including strengthening of cell walls and altered physiological processes including photosynthesis, energy reallocation and production of ROS. The enrichment of upregulated genes related to GO terms such as 'peroxidase activities' and 'small ion binding' indicates the activation of defense responses at 6 hpi. Peroxidases play an early role in plant defense by producing ROS, such as hydrogen peroxide, which can induce programmed cell death and are also indirectly involved in strengthening of cell walls and prevention of pathogen entry (Ali et al., 2018; Nanda et al., 2010). The early responses in the S genotype may denote direct, but weaker first responses, such as cell wall biogenesis (callose deposition) (Bani et al., 2018; Hématy et al., 2009), which the pathogen might have been able to counteract. Terms related to the disruption or alteration of photosynthesis and energy reallocation were also enriched in CDC Meadow at both 6 and 24 hpi indicating that growth and development processes are affected rapidly in susceptible genotypes. 'Response to abscisic acid' terms were enriched only in the susceptible CDC Meadow. Abscisic acid is involved in plant abiotic and biotic stress responses and is often associated with susceptibility to plant pathogens. For example, in cereal crops such as wheat and barley, abscisic acid acts as a susceptibility factor to pathogenic fungi (Qi et al., 2016; Ulferts et al., 2015).

Further investigation on the role of activation of abscisic acid-related pathways to susceptibility of CDC Meadow to *A. euteiches* would be an interesting avenue of research.

In PR genotypes at 24 hpi, enrichment of GO terms such as 'regulation of gene expression', 'regulation of macromolecule biosynthetic process', 'regulation of macromolecule metabolic process', and 'regulation of nitrogen compound metabolic process' suggests continuous activation of antimicrobial compound biosynthesis and reallocation of energy for resistance. Interestingly, in three PR genotypes at 24 hpi, enrichment of GO terms among downregulated genes such as 'inhibitor activity', 'enzyme inhibitor activity', 'monooxygenase activity', 'oxidoreductase activity', and 'heme binding activity', seems to indicate a downregulation of defense responses. The latter changes may also be due to the pathogen trying to hijack host defense responses in its favor. The simultaneous upregulation of diverse defense responses and a reduction in certain defense-related biochemicals suggest an active and dynamic interaction between plant defense and pathogen counter-defense mechanisms at this time point. Additionally, host defense and counter-defense this early after inoculation confirms the partially resistant quality of these selected pea genotypes.

Overall, our data suggest that PR genotypes initiate an early defense characterized by a burst in ROS production, activation of ET-pathway, activation of post-translational modifiers including kinases and F-box proteins, enhanced transcriptional regulation, and initiation of defense related secondary metabolites.

4.4.2 Mounting of early reactive oxygen species burst

The upregulation of genes such as thylakoid-bound ascorbate peroxidase, (detoxifies ROS) and lignin biosynthetic peroxidase (catalyze the oxidative depolymerization of lignin in the

presence of ROS) at 6 hpi in PR genotypes PI 660736, PI 660729, PI 557500, 5001 likely reflects early redox-balancing activities in response to *A. euteiches*. Rather than acting directly against the pathogen, these genes contribute to mitigating ROS toxicity and maintaining cellular function during the oxidative burst triggered by infection. The production of ROS is one of the earliest events in plant defense responses after pathogen recognition, and is mostly involved in hypersensitive response (HR) or programmed cell death to prevent spread of the pathogen to the surrounding cells (Nimchuk et al., 2003). In the model legume, *Medicago truncatula*, ROS accumulated either directly following *A. euteiches* infection through the perception of an *A. euteiches*-derived elicitor (Nars et al., 2013; Trapphoff et al., 2009) or indirectly through the *A. euteiches*-mediated decrease in root nitrate content (Thalineau et al., 2016). Similarly, hydrogen peroxide ROS was a major component of partial resistance to ARR in *M. truncatula* (Badis et al., 2015; Djébali et al., 2011).

4.4.3 JA and ethylene pathways respond early in PR lines

Our data suggests that ethylene signaling pathway activation may be an important component of resistance in the PR lines. Activation of ET signaling pathways enhances resistance to hemibiotrophic pathogens such as *A. euteiches*, but reduces resistance to biotrophic pathogens (Shu et al., 2024). At 6 hpi, enrichment of defense-related GO terms in BP category such as ‘ethylene-activated signaling pathway’ occurred in PR genotypes. Studies show that ERF, the major TFs regulated by the ET signaling pathway, induced resistance to hemibiotrophic pathogens in rice, wheat and soybean (Gao et al., 2022; Hawku et al., 2021; Tezuka et al., 2019). Upregulation of ERF genes in PR genotypes at 6 hpi suggests that pea genotypes similarly initiate resistance to *A. euteiches* by activating ET signaling pathways.

Changes to the JA pathway were also suggested by the data. Genes encoding JA biosynthetic enzyme were increased in PR lines, suggesting that JA is important to the resistance response. Allene oxide cyclase (AOC) is an essential enzyme in the JA biosynthetic pathway in plants, catalyzing the conversion of an unstable fatty acid precursor, known as 12-oxo-phytodienoic acid (12-OPDA), into the intermediate compound, allene oxide, which is then further converted into JA through a series of enzymatic reactions (Mueller, 1997). In PR genotypes, AOC encoding genes were induced at 6hpi, whereas other JA biosynthetic components showed no differential expression. A related study also reported that genes involved in JA biosynthesis were predominantly associated with oxidation–reduction processes.(Wu et al., 2022).

In contrast, differential expression of JAZ genes in PR lines is consistent with suppression of JA following *A. euteiches* inoculation. Several JAZ genes were upregulated at 6 hpi in three PR genotypes, and one JAZ gene was upregulated in all four PR genotypes, with no JAZ induction in the S genotype. JAZ proteins function as repressors of various TFs (e.g., certain members of Myb, NAC, ERF, and WRKY families) in JA signaling cascades. JA leads to ubiquitination and subsequent degradation of JAZ via the 26S proteasome, by facilitating the interaction between JAZ proteins and F-box protein COI1 (Figure 4.4), which is a component of the SCFCOI1 ubiquitin E3 ligase complex (Pauwels & Goossens, 2011; Ruan et al., 2019). JA-induced degradation of JAZ proteins releases TF repression, enabling specific downstream reactions. JAZ proteins can also act as inducers of MAP kinase signaling cascades (Li et al., 2017), calcium fluxes (Kenton et al., 1999), and other signaling cascades, such as those involving ET, SA, and abscisic acid (Ruan et al., 2019; Santner et al., 2009). Classically, JA plays a positive role in defense against necrotrophic pathogens in plants (Ghozlan et al., 2020;

Macioszek et al., 2023) whereas biotrophs, hemibiotrophic fungi, and oomycetes typically suppress JA-mediated responses (Antico et al., 2012; Thomma et al., 1998; Zhou et al., 1998). The upregulation of *JAZ* genes in PR genotypes following inoculation with the hemibiotrophic pathogen *A. euteiches* may be due to a pathogen induced suppression of JA pathways, or indicative of a JAZ-mediated defense pathway.

JA pathways interact with both SA and ABA mediated signaling. Although SA and JA pathways are known to be mutually antagonistic, we did not observe direct activation of SA pathways in our study. In contrast, an ABA-inducible bHLH factor (AIB) was upregulated in all PR genotypes at 6 hpi. AIB proteins possess a DNA binding domain of the bHLH type and have a positive role in ABA signaling in Arabidopsis (Kim & Kim, 2006; Li et al., 2007). In contrast, AIB/JA-associated MYC2-like1 (JAM1) acts as a repressor to negatively regulate JA signaling in Arabidopsis (Nakata et al., 2013). The upregulation of JAZ repressors and AIB in PR genotypes is interesting because both are negatively associated with JA signaling pathways.

We have developed a putative biological pathway (Figure 4.4) suggesting how JAZ proteins and TFs might together play a role in ARR tolerance in pea based on our findings and previous studies in other plant species. Further investigation of JAZ proteins in PR genotypes will be necessary to fully elucidate their functions in pea-ARR interactions.

4.4.4 Protein phosphorylation and degradation are activated in PR genotypes

We observed that genes associated with hormone-activated signaling, protein kinase activity, and phosphorylation were upregulated in PR lines. These pathways are known to link pathogen infection to defense response via activation of transcriptional response. For example, in the *N. benthamiana-Phytophthora infestans* (an oomycete) interaction, various MAPKs are

activated after inoculation and activate downstream defense response (Takahashi et al., 2007; Yu et al., 2012). Furthermore, many TFs, such as WRKY family, are substrates of MAPK cascades and are phosphorylated by MAPKs such as salicylic acid (SA)-induced protein kinase (SIPK), wound-induced protein kinase (WIPK), and NTF6 for *RBOHB* activation in *N. Benthamiana* (Adachi et al., 2015). Consistent upregulation of gene sets related to MAPK pathways and plant-pathogen interactions in PR genotypes, as opposed to CDC Meadow at 24hpi, further confirms stronger defense responses occur in PR genotypes.

F-box proteins, which act as substrate receptors of SCF E3 ubiquitin ligases, also play a crucial role in protein regulation through triggering protein degradation in the ubiquitin proteosome pathway (Gagne et al., 2002). Two F-box like encoding genes were induced at 6 hpi across all PR genotypes and a third at 24 hpi, but not in the S genotype. By 24 hpi, two of these shifted to downregulation, indicating rapid, time-locked control of F-box mediated protein degradation. In many plants including Arabidopsis, tobacco and rice, F-box proteins modulate immunity through regulation of secondary metabolite biosynthetic pathways and phytohormone signaling (Abd-Hamid et al., 2020). For example, F-box protein COI1 (coronatine insensitive1) promotes degradation of JAZ repressors, thus activating JA-responsive genes that enhance defense against necrotrophic pathogens in Arabidopsis (Melotto et al., 2008; Yan et al., 2009).

4.4.5 Transcriptional responses coordinate early defense

PR genotypes are characterized by changes to transcription factor expression, consistent with large scale regulatory changes to gene expression characterizing the resistance response. In PR genotypes, enrichment of ‘DNA binding transcription factor activities’ indicate that TFs and regulators play key roles in response to *A. euteiches*. All four partially resistant genotypes

exhibited a higher cumulative number of *A. euteiches*-responsive DEGs than the S genotype, which indicates that PR genotypes prepare for relatively robust defense responses by activating TFs which then cause large scale changes to gene expression to strengthen immune responses.

WRKY transcription factors, which are commonly associated with abiotic or biotic stress, and may be substrates of MAPKs, were found to be differentially expressed in the PR genotypes. Several WRKY-encoding genes were upregulated at 6 hpi in the four PR genotypes, one of which was also upregulated in the S genotype at 6 hpi. Two WRKY-encoding genes were downregulated in all PR genotypes at 24 hpi, while another two WRKY-encoding genes were upregulated at 24 hpi in all PR genotypes but not in the S genotype. This pattern of early broad activation followed by PR-specific tuning supports a model (Figure 4.4) in which WRKYs act as central nodes that coordinate downstream defenses (cell wall fortification, phenylpropanoid metabolism, and hormone cross-talk) and help sustain an effective response to *A. euteiches* (Bakshi & Oelmüller, 2014; Eulgem & Somssich, 2007; Wani et al., 2021). In contrast to our study, six WRKY TFs were previously found to be upregulated at 20 hpi in the susceptible genotype 'Linnea' following *A. euteiches* infection (Kälin et al., 2024). This difference in response might be due to different genotype, definition of susceptibility, environmental conditions (Bruce, 2014), different pathogen isolates (Dolatabadian & Fernando, 2022) and age of seedling during inoculation (Carella et al., 2015) as seen in various other crops. Collectively, our results extend to ARR the established role of WRKYs in plant-oomycete interactions (Cui et al., 2019; Knoth et al., 2007; Merz et al., 2015), and suggest that temporally phased WRKY regulation is a distinguishing response of PR genotypes to *A. euteiches*.

Myb TFs, which are also associated with defense responses, were upregulated in PR genotypes at 6 hpi, but not in the S genotype. Myb51 is associated with wound response or

resistance against insect damage (Cheong et al., 2002; Johnson & Dowd, 2004), and its ortholog (Psat5g161000) was upregulated in PR genotypes at 6 hpi indicating it may also be involved in response to *A. euteiches*. Myb TFs regulate many defense responses including ROS accumulation (Yao et al., 2020); secondary metabolite production via phenylpropanoid pathway (Cao et al., 2020); lignin biosynthesis (Chezem et al., 2017; Kim et al., 2020; Zhu et al., 2022); production of antimicrobial compounds such as phytoalexins (Zhang et al., 2024); phenylpropanoid metabolism (Liu et al., 2015) and modulate interactions between JA and SA pathways (Shim et al., 2013).

In contrast to our results, Kälin et al. (2023) observed upregulation of several Myb-like TFs in the susceptible pea cultivar 'Linnea' upon *A. euteiches* infection, indicating that the timing, specific Myb family members involved, or experimental conditions may influence Myb TF expression patterns between genotypes. Together with contrasting WRKY expression in other studies, this result underscores the complexity of pea immune response to *A. euteiches*, suggesting that TF activation may be context-dependent, varying with genotype, infection timing, or environmental factors.

4.4.6 Pathway enrichment: secondary metabolite pathways dominate in PR lines

In PR genotypes, the robust enrichment of genes (e.g. defense response, phenylpropanoid metabolism, and cell-wall biogenesis) and pathways (e.g. metabolic pathways, biosynthesis of secondary metabolites, phenylpropanoid biosynthesis, and flavonoid biosynthesis) compared to CDC Meadow indicate that these genotypes are initiating biochemical and signaling defenses against the pathogen. Plant secondary metabolites (alkaloids, terpenes and phenolic compounds) predominantly function in protection of the plant against pathogens, pests and abiotic stress due

to their direct and indirect antimicrobial properties (Link et al., 1929; Mansfield, 2000; Wink, 2008; Zaynab et al., 2018). Phenylpropanoid biosynthesis is essential for production of a wide range of compounds involving lignin, flavonoids and other phenolics which are involved in biotic and abiotic responses (Dixon et al., 2002; Li et al., 2020; Yadav et al., 2020).

In particular, multiple genes related to biosynthesis of the phytoalexin pisatin were upregulated in the PR genotypes. Pisatin, the principal phytoalexin of *P. sativum*, contributes to basal and induced defense responses against *A. euteiches*. Pisatin, the primary phytoalexin produced by *P. sativum*, has robust antimicrobial properties and plays a role in both basal and induced defense to *A. euteiches* (Cannesan et al., 2011; Hosseini et al., 2015; Selim et al., 2017). Pea root border cells responded to *A. euteiches* inoculation by significantly increasing the production and secretion of pisatin into the rhizosphere (Hosseini et al., 2015), and high levels of pisatin secretion have been shown to completely prevent mycelia from infecting root cells (Hosseini et al., 2015; Wu & VanEtten, 2004). Multiple genes related to pisatin biosynthesis-related secondary metabolites were differentially expressed in all PR genotypes with most genes upregulated in PR genotype PI 660736 at 6 hpi and 24 hpi (Figure 4.3). The differential expression of pisatin-related genes was supported by the accumulation of high levels of pisatin and associated metabolites such as *trans*-cinnamate, 4-coumaroyl-CoA, and daidzein in roots at 2 hpi and 24 hpi in PR genotype PI 660736, but not the S genotype, indicating that pisatin may play a role in partial resistance to ARR in PR genotypes. Besides pisatin and associated metabolites, other metabolites such as soyasaponin I, dehydrosoyasaponin I, and soyasaponin βg were also identified to be involved in partial resistance of genotype PI 660736 and PI 557500 (Goyal et al., 2024). This study demonstrated the potent *in vitro* inhibitory activity of saponins on

A. euteiches oospore germination prior to zoospore production, highlighting their potential role in pre-infection stage resistance in pea. These results highlight the importance of root exudate composition in plant defense against pathogens, and suggest that breeding or genetic engineering to enhance the production of phytoalexins such as pisatin and saponins could be extremely valuable.

4.4.7 Defense genes and their association with disease related QTL

A proportion of the defense-related genes identified in our dataset mapped to previously identified disease resistance QTLs. Genes encoding WRKY family transcription factor, disease resistance-responsive, dirigent domain protein, MAPKKK and AP2-like ethylene-responsive transcription factor were mapped within the major-effect QTL intervals (*Ae-Ps4.5* (LGIV) and *Ae-Ps7.6* (LGVII)) that were associated with significantly reduced disease severity in different pea genetic backgrounds (Clément Lavaud et al., 2015). Candidate genes that align with DEGs found in our dataset, such as receptor-like kinases and defense regulators, were recently identified by high-resolution mapping (Lavaud et al., 2024; Leprévost et al., 2023) for minor-effect QTL (e.g. *Ae-Ps2.2* (chr6/LGII), *Ae-Ps3.1*(chr5/LGIII) and *Ae-Ps5.1* (chr3/LGV)), which had some effect on resistance (Lavaud et al., 2016; Clément Lavaud et al., 2015). However, many defense-related DEGs identified by our study did not fall within previously defined QTL regions, suggesting that additional, uncharacterized loci contribute to partial resistance.

4.5 CONCLUSIONS

Our findings present a new perspective on ARR defense in pea, highlighting many defense-related genes, such as those encoding PRRs, MAP kinases, TFs (WRKY, NAC-like, ERF), JAZ proteins, AOC, and pisatin-related biosynthetic enzymes. The differential expression

of these genes between *A. euteiches*-inoculated and mock-treated root samples was time-dependent, with maximal changes observed at 6 and 24 hpi in both the PR genotypes and the S one, suggesting that these two time points are crucial for the further exploration of host response and disease resistance mechanisms in our experimental design. Among all identified defense-related genes, many JA/ET pathway-related genes (mainly JAZ repressors and AOC) and ERF were upregulated following inoculation in PR genotypes, but not the susceptible genotype. Interestingly, these shared responses across PR genotypes likely arise from close genetic relatedness due to common ancestry (Kraft, 1992; McGee et al., 2012). Furthermore, this study underscores the importance of pisatin as an important phytoalexin in the defense of pea against *A. euteiches*. Further research to elucidate the precise role of various candidate genes distinguished in the current study will provide a deeper understanding of ARR response mechanisms in pea and facilitate breeding efforts in the future.

Acknowledgements

Funding was provided by the AAFC CAP Pulse Cluster with contributions from Agriculture and Agri-food Canada, Alberta Pulse Growers, Saskatchewan Pulse Growers, and Manitoba Pulse and Soybean Growers. We thank Dr. Sabine Banniza, University of Saskatchewan for providing the *Ae1* isolate. The authors acknowledge Christine Vucurevich, Eric Amundsen, Daria Ryabova and all members of the pulse pathology lab, AAFC Lethbridge for their technical assistance.

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TABLES AND FIGURES

Table 4.1. List of pea genotypes used to define Pea ARR response via RNA-seq

Genotype	Description	References
PI 660729	Parent is 90-2131 (a breeding genotype developed by the USDA-ARS), QTL *Ae-Ps7.6* present	McGee et al, 2012
5001	*Fusarium* spp. partial resistance (Fus PR)	Porter et al, 2014
PI 660736	Parent is 90-2131, QTL *Ae-Ps7.6* present	McGee et al., 2012
PI 557500 (90-2079)	Fus PR, Fusarium wilt resistant, QTL *Ae-Ps4.5*	Lavaud et al., 2015
CDC Meadow	Susceptible commercial cultivar	

Table 4.2. Putative roles of defense-related differentially expressed genes

Genes	Description	References
Psat7g260120	WRKY transcription factor	Adachi et al., 2015
Psat7g221800	AP2-like ethylene-responsive transcription factor	Shu et al., 2024
Psat7g203360	MAP kinase I	Meng and Zhang, 2013
Psat7g199960	disease resistance-responsive, dirigent domain protein	Paniagua et al., 2017
Psat7g062920	cysteine-rich RLK (receptor-like kinase) protein	Wang et al., 2019
Psat7g049280	DNA-binding domain protein	
Psat7g036480	WRKY family transcription factor	Adachi et al., 2015
Psat6g148680	phosphoglycerate mutase family protein	
Psat6g049880	ethylene response factor	Shu et al., 2024
Psat6g026720	WRKY family transcription factor	Adachi et al., 2015
Psat6g026680	WRKY family transcription factor	Adachi et al., 2015
Psat6g003880	LRR and NB-ARC domain disease resistance protein	Wang et al., 2019
Psat6g000240	4-coumarate-CoA ligase-like protein	Lavhale et al., 2021
Psat5g285560	myb transcription factor	Cao et al., 2020
Psat5g260120	F-box plant-like protein	Melotto et al., 2008
Psat5g250400	lignin biosynthetic peroxidase	Zhu et al., 2022
Psat5g213640	LRR and NB-ARC domain disease resistance protein	Wang et al., 2019
Psat5g161000	MYB transcription factor MYB51	Cao et al., 2020
Psat5g121120	myb transcription factor	Cao et al., 2020
Psat5g086720	thylakoid-bound ascorbate peroxidase	
Psat5g068680	WRKY family transcription factor	Adachi et al., 2015
Psat4g225000	ethylene response factor	Shu et al., 2024
Psat4g211280	2-hydroxyisoflavanone dehydratase	
Psat4g160360	WRKY family transcription factor	Adachi et al., 2015
Psat4g104200	disease resistance-responsive, dirigent domain protein	Paniagua et al., 2017
Psat4g103120	F-box protein	Melotto et al., 2008
Psat4g100080	naringenin 3-dioxygenase (flavanone-3-hydroxylase)	
Psat4g088560	N/A	
Psat4g086600	MAP kinase kinase kinase	Meng and Zhang, 2013
Psat4g086560	MAP3K-like kinase	Meng and Zhang, 2013
Psat4g080720	myb transcription factor	
Psat4g073120	ethylene response factor	Shu et al., 2024
Psat4g018840	jasmonate zim-domain protein	Pauwels and Goossens, 2011
Psat3g190720	allene oxide cyclase	Mueller, 1997
Psat3g190560	allene oxide cyclase	Mueller, 1997
Psat3g149240	ethylene response factor	Shu et al., 2024

Psat3g107040	disease resistance protein (TIR-NBS-LRR class), putative	Wang et al, 2019
Psat3g074160	NB-ARC domain disease resistance protein	Bonhomme et al., 2019
Psat3g067360	disease resistance response protein	(Paniagua et al., 2017) Paniagua et al., 2017
Psat3g056880	myb transcription factor	Cao et al., 2020
Psat3g031200	F-box plant protein, putative	Melotto et al., 2008
Psat3g008320	acid phosphatase/vanadium-dependent haloperoxidase	
Psat2g173520	NAC transcription factor-like protein	(Yuan et al., 2019) Yuan et al., 2019
Psat2g140480	disease resistance protein (CC-NBS-LRR) family protein	Paniagua et al., 2017
Psat2g069120	flavonol synthase/flavanone 3-hydroxylase	
Psat2g062120	F-box/LRR protein, putative	Melotto et al., 2008
Psat2g056200	MAP kinase kinase	Meng and Zhang, 2013
Psat2g042600	myb transcription factor	Cao et al., 2020
Psat1g202400	NAC transcription factor-like protein	Yuan et al., 2019
Psat1g195000	jasmonate zim-domain protein	Pauwels and Goossens, 2011
Psat1g194920	jasmonate zim-domain protein	Pauwels and Goossens, 2011
Psat1g179200	F-box plant-like protein, putative	Melotto et al., 2008
Psat1g156720	pathogenesis-related protein bet V I family protein	Colditz et al.,, 2007
Psat1g136080	jasmonate zim-domain protein	Pauwels and Goossens, 2011
Psat0s766g0040	jasmonate zim-domain protein	Pauwels and Goossens, 2011
Psat0s416g0080	ethylene response factor	Shu et al., 2024
Psat0s415g0080	ethylene-responsive transcription factor	Shu et al., 2024
Psat0s1953g0120	myb transcription factor	Cao et al., 2020
Psat0s1094g0040	ABA-inducible bHLH-type transcription factor	Nakata, 2013

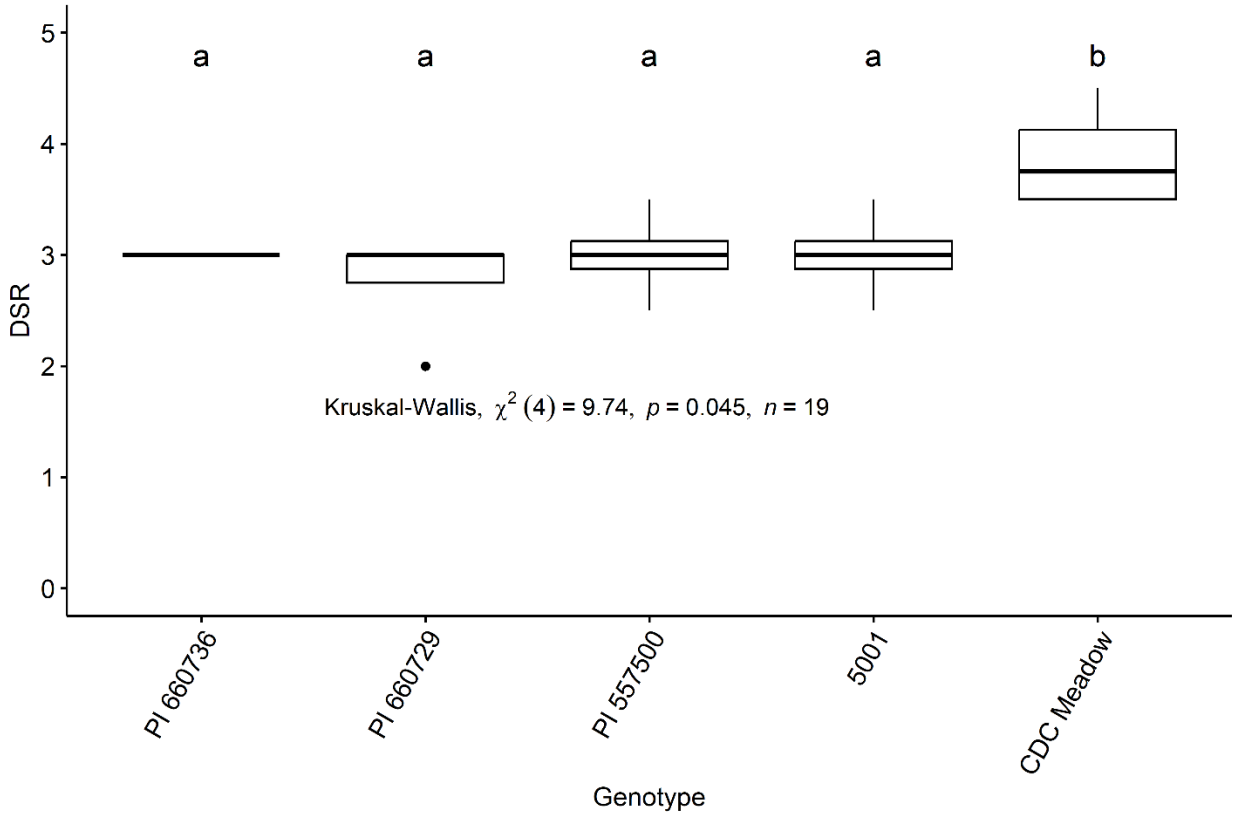

Figure 4.1. Box and whisker plot of disease severity ratings (DSR) in response to *A. euteiches* isolate *Ae1* inoculation, assessed at 14 dpi in greenhouse conditions. The statistical significance was determined based on Kruskal Wallis test followed by Dunnet test to adjust for the multiple comparison effects. Boxes with different small letters on the top indicates the difference in DSR at $P < 0.05$.

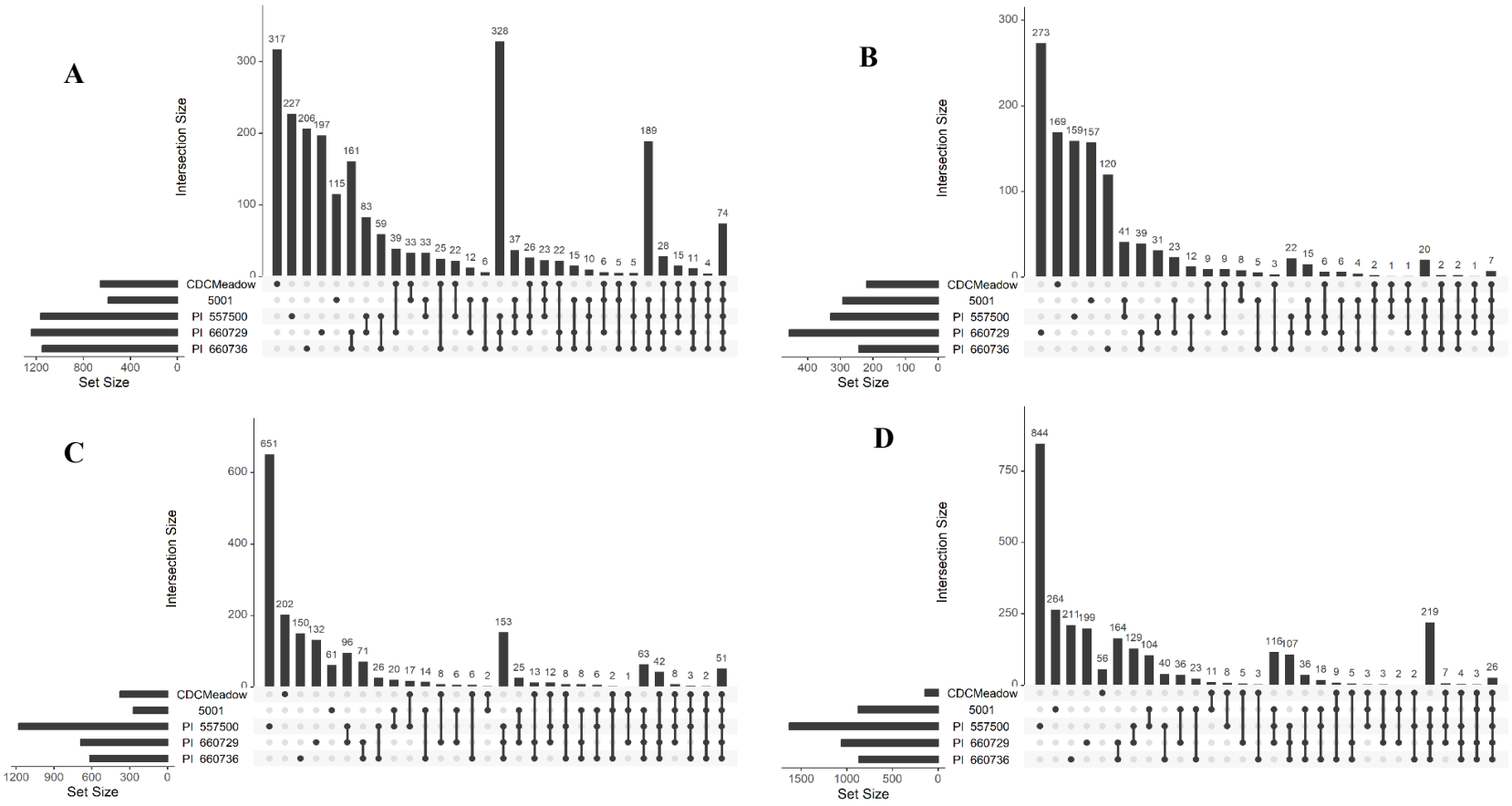

Figure 4.2. Upset plots showing a summary of upregulated (A-6hpi, C-24hpi) and downregulated (B-6hpi, D-24hpi) genes in all genotypes. The plot illustrates the unique and overlapping sets of differentially expressed genes (DEGs) identified in all genotypes. The horizontal bars on the left represent the total number of DEGs in each genotype, the dots indicate the number of DEG unique to each genotype, while the vertical bars depict the number of DEGs shared among specific intersections of genotypes, as indicated by the connected dots below.

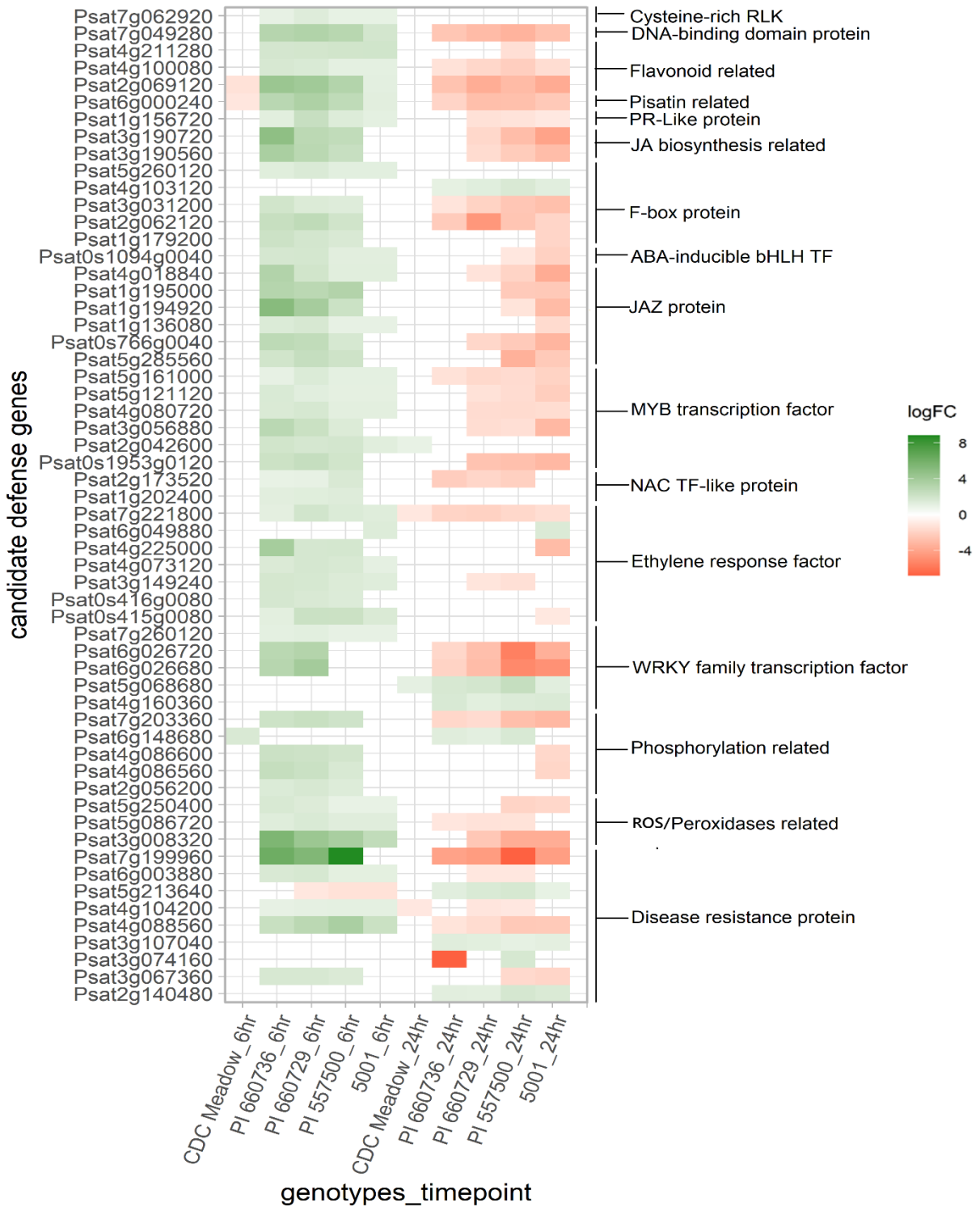

Figure 4.3. Heatmap showing important defense-related genes differentially expressed at 6 hpi or 24 hpi in all the genotypes. The DEGs from different pathways were chosen based on their role in model crops' defence responses. Annotations for each gene are listed in Table 4.2.

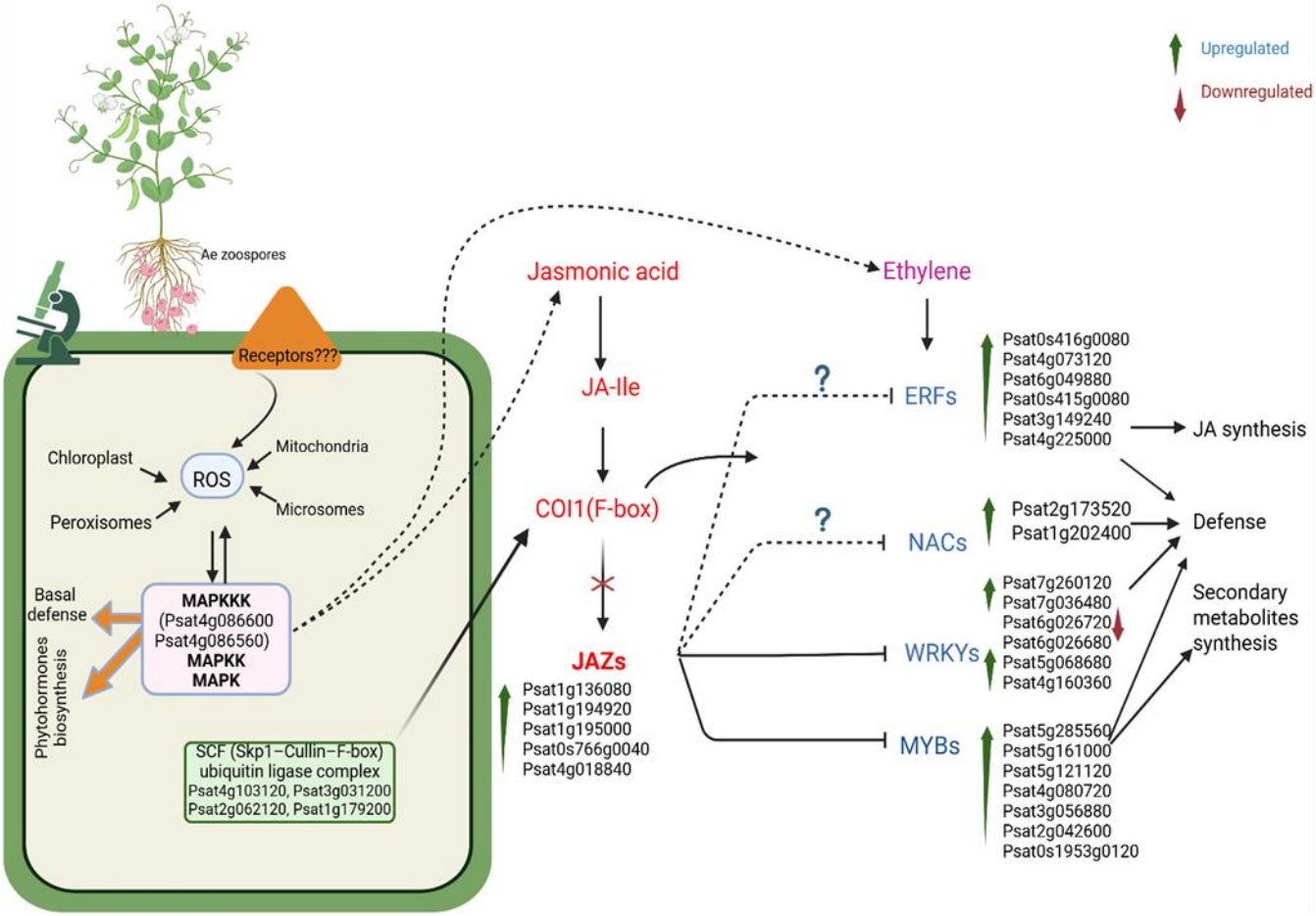

Figure 4.4. Overview of putative biological roles and molecular functions of important defense-related genes upregulated at 6hpi in PR genotypes: PI 660736, PI 660729, PI 557500, and 5001.

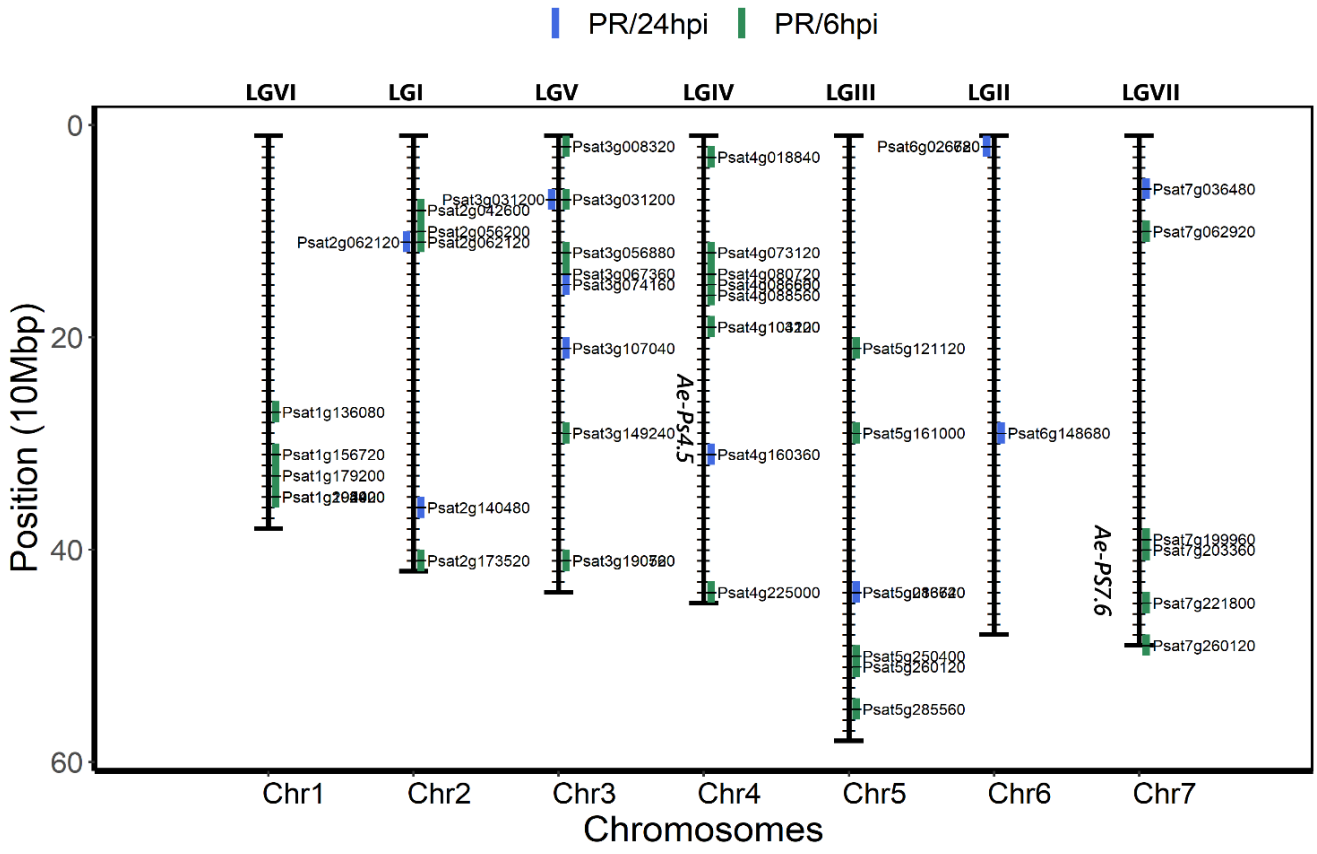

Figure 4.5. Pea chromosome map showing locations of defense-related DEGs identified as differentially expression at 6 hpi (green) or 24 hpi (blue) in PR genotypes (PI 660736, PI 660729, PI 557500, and 5001). The short bars on the left-hand side represent down-regulated genes and on the right represent up-regulated genes. The top X-axis denotes linkage groups. Two major QTLs (*Ae-Ps4.5* and *Ae PS7.6*) have been physically positioned on their respective chromosomes based on previously published studies (Hamon et al., 2013; Lavaud et al., 2024).

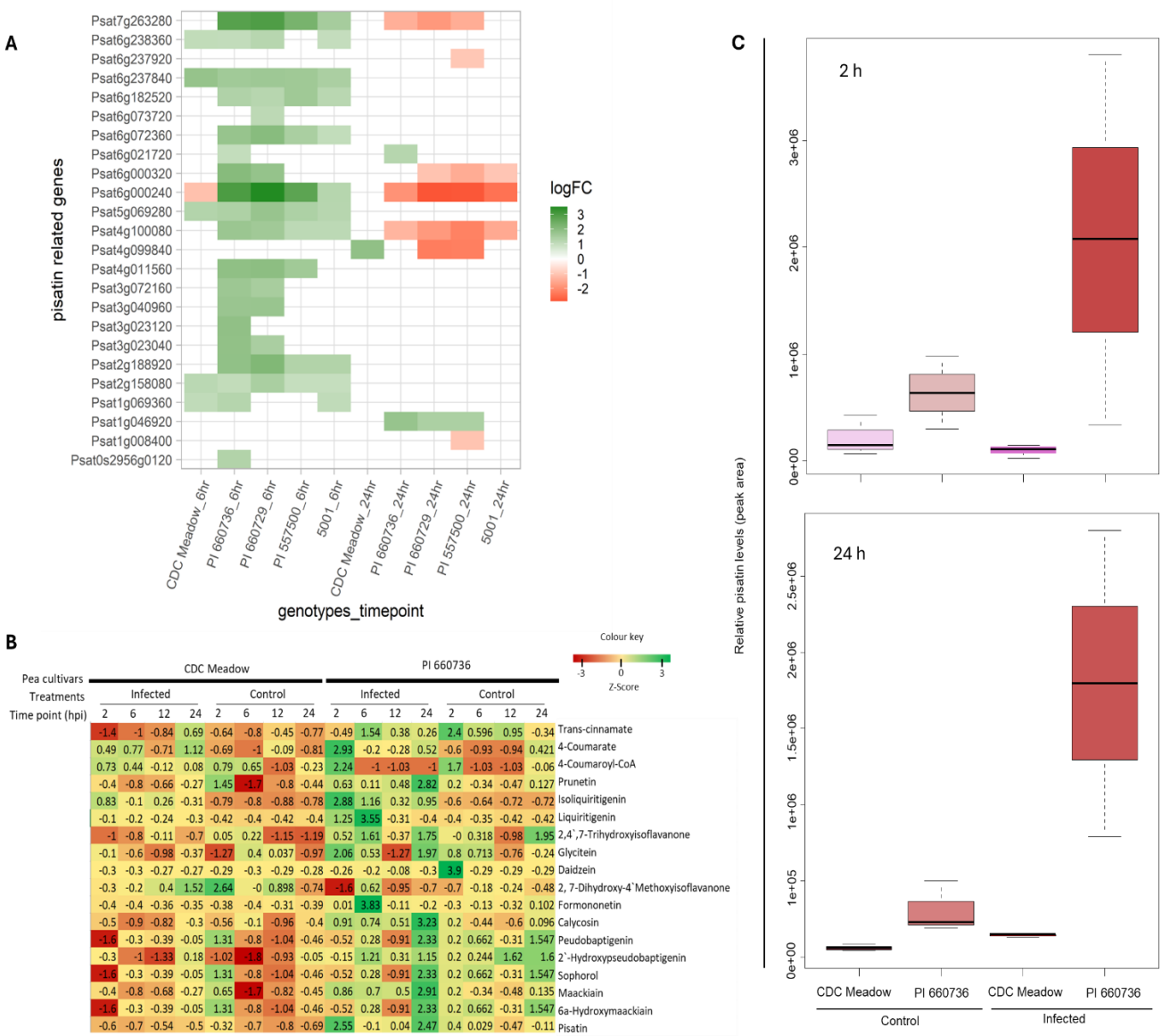

Figure 4.6. A. Heatmap showing the expression of pisatin-related genes between *A. euteiches* inoculated and mock-inoculated pea genotypes at 6 hpi and 24 hpi. B. Heatmap of metabolic changes in *P. sativum* roots at 2, 6, 12 and 24 hpi for two cultivars: CDC Meadow (S) and PI 660736 (PR). The metabolites are organized sequentially along the biosynthesis of the phytoalexin pisatin (from precursors to end product). Each cell represents the Z-score normalized peak areas. C. Relative pisatin levels in P. sativum root at 2 and 24 h post-inoculation or mock inoculation (control) with *A. euteiches*. Comparative pisatin levels in the roots of CDC Meadow (S) and PI 660736 (PR) are shown.

CHAPTER 5 : RNA SEQ STUDY OF PARTIALLY RESISTANT AND SUSCEPTIBLE PEA GENOTYPES DURING INTERACTIONS WITH *FUSARIUM AVENACEUM*, A MAJOR CAUSAL AGENT OF PEA ROOT ROT

ABSTRACT

Fusarium root rot (FRR), caused by *Fusarium avenaceum* (*Fave*), is a devastating disease affecting pea (*Pisum sativum L.*) production, particularly in western Canada, where yield losses can reach up to 100%. Despite its economic impact, the molecular mechanisms underlying pea resistance to *Fave* remain poorly understood. This study employed RNA-seq to investigate the transcriptomic responses of partially resistant (PR) and susceptible (S) pea genotypes during *Fave* infection. Four genotypes, three PR (5001, Carman, K2) and one S (CDC Meadow) were inoculated with *Fave* macroconidia, and root samples were collected at 2 hours, 3 days, 6 days, and 9 days post-inoculation (hpi/dpi). RNA-seq analysis revealed 635 differentially expressed genes (DEGs) between inoculated and mock-treated plants, with more genes downregulated than upregulated across most time points. Gene Ontology (GO) enrichment and pathway analyses highlighted the activation of defense-related processes, including secondary metabolite biosynthesis, phenylpropanoid biosynthesis, and nitrogen metabolism, particularly in PR genotypes. Key defense-related genes, such as defensins, leghemoglobins, and nodulin-like proteins, were significantly upregulated in PR genotypes, suggesting their roles in antimicrobial defense and oxidative stress management. Conversely, the downregulation of germin-like proteins in the susceptible genotype CDC Meadow may indicate a susceptibility factor facilitating pathogen colonization. This study provides novel insights into the molecular basis of partial resistance to *Fave* in pea, identifying candidate genes and pathways that could inform breeding strategies for developing FRR-resistant cultivars. These findings underscore the

complexity of pea-*Fave* interactions and lay the groundwork for future functional studies to validate the roles of identified genes in disease resistance.

5.1 INTRODUCTION

Field pea (*Pisum sativum* L., 2*n*=14) is a major legume crop worldwide due to its plethora of benefits. It serves as a vital source of protein and fiber for both humans and animals, has a low glycemic index, and provides essential micronutrients and amino acids such as lysine and tryptophan, which are often deficient in cereal grains (Parihar *et al.*, 2021, Amarakoon *et al.*, 2012). Pea is a short-season crop with high yield potential and excellent cold tolerance, making it adaptable to diverse growing conditions. Additionally, pea enhances soil fertility through biological nitrogen fixation, a process that reduces the need for external fertilizers. On average, field peas contribute approximately 40–100 kg of nitrogen per hectare per year under optimal conditions, although this value can vary depending on factors such as crop rotation practices and environmental conditions. (Stevenson & Kessel, 1996, Chapagain & Riseman, 2014).

The primary pea-growing regions include North America, Europe, Australia, and Asia, with Canada leading global production (AAFC, 2024). However, pea productivity in western Canada, often referred to as the legume basket of the country, faces significant challenges due to root rot, commonly known as the Pea Root Rot Complex (PRRC, Hwang & Chang, 1989, Bailey, 2003, Foroud *et al.*, 2014, Safarieskandari *et al.*, 2021).

PRRC is caused by a range of fungal and oomycete pathogens, including *Fusarium* spp., *Aphanomyces euteiches*, *Rhizoctonia solani*, and *Pythium* spp., with *Fusarium avenaceum* (*Fave*) being the most aggressive and predominant among the *Fusarium* species (Chatterton *et al.*, 2015, Chatterton *et al.*, 2019, Safarieskandari et al., 2021, Feng *et al.*, 2010, Esmaeili Taheri *et al.*, 2017). Over the past 10–15 years, pulse disease surveys conducted in the Canadian prairies have shown a steady increase in root rot incidence and prevalence caused by *Fave*, making it a significant threat to the Canadian pulse industry. In severely infested fields, yield losses can

reach up to 100% (Chatterton et al., 2019, Wu *et al.*, 2018). Despite its dominance and aggressiveness, *Fave* has received limited research attention compared to other pathogens in PRRC. This is largely due to challenges such as inconsistent infection, variability in disease development, and symptoms that differ between field and greenhouse conditions. These factors complicate efforts to study and manage the disease effectively.

The symptoms of Fusarium root rot (FRR) in peas initially appear as reddish-brown streaks on primary and secondary roots, which later merge into dark or black discoloration extending to the epicotyl region (Coleman, 2016, Safarieskandari et al., 2021). Further, it restricts nutrient and water flow to the aerial parts of the plant, resulting in stunted growth, chlorosis, and significant yield loss. *Fave* has a wide host range encompassing over 80 plant genera, including legumes (Kollmorgen, 1974, Feng et al., 2010), cereals (Backhouse *et al.*, 2004, Nielsen *et al.*, 2011), vegetables (Gachango *et al.*, 2012), oilseed (Fernandez, 2007), fruits (Kou *et al.*, 2014) and ornamentals (Kopacki & Wagner, 2006). Depending on the plant species, *Fave* can infect various parts, such as roots, tubers, stems, heads, and fruits. *Fave* thrives in cool, wet conditions and overwinters in soil and crop residues as mycelium. Its broad host range and adaptability to diverse environmental conditions enable it to persist in agricultural fields. Management of pea FRR is challenging due to the lack of effective chemical treatments and resistant cultivars. Crop rotation, although a potential strategy, is often ineffective because of the extensive host range of *Fave* and the presence of volunteer host plants in the field. Consequently, FRR poses a significant threat to pea production in western Canada and globally, requiring urgent attention for sustainable management solutions.

Resistance studies for FRR in pea are limited, and an understanding of the resistance response in this interaction is crucial to develop effective management strategies. RNA

sequencing (RNA-seq) provides a genome-wide view of gene expression, enabling discovery of defense-related genes and pathways in plant–pathogen interactions and supporting strategies for yield protection and sustainable agriculture (Kawahara *et al.*, 2012, Kälin *et al.*, 2024, Zhang *et al.*, 2022). RNA-seq is also a powerful tool to study partially resistant pea genotypes and uncover the mechanisms behind partial resistance. With the availability of a reference *Pisum sativum* genome (Kreplak et al., 2019, Yang et al., 2022) and its extensive gene annotations, RNA-seq provides an invaluable resource for exploring the transcriptomic response to *Fave* inoculation. In this study, we used RNA-seq to investigate defense responses in pea-*Fave* interactions, comparing susceptible (S) and partially resistant (PR) genotypes following inoculation with *Fave* macroconidia.

5.2 MATERIALS AND METHODS

5.2.1 Fave macroconidia production

Fave isolate, 'Fa 1306.08,' sourced from field pea surveys in Alberta and Saskatchewan, was selected for inoculum preparation due to its moderate-to-high aggressiveness (Safarieskandari et al. 2021). The isolate was maintained on potato dextrose agar (PDA) amended with penicillin and streptomycin (150 mg/L each) at room temperature under ambient light. To preserve its virulence, the fungal culture was refreshed every four weeks and passed through the host after every sixth transfer. For inoculum preparation, a 5 mm mycelial plug from the actively growing edge of a 7–10-day-old PDA culture was transferred into a 250 mL Erlenmeyer flask containing 100 mL of liquid carboxymethylcellulose (CMC) medium (Cappellini & Peterson, 1965). The flask was incubated at 25°C on a shaker (170–190 rpm) for 7–10 days until a cloudy suspension with visible mycelial chunks formed. The culture was shaken vigorously to release macroconidia, which were then filtered through four layers of

cheesecloth to remove mycelia and agar. Macroconidia were collected by centrifugation at 4000 rpm for 10 minutes, washed three times with sterile distilled water (SDW), and resuspended in SDW. The concentration of macroconidia was determined using a hemocytometer, and the suspension was diluted to the desired concentration.

5.2.2 Plant material, inoculation, root sampling for RNA

Four pea genotypes with different susceptibilities to *Fave* were selected for the RNA-seq experiment: K2, 5001, and Carman (designated as partially resistant, PR) and CDC Meadow (designated as susceptible, S). Seeds were surface-sterilized using 10% bleach and sown in rootrainer cells (Rootrainer International, Canada) filled with sterilized vermiculite. Plants were grown under standard greenhouse conditions at 22°C/16°C (day/night) and 16-hour photoperiod, maintaining moderate moisture levels through daily watering.

At 14 days post-germination, rootrainers were immersed in a 2L suspension containing 50,000 macroconidia/mL for one hour to ensure sufficient conidia adhered to the root surface for infection. Mock-inoculated plants were treated similarly with double-distilled water (ddH_2O). Treatments were arranged in a randomized split-plot design with three replications, where germplasm served as the main factor and sampling time as the sub-plot factor. Roots were sampled for RNA extraction at 2 hours post-inoculation (hpi), 3 days post-inoculation (dpi), 6 dpi, and 9 dpi, with three replicates per time point, based on preliminary findings that infection in the pea-*Fave* interaction is detectable between 4–6 dpi (Chapter 3). Roots were washed, flash-frozen in liquid nitrogen, and stored at -80°C until RNA extraction.

At twenty-one days after inoculation, a set of plants were also rated for their disease severity on a scale of 1-7, where a rating score of 1 denotes a healthy plant, 2 denotes small reddish-brown lesions at the seed attachment point, 3 denotes coalescing of localized

root/epicotyl lesions approximately 180° around the tap root, 4 denotes lesions extending and completely encircling the tap root, 5 denotes increasingly discoloured and extended epicotyl lesions, 6 denotes epicotyl lesions encircling the epicotyl and extending up to the green stem, and 7 denotes a completely decayed root system (Schneider & Kelly, 2000, Safarieskandari et al., 2021).

5.2.3 RNA extraction, library preparation, and sequencing

To analyze gene expression patterns in partially resistant (PR) versus susceptible (S) genotypes, RNA-seq was conducted. Total RNA was extracted from the roots of three PR genotypes (5001, Carman, K2) and one S genotype (CDC Meadow) following inoculation with *Fave* macroconidia or mock treatment. A total of 96 samples, consisting of inoculated and mock-treated groups across four genotypes at four time points (2 hpi, 3 dpi, 6 dpi, and 9 dpi) with three biological replicates (4 genotypes × 3 replicates × 4 time points × 2 treatments = 96 samples), were processed for sequencing.

RNA was isolated using the RNeasy Plant Mini Kit (Qiagen Inc., Toronto, ON, Canada) following the manufacturer's protocol, with residual DNA removed using RNase-free DNase I (Thermo Fisher Scientific, Ottawa, ON). DNA removal was confirmed via PCR using Invitrogen Platinum PCR SuperMix High Fidelity (Thermo Fisher Scientific) and gel electrophoresis. RNA quality was assessed using a Bioanalyzer and the Agilent RNA 6000 Nano Kit (Agilent Technologies, Mississauga, ON, Canada). RNA quantity and integrity were further verified using the 5K/RNA/Charge Variant Assay LabChip GX and RNA Assay Reagent Kit (PerkinElmer, Woodbridge, ON, Canada).

Library preparation and sequencing were outsourced to Genome Québec Centre D'Expertise et de Services (Montreal, QC, Canada). For library preparation, 250 ng of total RNA

underwent mRNA enrichment using the NEBNext Poly(A) Magnetic Isolation Module (New England BioLabs, Whitby, ON, Canada). cDNA synthesis was performed with NEBNext RNA First Strand Synthesis and Ultra Directional RNA Second Strand Synthesis Modules. Subsequent steps used the NEBNext Ultra II DNA Library Prep Kit for Illumina. Libraries were quantified with the Kapa Illumina GA with Revised Primers-SYBR Fast Universal Kit (Kapa Biosystems, Toronto, ON, Canada), and fragment size was confirmed using a LabChip GX (PerkinElmer). Sequencing was carried out on the Illumina NovaSeq 6000 S4 platform, generating 100-bp paired-end reads.

5.2.4 Bioinformatic and statistical analysis of RNA-Seq data

The reference genome and corresponding annotation files for *P. sativum* (CAAS_Psat_ZW6_1.0) was obtained using the NCBI datasets tool (O'Leary et al., 2024) under accession number GCF_024323335.1. The genome was indexed using STAR (Dobin et al., 2013). Quality assessment of raw RNA-seq reads was performed using FastQC (Andrews, 2010), and summary reports were generated with MultiQC (Ewels et al., 2016). Adaptor removal and quality filtering were carried out with BBDuk (Bushnell, 2014) using a phred score threshold of <50. Cleaned reads were aligned to the indexed *P. sativum* reference genome using STAR. Aligned BAM files were indexed using SAMtools (Danecek et al., 2021). Gene-level quantification was performed using FeatureCounts (Liao et al., 2013).

All downstream analysis was performed in R (version 4.3.1). Raw counts were first normalized to counts per million (CPM) using edgeR (Robinson et al., 2009), and normalized library sizes were plotted with ggplot2 (Wickham, 2011) to assess sequencing depth and potential biases across treatment groups. A variance stabilizing transformation (VST) was applied to the count data via DESeq2 (Love et al., 2014), and Principal Component Analysis

(PCA) was performed to explore sample clustering and overall expression patterns. The first two principal components, accounting for a substantial proportion of variance, were visualized using ggplot2. Heatmaps of the 350 most variable genes were generated using the pheatmap. Prior to model fitting, genes with low counts were filtered out to improve statistical power. The model was then fitted using DESeq2, and multiple contrasts were defined. Two primary sets of comparisons were made:

1. Within each genotype and at each time point, differences between inoculated and control (mock-inoculated) samples were tested.
2. Within each treatment group and at each time point, the susceptible genotype (CDC Meadow) was compared to each partially resistant genotype (K2, 5001, and Carman).

A power analysis was conducted using RNAseqPower (Hart et al., 2013) to estimate the detectable effect sizes given the sample size and variability. Significance thresholds were set at a false discovery rate (FDR) < 0.05 and an absolute $\log_2$ fold-change > 1.5. Differential expression results were summarized by enumerating upregulated and downregulated genes for each contrast, and volcano plots were generated using the EnhancedVolcano package (citation) to visually depict significant changes. Upset plots were created using the R package 'UpsetR'(Conway *et al.*, 2017), while heatmaps and the mapping of DEGs onto chromosomes were created using the R package ggplot2 (Wickham, 2011). GO term enrichment analysis was performed using g:profiler (Raudvere *et al.*, 2019) at a threshold of $P<0.05$. Pathway analysis was performed using the iDEP web application (Ge *et al.*, 2018). The sequence data generated in this study are available at the National Center for Biotechnology Information (NCBI) Sequence Read Archive. BioProjectID will be updated once completed.

5.3 RESULTS

5.3.1 Disease severity ratings

The genotypes 5001, Carman, K2, and CDC Meadow were grown in the greenhouse until 21 dpi to confirm their phenotypes against *Fave*. The PR-designated genotypes showed numerically lower disease severity ratings (DSR) compared to the S genotype CDC Meadow, with Carman and K2 exhibiting significantly reduced disease pressure ($P < 0.05$, Figure 5.1). These findings validated the partial resistance of at least two genotypes to *Fave*. However, all four genotypes were included in the RNA-seq analysis with the expectation that comparisons across genotypes with varying resistance levels would provide a more comprehensive understanding of pea-*Fave* interactions.

5.3.2 RNA-seq analysis of PR and S pea genotypes

An average of over 75 million reads per sample was obtained and over 93% of the raw reads were mapped (MultiQC, Supplementary file 3) to the pea reference genome. A total of 635 genes were differentially expressed between *Fave* inoculated and mock-treated samples with an FDR below 0.05 and a $\log_2$ fold-change greater or lower than 1 (Raudvere et al., 2019). PCA of the gene count matrix showed that transcriptional variation was primarily structured by sampling time and genotype, with only modest separation between *Fave* inoculated and control samples across most genotypes (Figure 5.2). For all genotypes and time points after inoculation, except Carman at 9 dai, the number of upregulated genes was lower than downregulated (Figure 5.3). Common and exclusives genes for both upregulated (Figure 5.4) and downregulated genes (Figure 5.5) categories for each genotype and time points are represented in upset plots.

5.3.3 GO term enrichment analysis

To understand the host pathways involved in response to the *Fave* infection process, GO term enrichment analysis was done for upregulated and downregulated genes of each genotype at all time points (Supplementary files 4 and 5). At 2 hpi, GO terms 'regulation of hormone levels' and 'regulation of biological quality' were enriched among upregulated genes in PR genotype 5001 in biological process (BP) category. The term "phosphoric diester hydrolase activity" was enriched in molecular function (MF) category among upregulated genes in PR genotype Carman, whereas the GO term 'phosphorelay signal transduction system' was enriched among upregulated genes in CDC Meadow. No significant GO terms were enriched in other genotypes at 2hpi.

At 3dpi, GO terms such as 'manganese ion transmembrane transporter activity' were enriched among upregulated genes of PR genotype K2 in MF category. Similarly, terms such as 'intracellular manganese ion homeostasis', 'manganese ion homeostasis' were enriched in the BP category. Whereas no other significant terms were enriched among upregulated genes of any other genotypes at 3dpi. At 6dpi, GO term 'nitrogen cycle metabolic process' was enriched in BP category among upregulated genes of genotype 5001. In contrast, in S genotype CDC Meadow, terms such as 'oxidoreductase activity' in MF category and terms such as 'plastoglobule' were enriched in BP category among the upregulated genes at 6hpi. At 9dpi, terms such as 'P-type proton-exporting transporter activity' in MF category, and 'proton export across plasma membrane' in BP category were enriched among upregulated genes of PR genotype K2. Similarly in PR genotype 5001 terms such as 'oxygen carrier activity' in MF category and 'gas transport', 'nitrogen cycle metabolic process' were enriched among upregulated genes of genotype 5001.

Among the downregulated genes, at 2hpi, terms such as exocyst and cell cortex were enriched in S genotype CDC Meadow in the cellular compartment category. At 6hpi, terms such as 'DNA-binding transcription factor activity' and 'transcription regulator activity' were enriched in the MF category in genotype K2. At 9dpi, terms such as 'GTP cyclohydrolase activity' was enriched in MF category in genotype 5001. In S genotype CDC Meadow, terms 'signal transduction' and 'signaling' were enriched in BP category at 9dpi.

5.3.4 Pathway analysis

DEGs were analyzed further using iDEP for enriched pathway networks (Ge et al., 2018). At 2hpi gene sets upregulated in genotype 5001 were related to phenylpropanoid and secondary metabolite biosynthesis (Figure 5.6). Gene sets upregulated in K2 were related to carbon fixation in photosynthetic organisms and glyoxylate and dicarboxylate metabolism. In contrast, gene sets downregulated in K2 were related to plant-pathogen interaction (Figure 5.6).

At 3dpi, gene sets upregulated in genotypes 5001, Carman and K2 were related to metabolic pathways and biosynthesis of secondary metabolites and phenylpropanoid biosynthesis (Figure 5.7), while in CDC meadow, gene sets upregulated were related to homologous recombination and gene sets downregulated were related to protein processing in the endoplasmic reticulum (Figure 5.7).

At 6dpi, upregulated gene sets in Carman were related to cyanoamino acid metabolism. In contrast, in CDC Meadow, upregulated gene sets were related to nitrogen metabolism, biosynthesis of secondary metabolites, and metabolic pathways (Figure 5.8). Gene sets downregulated in Carman and K2 were associated with processes such as plant-pathogen interaction, while gene sets downregulated in CDC Meadow were related to protein processing in the endoplasmic reticulum (Figure 5.8).

At 9dpi, gene sets upregulated in K2 were related to processes such as phenylpropanoid isoquinoline and alkaloid biosynthesis and oxidative phosphorylation (Figure 5.9). Gene sets upregulated in Carman were related to ABC transporters. Gene sets upregulated in CDC Meadow were related to diterpenoid biosynthesis and alpha-linoleic acid metabolism, while downregulated genes in Carman were related to Alanine aspartame and glutamate metabolism.

In all other genotypes at all time points, no gene sets upregulated or downregulated were associated with any significant processes or pathways (Figure 5.9).

5.3.5 Candidate defense-related DEGs

Important defense-related genes based on previous studies on model crops were filtered and a heatmap for all the time points was generated. Defense-related genes were differentially expressed throughout different time points and genotypes. Highly regulated genes are represented in Figure 5.10 and listed in Table 5.1 with their putative roles. In S genotype CDC Meadow, gene Psat5g172760 encoding for nodulin-25-like, well known for its role in nodulation in legumes, was highly upregulated at 3dpi whereas genes such as Psat7g151720 germin-like protein were downregulated at 6dpi.

In PR genotype K2, genes Psat7g097160 encoding FK506-binding protein 4-like and Psat2g187880 encoding putative 12-oxophytodienoate reductase 11 which is known for its involvement in jasmonic acid (JA) were highly upregulated at 2hpi while at 3 and 6dpi no significant defense related genes were upregulated. At 9dpi genes such as Psat7g249480 encoding CASP-like protein 4B1 were highly upregulated in K2.

In genotype 5001, gene Psat7g125280 encoding thioredoxin M3, chloroplastic-like which is known to be involved in ROS management and oxidative stress response was highly upregulated at 6dpi. Similarly, Psat7g013000 encoding leghemoglobin Lb120-29 was also highly

upregulated at 6dpi and 9dpi. At 9dpi, genes Psat7g060760 and Psat5g300960 encoding cationic amino acid transporter 1-like and embryo-specific protein ATS3B-like, respectively, were upregulated.

In genotype Carman, genes such as Psat5g300920 encoding embryo-specific protein ATS3B-like were highly upregulated at 2hpi. At 3dpi, Psat5g147200 and Psat6g173720 peroxidase P7-like, leghemoglobin 29-like, 7-deoxyloganetin glucosyltransferase-like, and F-box/At1g57790-like respectively were highly upregulated. While genes Psat7g099000 and Psat3g032400 and encoding for 7-deoxyloganetin glucosyltransferase-like and F-box/At1g57790-like respectively were highly downregulated at 3dpi. At 6dpi gene Psat5g057280 encoding Defensin, known antimicrobial peptides which are involved in defense against various fungal pathogens were highly upregulated with log2fold change value of more than 16. Similarly, gene Psat6g242600 encoding protein NRT1/ PTR FAMILY 7.1-like was also highly upregulated. At 9dpi, gene Psat3g171520 encoding for probable 2-isopropylmalate synthase was highly upregulated. Similarly, an uncharacterized gene encoding leghemoglobin-1-like was also highly upregulated in Carman at 9dpi.

5.4 DISCUSSION

Root rot caused by the non-host specific pathogen *Fave* is a significant disease causing annual decreases in yield in Canada and worldwide. Management options are currently limited to classical strategies such as crop rotation with non-host crops (Safarieskandari et al., 2021, Chatterton et al., 2019, Esmaeili Taheri et al., 2017). Despite the rapid development of genetics and omics research in recent years in plant science, less attention has been given to the study of pea resistance to *Fave*. There is no information regarding defense and counter defense from both host and pathogen perspective on this host-pathogen interaction. To understand the timing of host

defense strategies and identify candidate genes for resistance against *Fave*, we performed a RNA-seq study of pea roots during the infection process. Interestingly, more genes were downregulated than upregulated at all time points except for Carman at 9dpi and there was not much difference in DEGs between partially resistant and susceptible genotypes. The lack of strong DEG differences between partially resistant and susceptible genotypes at this time point may indicate that resistance is governed less by transcriptional reprogramming and more by constitutive traits such as root morphology or chemical barriers already present prior to infection.

Go-term enrichment analysis revealed the upregulation of genes related to the biosynthesis of secondary metabolites and metabolic pathways in at least one of the PR genotypes at almost all time points after *Fave* inoculation whereas in the susceptible genotype CDC Meadow, this activation was observed only at 6 dpi. These observations suggest that defense includes activation of secondary metabolites such as phytoalexins, phenolics, saponins and terpenoids. These compounds are directly antimicrobial to *Fusarium* species (Perincherry et al., 2021, Fu et al., 2023, Ninkuu et al., 2023) and are known to affect the cell permeability of the microbes, and to alter the membrane proteins, resulting in changes to pH gradient, ATP production, and membrane bound enzymes. For example, α-Tomatine, a saponin found in tomatoes, activates monomeric G-protein signaling pathways and phosphotyrosine kinases in *Fusarium oxysporum* (Zaynab et al., 2018). α-Tomatine interacts with the fungal cell membrane, causing leakage of cellular components, which triggers a burst of reactive oxygen species (ROS) and elevated Ca^{2+} levels, ultimately compromising the pathogen's cellular integrity (Ito et al., 2007). Similarly, the phytoalexin zealexin in maize causes resistance against *F. graminearum* microspores (Huffaker et al., 2011). Interestingly, in PR genotypes K2 and Carmen, GO term ‘plant-pathogen interaction’ was enriched among downregulated genes at 6hpi. We hypothesise

that negative regulation of plant defense may be due to pathogen manipulation to suppress defenses or natural feedback mechanisms to prevent excessive or prolonged immune activation, which could otherwise harm the plant. This effect was not seen at 9dpi, and its short term nature further supports the hypothesis of natural feedback mechanism. In PR genotypes at early to late time points, enrichment of pathways such as phenylpropanoid biosynthesis indicates that an array of secondary metabolites, including lignin, flavonoids, phytoalexins, are crucial for host defense.

The high upregulation of genes encoding leghemoglobin and nodulin-like following inoculation suggests that the plant's response involves a unique interplay between its nitrogen-fixing symbiotic mechanisms and defense processes. Leghemoglobin (LegHb) is a heme-containing protein predominantly found in leguminous plants, where it facilitates efficient nitrogen fixation in root nodules of nitrogen fixing plants (Ott *et al.*, 2005, Hoy & Hargrove, 2008). Non-symbiotic LegHb are detected in many non-legume and legume plants including pea (Anderson *et al.*, 1996) and its oxygen-binding properties might help manage oxygen levels and indirectly mitigate oxidative stress. The significant upregulation of LegHb might indicate i) the indirect activation of the symbiotic response; ii) a general response to stress involving root associated pathogens; iii) a response to oxidative stress caused by reactive oxygen species production during a pathogen attack; iv) a defense response to ensure optimum O_2 diffusion to root cells to provide sufficient energy for defense; or v) pathogen manipulation of host gene expression to facilitate proliferation, an idea supported by the observation that nodulin-25-like (also involved in nodulation) was upregulated at an early time point in the susceptible genotype and later time points in the PR genotypes.

Defensin was another important defense component which was highly upregulated in PR genotype 'Carman' at 6dpi. Carman demonstrated strong partial resistance to *Fave* in greenhouse and field conditions. Defensins are an integral part of plant innate immunity. They are highly stable small peptides that pose direct antimicrobial activity to a wide range of pathogens and chemical substances such as fungi, bacteria and proteinase inactivation (Stotz *et al.*, 2009, Lacerda *et al.*, 2014). For example, defensins in *Medicago* spp. (such as MsDef1and MtDef4) are potent inhibitors of fungi *F. graminearum* and *F. oxysporum* (Ramamoorthy *et al.*, 2007, Velivelli *et al.*, 2020, Sathoff *et al.*, 2019, Sagaram *et al.*, 2011). The defensin upregulated in our study is "defensin Tk-AMP-D1.1-like", an analogue to defensin Tk-AMP-D1 from *Triticum kiharae,* which has antifungal activity against *Fusarium* species such as *F. graminearum* and *F. verticillioides* (Odintsova *et al.*, 2008). The strong upregulation of this gene in our study indicates that this might also have antifungal activity against *Fave*.

Interestingly, in susceptible cultivar "CDC Meadow", two genes from germin-like protein subfamily (GLPs) were downregulated at 6hpi. GLPs, are associated with regulation function such as germination, pollen development, and embryogenesis, and are known to change expression during plant-pathogen interactions. For example, a germin-like gene was highly induced in wheat treated with *F. graminearum* produced deoxynivalenol (DON) (Desmond et al., 2008, Boddu et al., 2006). Although GLPs do not show direct antimicrobial function, many GLPs show oxalate oxidase (OxO) or superoxide dismutase (SOD)-like activity and are involved in both structural and biochemical defenses in plants via reactive oxygen metabolism while playing roles in physiological processes (Dunwell *et al.*, 2008). For example, expression of the germin-like gene *OsRGLP1* from rice in *Medicago truncatula* led to increased hydrogen peroxide (H_2O_2) accumulation and enhanced SOD activity, indicating elevated reactive oxygen

species production (Sultana *et al.*, 2016). Further, these plants showed significant resistance to *Fusarium oxysporum* infection, exhibiting fewer disease symptoms and no yield reduction. This study demonstrated that the elevated presence of H_2O_2 in the apoplast, mediated by GLP proteins, plays multiple roles in pathogen defense, including reinforcing the cell wall, inactivating haustoria and appressoria, and inducing the expression of defense-related genes. The antifungal activity of *GLP* is closely associated with the activation of defense pathways regulated by salicylic acid (SA) and jasmonic acid (JA), leading to increased expression of pathogenesis-related proteins and plant defensins (Sultana et al., 2016, Govindan *et al.*, 2024). In our case, the downregulation of GLPs at 6 hpi in the susceptible cultivar CDC Meadow may indicate that they are a susceptibility factor whose downregulation facilitates colonization of the plants. Six hours after inoculation is a crucial time point in terms of plant infection and pathogen detection in the case of pea-*Fave* interaction as described in Chapter 3.

In conclusion, this study provides significant insights into the molecular responses of pea plants to *F. avenaceum* infection, highlighting differences in defense mechanisms between partially resistant and susceptible genotypes. The observed temporal patterns of gene expression, including the activation of pathways such as secondary metabolite biosynthesis and phenylpropanoid biosynthesis, emphasize the dynamic nature of plant defense. Key findings, such as the upregulation of leghemoglobin, defensin, and nodulin-like genes, suggest a multifaceted interplay of symbiotic, oxidative stress management, and direct antimicrobial responses in partially resistant genotypes. Conversely, the downregulation of germin-like proteins in the susceptible cultivar underscores their potential role as susceptibility factors. These findings contribute to a deeper understanding of the complex host-pathogen interactions and provide valuable candidate genes for breeding programs aimed at enhancing resistance to root rot

in peas. Further studies are warranted to elucidate the exact mechanisms underlying these gene regulations and to validate their functional roles in conferring resistance to *Fave*.

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TABLES AND FIGURES

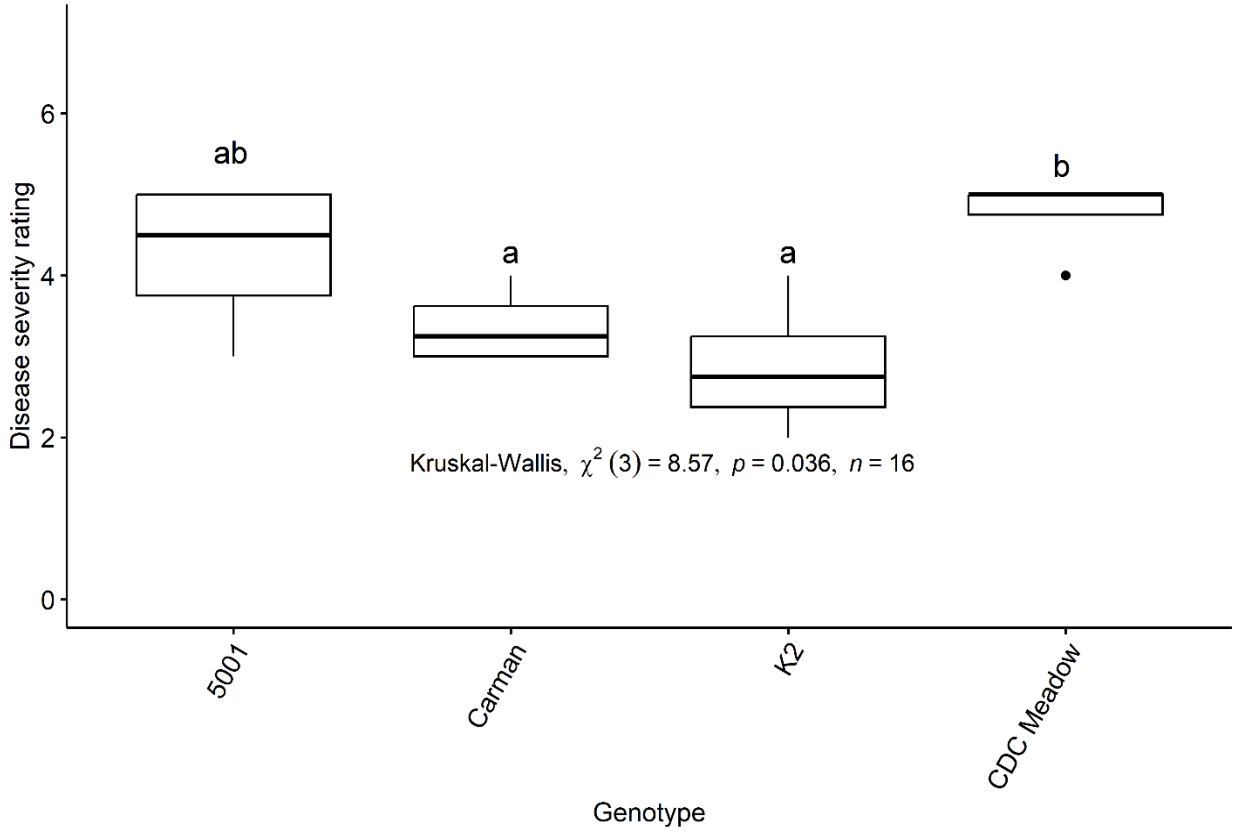


Figure 5.1. Box and whisker plot of disease severity ratings (DSR) in response to F. avenaceum at 21 dpi in greenhouse conditions. The statistical significance was determined based on Kruskal Wallis test followed by Dunnet test to adjust for the multiple comparison effects. Box with different letters on the top indicates the difference in DSR at $p < 0.05$.

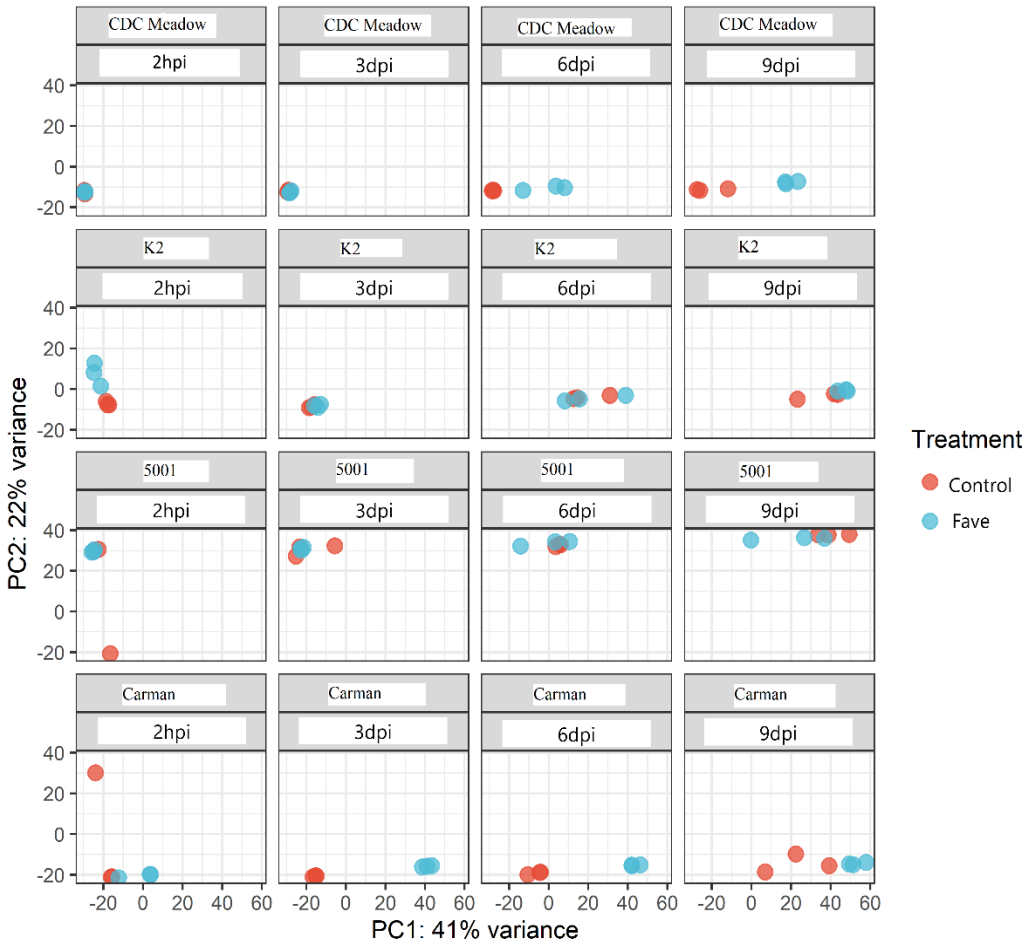


Figure 5.2. Principal component analysis (PCA) of gene count matrix in four genotypes (CDC Meadow, K2, 5001 and Carman) across both treatments (F. avenaceum inoculated (Fave) and mock-inoculated (Control) and all sampling times (2hpi, 3dpi, 6dpi, 9dpi).

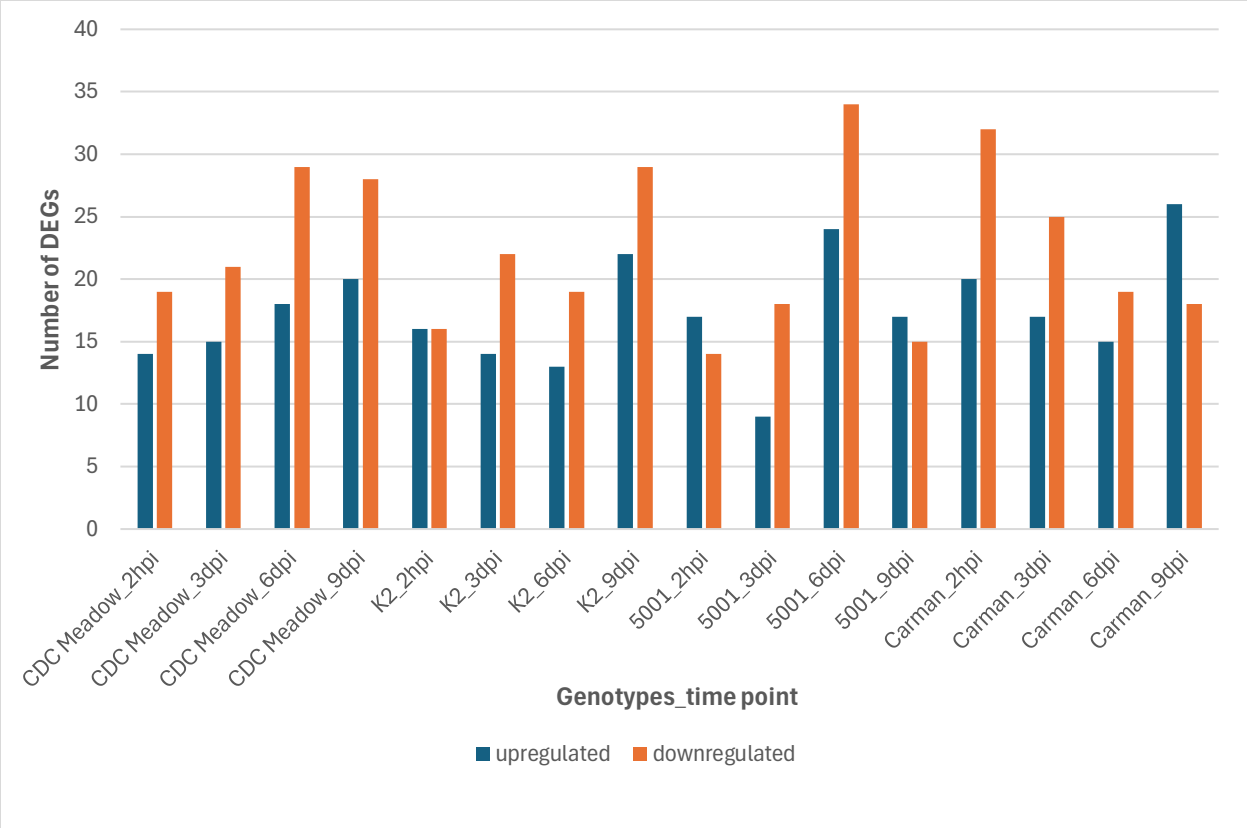


Figure 5.3. Number of upregulated (blue) and downregulated (orange) genes from all the genotypes (CDC Meadow, K2, 5001 and Carman) at all the time points (2hpi, 3dpi, 6dpi, 9dpi).

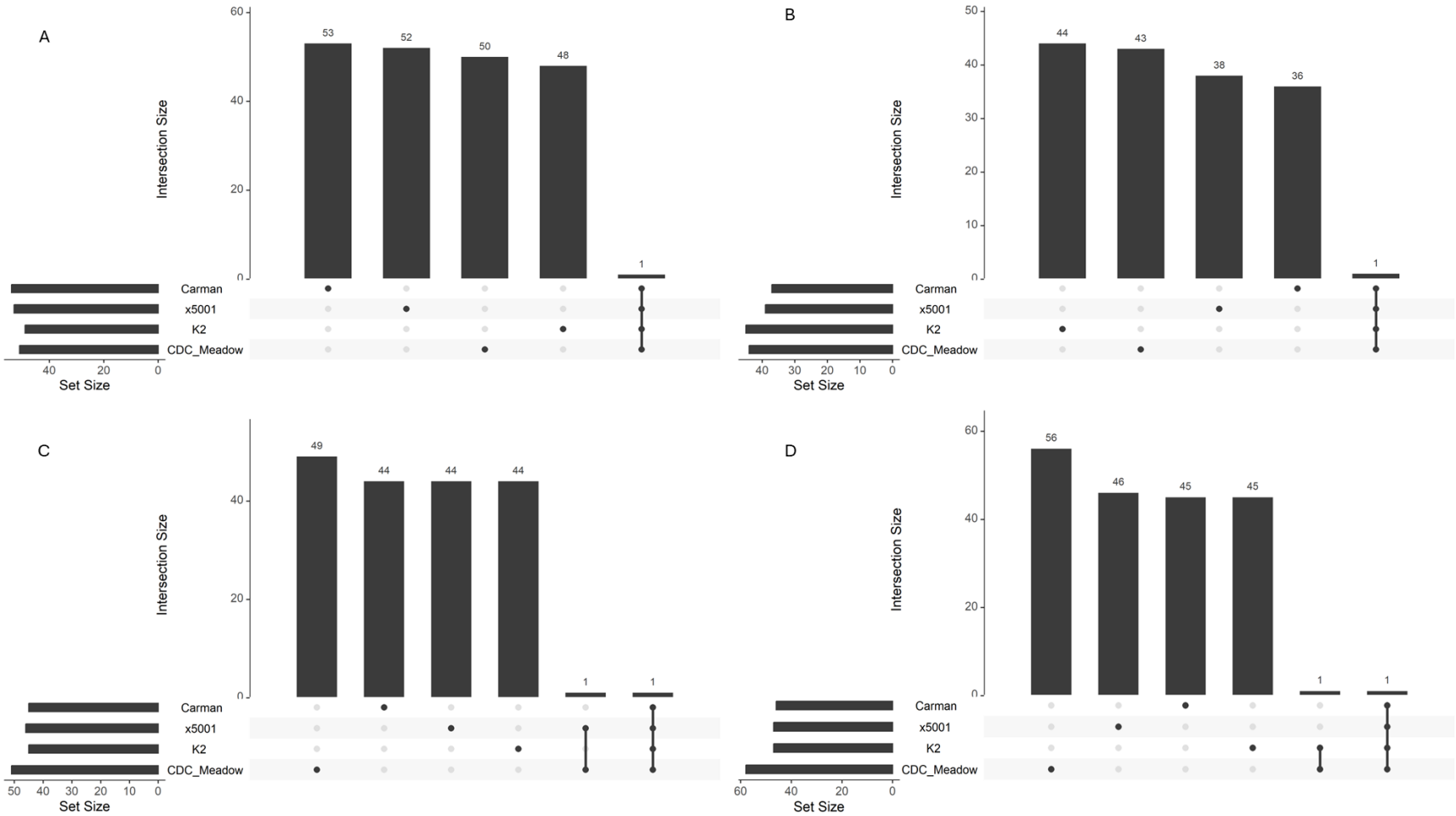


Figure 5.4. Upset plots summarizing upregulated genes (A: 2hpi, B: 3dpi, C: 6dpi, D: 9dpi) in all genotypes. The plot illustrates the overlap and unique sets of differentially expressed genes (DEGs) identified in all genotypes. The horizontal bars on the left represent the total number of DEGs in each genotype, while the vertical bars depict the number of DEGs shared among specific intersections of genotypes, as indicated by the connected dots below.

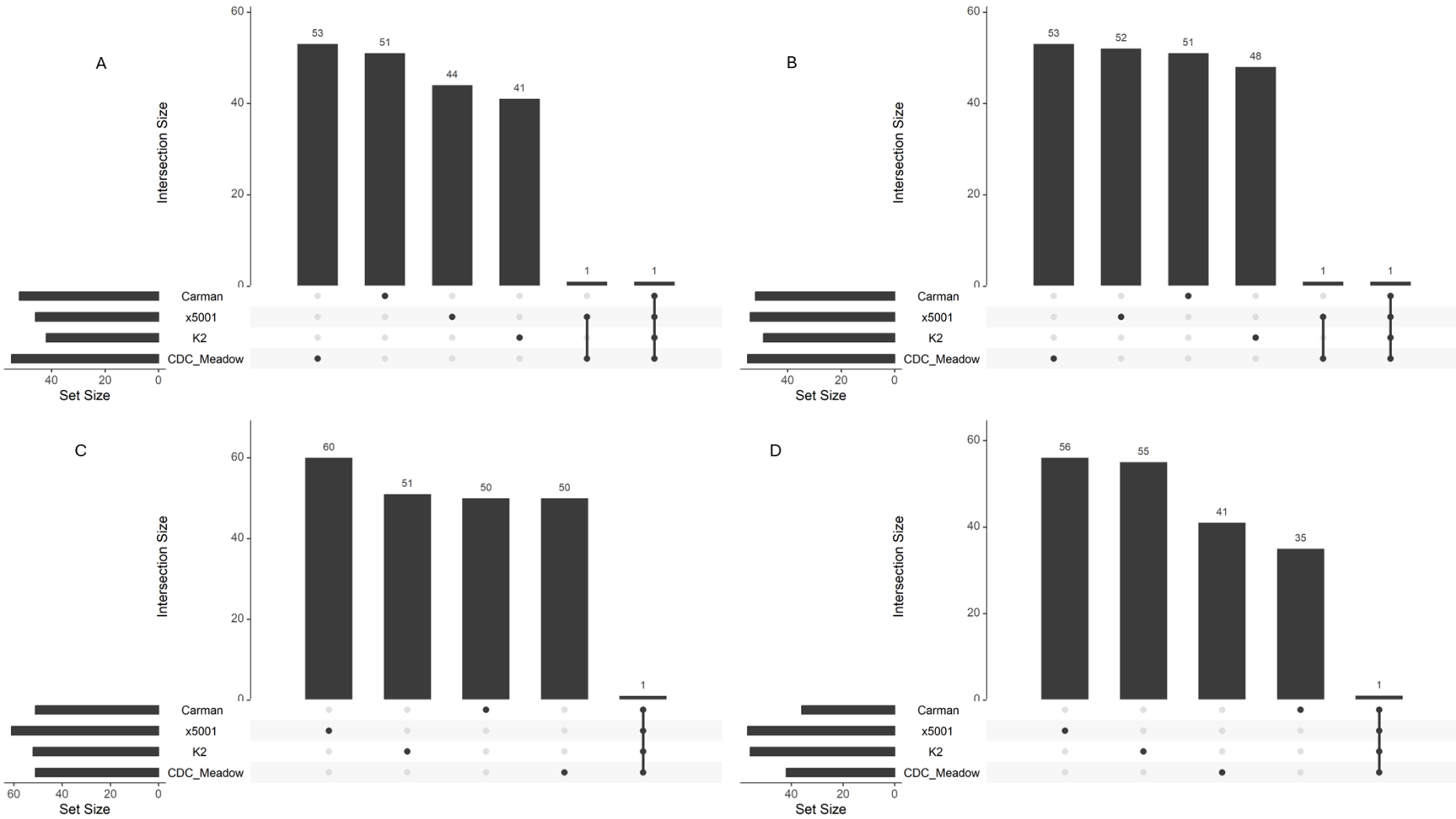


Figure 5.5. Upset plots showing a summary of downregulated genes (A: 2hpi, B: 3dpi, C: 6dpi, D: 9dpi) in all genotypes. The plot illustrates the overlap and unique sets of differentially expressed genes (DEGs) identified in all genotypes. The horizontal bars on the left represent the total number of DEGs in each genotype, while the vertical bars depict the number of DEGs shared among specific intersections of genotypes, as indicated by the connected dots below.

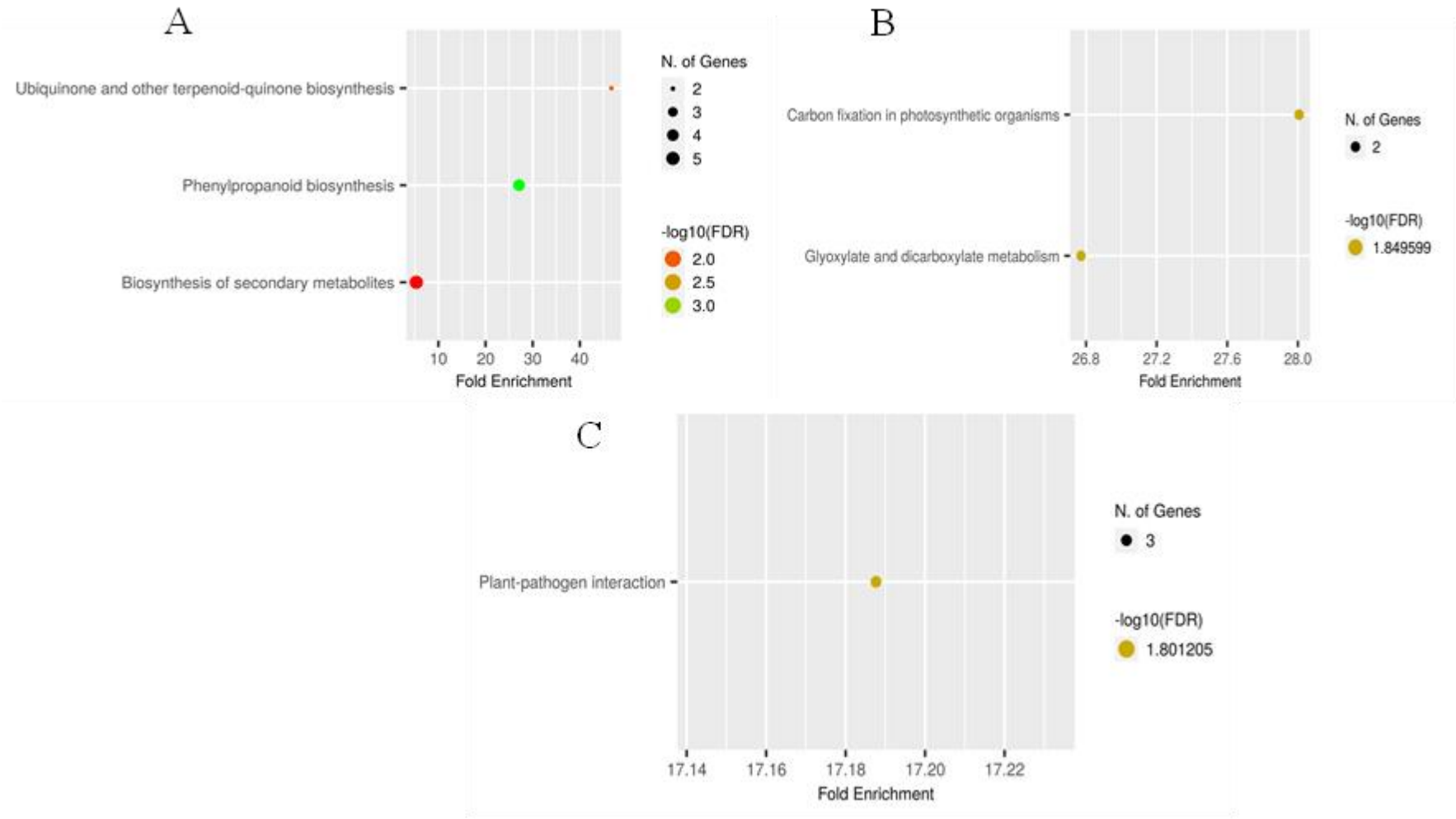


Figure 5.6. Dot plots showing a summary of the enriched pathway network of upregulated genes in PR genotypes 5001 (A), K2 (B) at 2hpi. GO pathway enrichment among downregulated genes at 2hpi in PR genotype (K2).

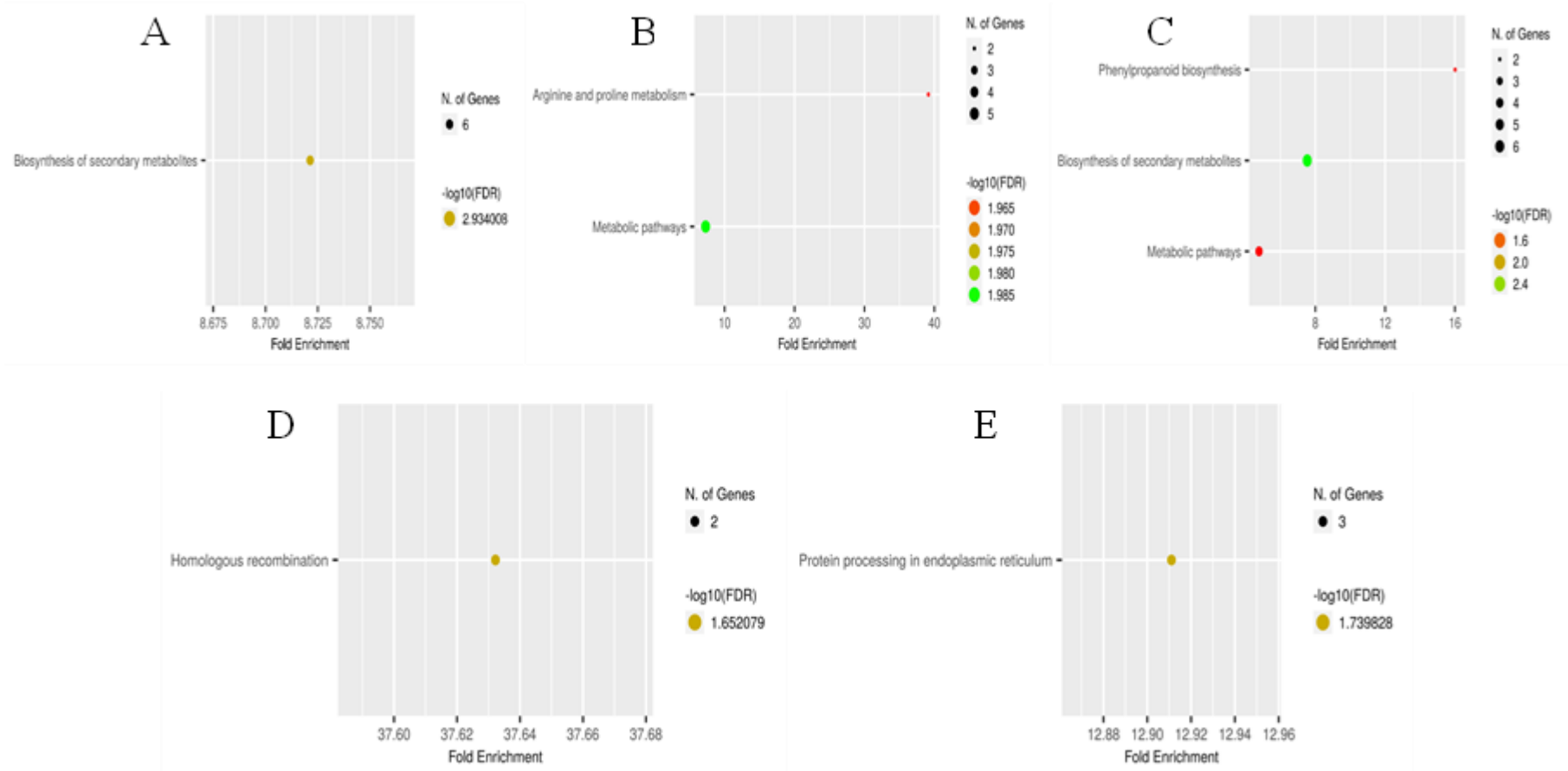


Figure 5.7. Dot plots showing a summary of the enriched pathway network of upregulated genes in PR genotypes at 3dpi, 5001 (A) Carman (B), K2 (C), and S genotype CDC Meadow (D). GO pathway enrichment among downregulated genes at 3dpi CDC Meadow (E).

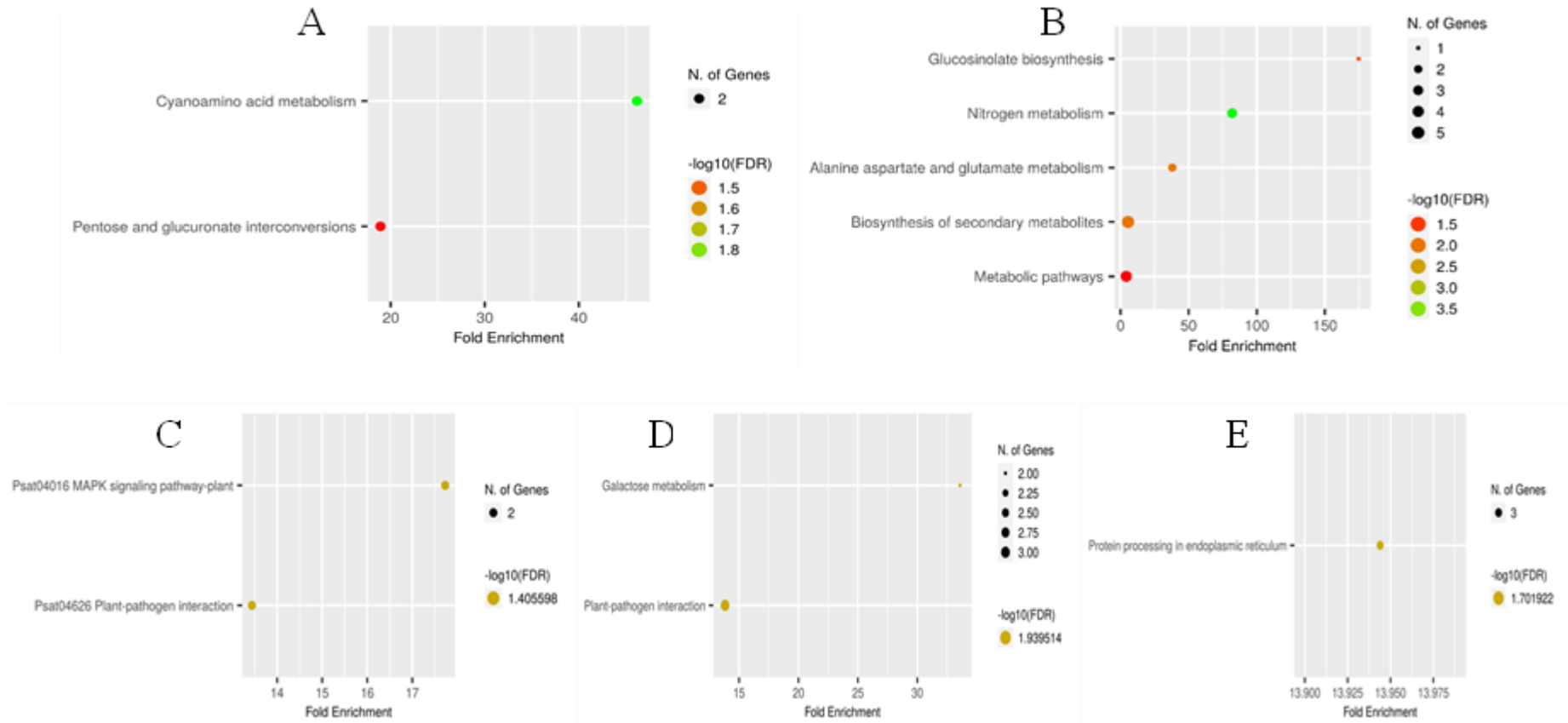


Figure 5.8. Dot plots showing a summary of the enriched pathway network of upregulated genes in PR genotype Carman (A), and susceptible genotype CDC Meadow (B)at 6dpi and among downregulated genes Carman (C), K2 (D), CDC Meadow (E) at 6dpi.

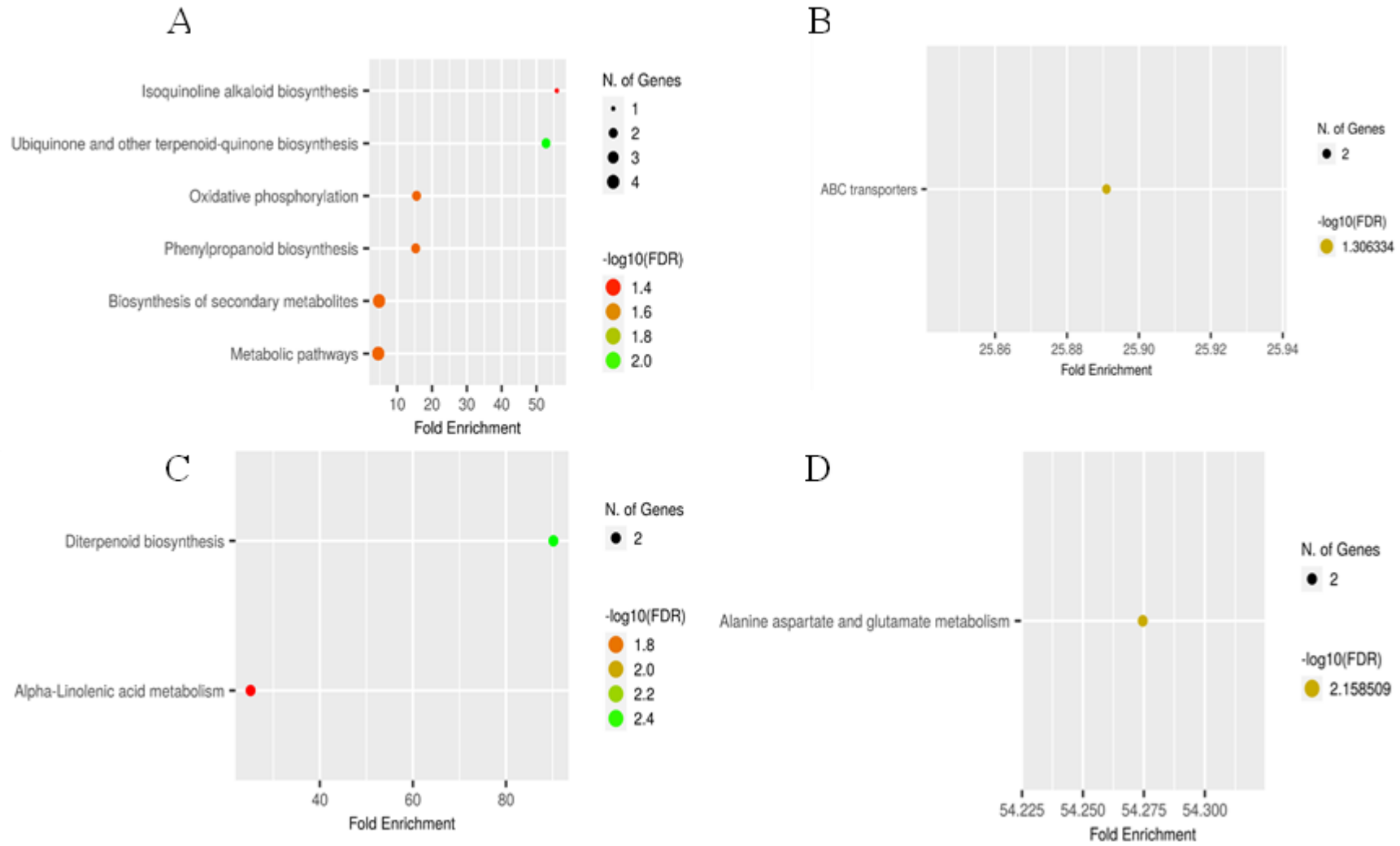


Figure 5.9. Dot plots showing a summary of the enriched pathway network of upregulated genes in PR genotype at 9dpi, K2 (A), Carman (B), CDC Meadow (C) and among downregulated genes Carman (D) at 9dpi.

Table 5.1. Highly regulated candidate genes with their annotation

Genotype	Time point	Gene	Description	log2FC	remarks
CDC Meadow	3dpi	Psat5g172760	nodulin-25-like	12.63	up
CDC Meadow	6dpi	Psat7g151720	germin-like protein subfamily 1 member 17	-9.37	down
CDC Meadow	6dpi	127106330	germin-like protein subfamily 1 member 17	-10.63	down
K2	2hpi	Psat7g097160	FK506-binding protein 4-like	12.12	up
K2	2hpi	Psat2g187880	putative 12-oxophytodienoate reductase 11	8.13	up
K2	9dpi	Psat7g249480	CASP-like protein 4B1	11.18	up
K2	9dpi	127080057	carbonic anhydrase 2-like	9.82	up
5001	6dpi	Psat7g125280	thioredoxin M3, chloroplastic-like	11.21	up
5001	6 dpi	Psat7g013000	leghemoglobin Lb120-29	11.29	up
5001	9dpi	Psat7g060760	cationic amino acid transporter 1-like	9.53	up
5001	9dpi	Psat5g300960	embryo-specific protein ATS3B-like	16.19	up
5001	9dpi	Psat2g039800	leghemoglobin Lb120-8	13.02	up
Carman	2 hpi	Psat5g300920	embryo-specific protein ATS3B-like	14.88	up
Carman	2 hpi	Psat5g300880	embryo-specific protein ATS3B-like	11.277	up
Carman	3dpi	Psat5g147200	peroxidase P7-like	8.81	up
Carman	3dpi	Psat6g173720	leghemoglobin 29-like	16.57	up
Carman	3dpi	Undefined	probable purine permease 11	7.26	up
Carman	3dpi	Psat7g099000	7-deoxyloganetin glucosyltransferase-like	-9.82	down
Carman	3dpi	Psat3g032400	F-box/At1g57790-like	-11.53	down
Carman	6dpi	Psat5g057280	defensin Tk-AMP-D1.1-like	16.08	up
Carman	6dpi	Psat6g242600	protein NRT1/ PTR FAMILY 7.1-like	9.71	up
Carman	9dpi	Psat3g171520	probable 2-isopropylmalate synthase	16.81	up
Carman	9dpi	Undefined	leghemoglobin-1-like	14.06	up

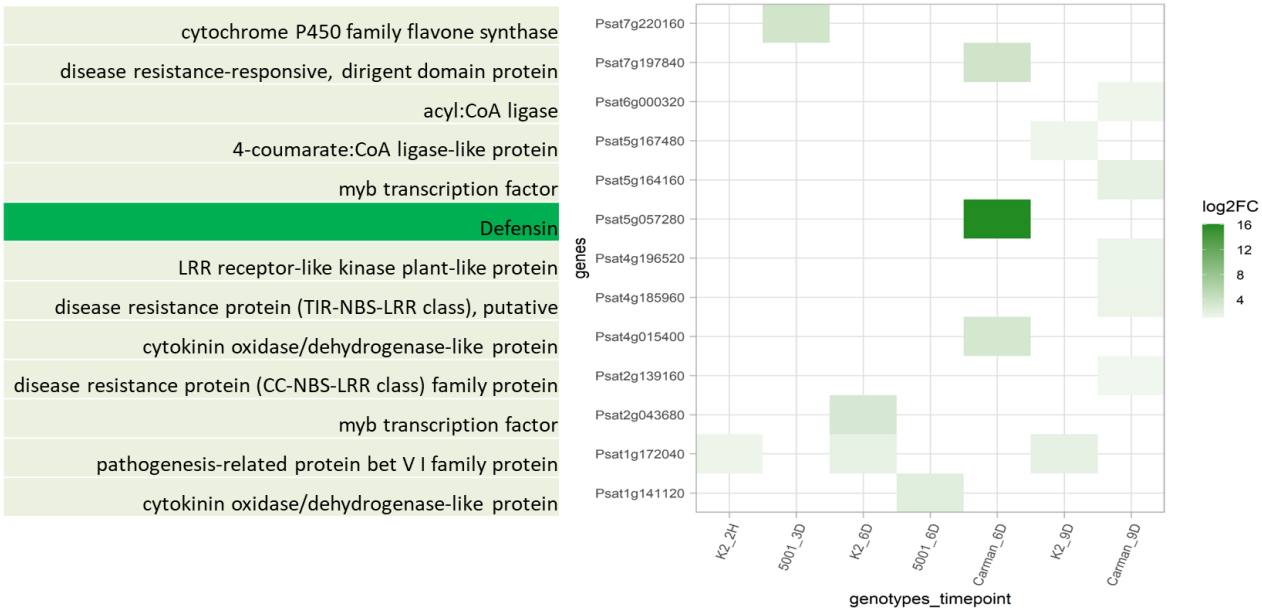


Figure 5.10. Heatmap with important defense-related genes at different time points where 2H:2hpi, 3D: 3dpi, 6D:6dpi and 9D: 9dpi.

CHAPTER 6 : CONCLUSION

Pea root rot remains a persistent challenge for pea production in western Canada, particularly in Saskatchewan and Alberta, where up to 70% of fields may be affected by *Aphanomyces euteiches* during wet summers. Considering the yearly challenge for Canadian farmers and limited effectiveness of current management practices, this thesis dissects pathogen colonization and host defense mechanisms at the molecular level with the ultimate goal of supporting resistance breeding and improved field-level management strategies.

Chapter 2 tested different inoculation methods for *Fusarium avenaceum* under controlled conditions to ensure reproducible disease development for screening pea germplasm. Several inoculation approaches were compared across pea genotypes with varying resistance levels. Soil inoculation with *Fave* conidia was the most reliable and biologically relevant method, providing consistent disease severity and mimicking natural infection. In contrast, classical seed soak methods, while producing high disease pressure, negatively impacted emergence, making the plants unsuitable for large-scale screening. Alternative methods, including modified seed and root soak techniques, were found to be valuable for controlled host-pathogen interaction studies. These findings establish a standardized approach for phenotyping partial resistance to *Fave*, critical for advancing resistance screening efforts.

Chapter 3 defined critical time points for both *A. euteiches* and *F. avenaceum* colonization and symptom development using hydroponic and greenhouse systems. Disease progression data and qPCR quantification demonstrated that a latent period of 2–4 days follows

initial colonization before visible symptoms emerge, with timing influenced by cultivar and pathogen. The study identified 2–24 hpi (for *A. euteiches*) and up to 6 dpi (for *F. avenaceum*) as key windows for capturing early defense responses. This work established a temporal framework essential for early-stage defense studies including downstream transcriptomic and metabolomic analyses.

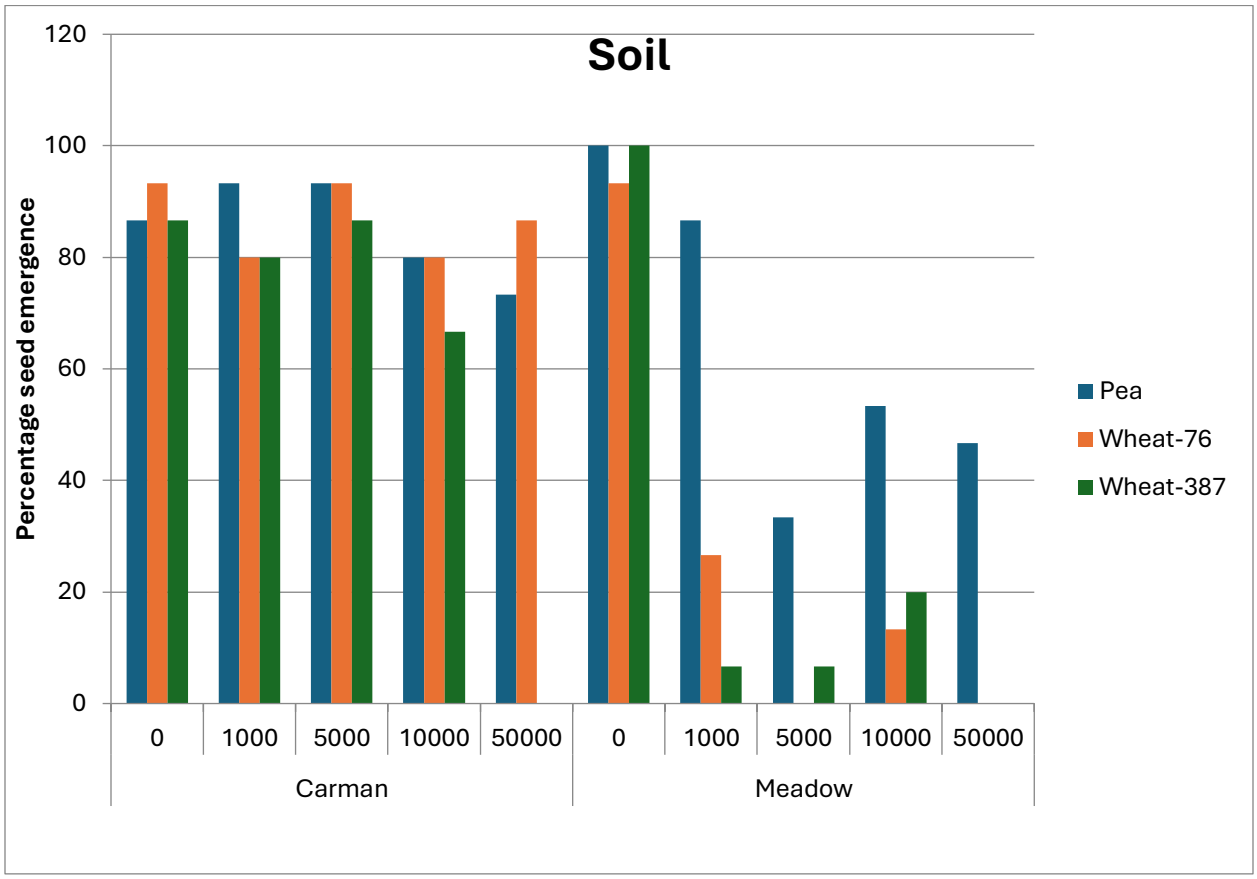
Chapter 4 explored the transcriptomic and metabolomic responses of partially resistant (PR) and susceptible pea genotypes to *A. euteiches*. RNA-seq and targeted metabolite profiling revealed that PR genotypes exhibit strong transcriptional activation of key defense pathways, including MAPK signaling, transcription factors (e.g., WRKY, MYB), and phytoalexin, including pisatin, biosynthesis. Hormonal pathways related factors, particularly ethylene (ERF) and jasmonic acid signaling (AOC and JAZ), were prominently activated in PR lines. Metabolomics confirmed rapid accumulation of pisatin and its precursors, especially in PI 660736. Importantly, many differentially expressed genes (DEGs) mapped outside known QTLs (e.g., *Ae-Ps4.5*, *Ae-Ps7.6*), suggesting novel loci contributing to resistance. These findings provide molecular targets for resistance breeding and highlight the importance of early, coordinated defense activation in limiting *A. euteiches* colonization.

Chapter 5 extended the transcriptomic investigation to *F. avenaceum*, profiling PR (5001, Carman, K2) and susceptible (CDC Meadow) genotypes. A total of 635 DEGs were identified across time points, with a general trend of downregulation in susceptible lines. In PR genotypes,

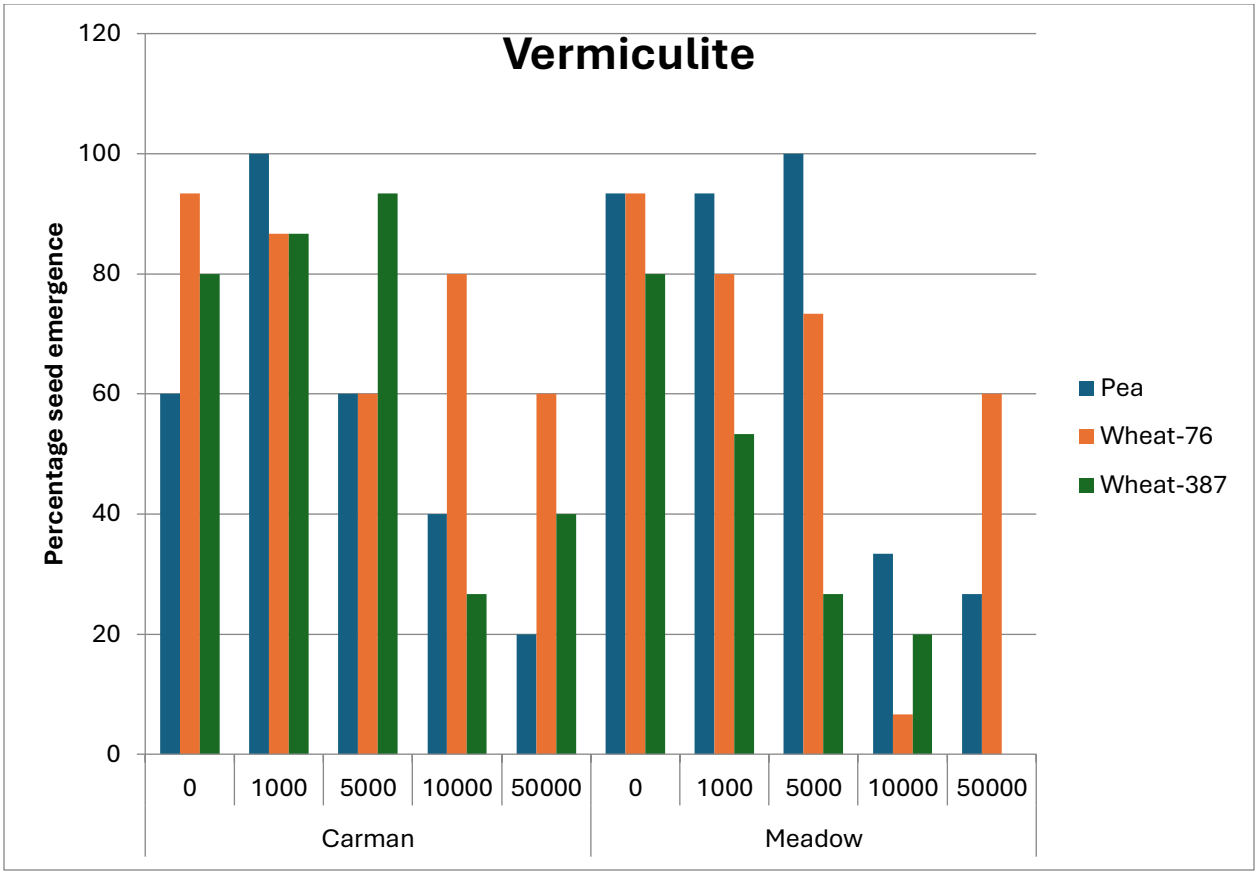
early upregulation of genes involved in secondary metabolism, nitrogen homeostasis, and oxidative stress management was observed. Notably, defensins and leghemoglobins were strongly induced in PR lines, suggesting roles in pathogen restriction and redox balance. In contrast, germin-like proteins (GLPs) were downregulated early CDC Meadow, which may impair ROS-based defense. These insights suggest that PR genotypes mount a more robust and timely defense response, and identify key candidate genes, including defensins, leghemoglobins, and GLPs, for further functional validation and breeding applications. Notably, the defense responses activated against *A. euteiches* and *F. avenaceum* showed limited overlap, underscoring the distinct nature of these two pathosystem.

Altogether, this work advances our understanding of host-pathogen interactions in the pea root rot complex. By integrating phenotypic, transcriptomic, and metabolomic data across genotypes and pathogens, it identifies critical defense components and time points associated with resistance expression. These findings directly support the development of molecular markers and breeding strategies to improve root rot resistance in pea cultivars adapted to Canadian environments.

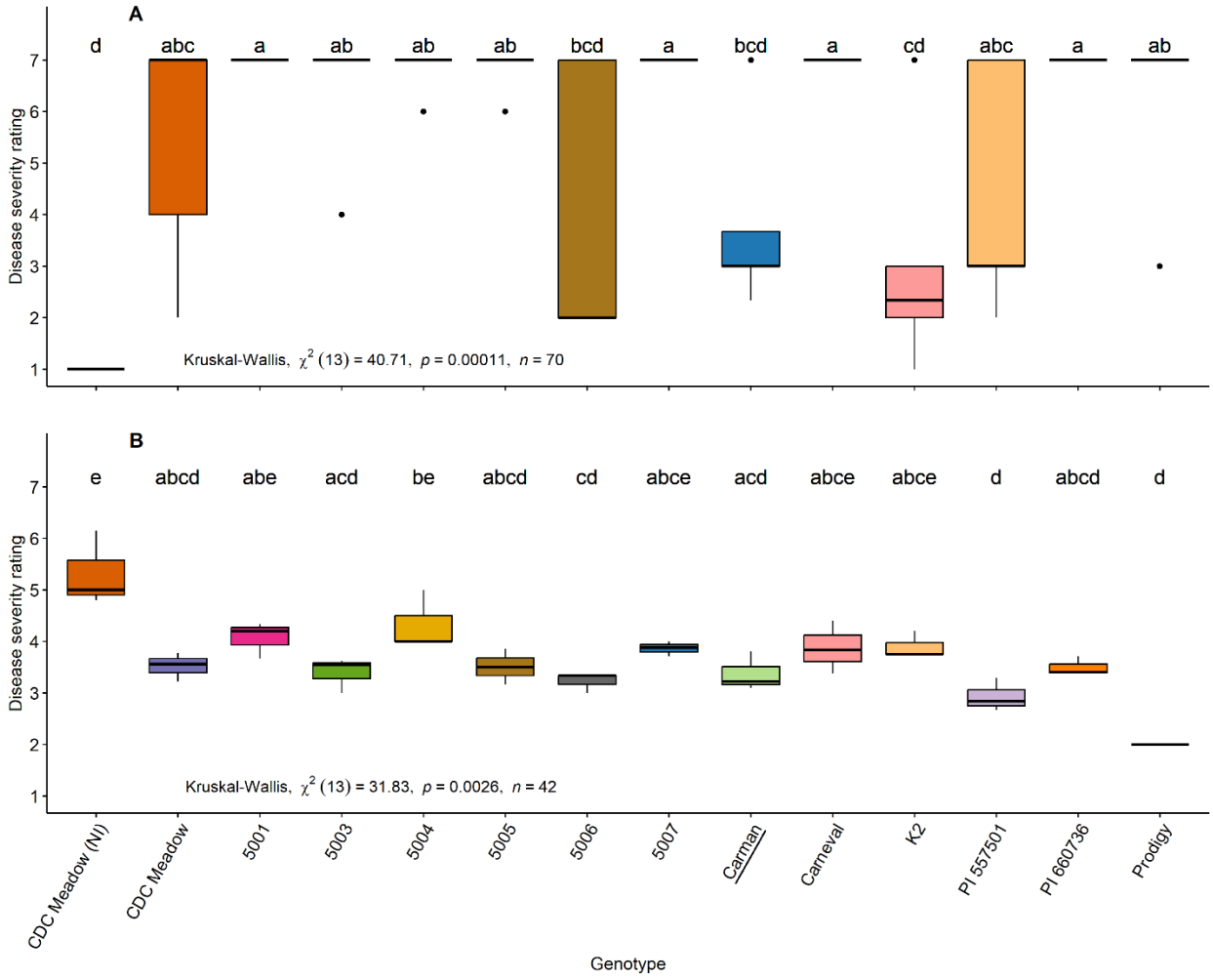
APPENDIX



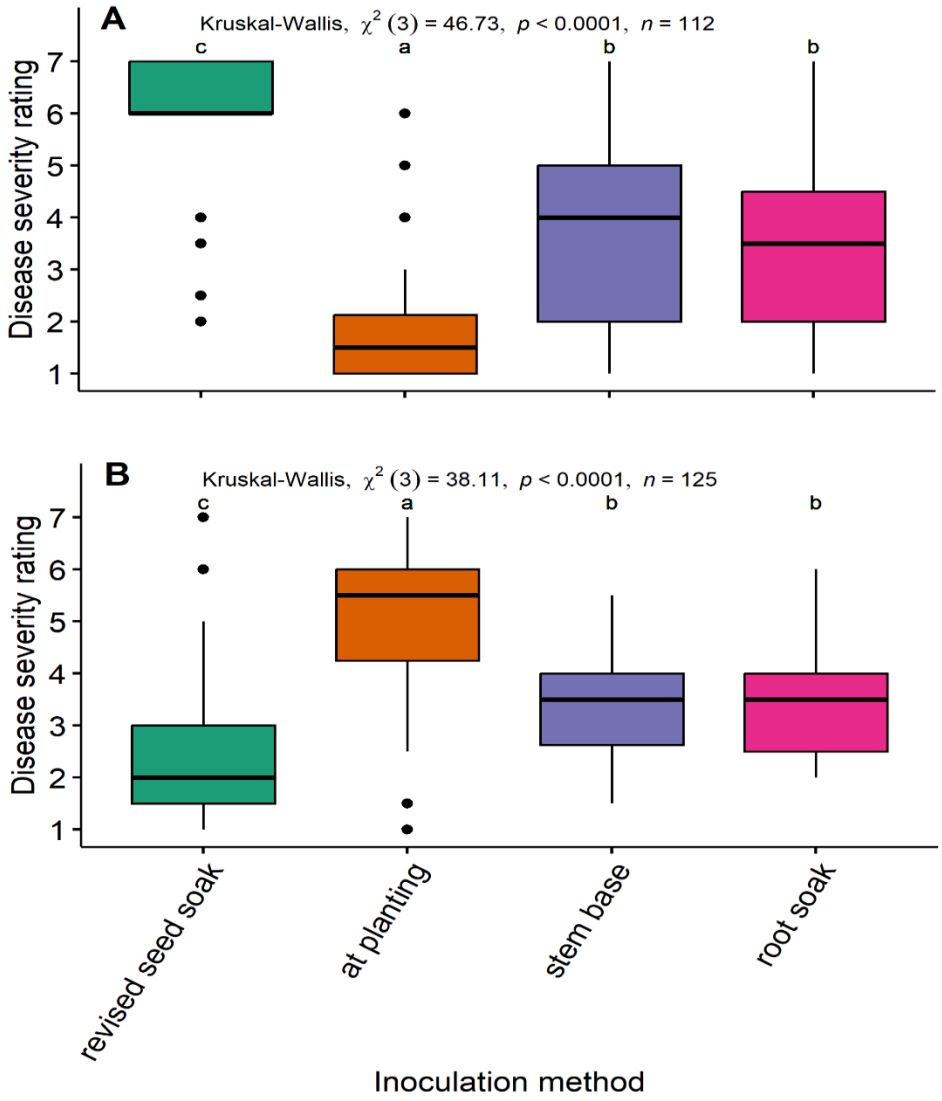
Supplementary Figure 1. Percentage Carman and CDC Meadow seed emergence following treatment with different concentrations (0, 1000, 5000, 10000 and 50000 macroconidia/ml) of three different *F. avenaceum* isolates; 1306.08 (Pea), Fa DAOM 2420768 (Wheat-76), and Fa DAOM 242378 (Wheat-387) and grown in soil. Y axis represents the percentage emergence.



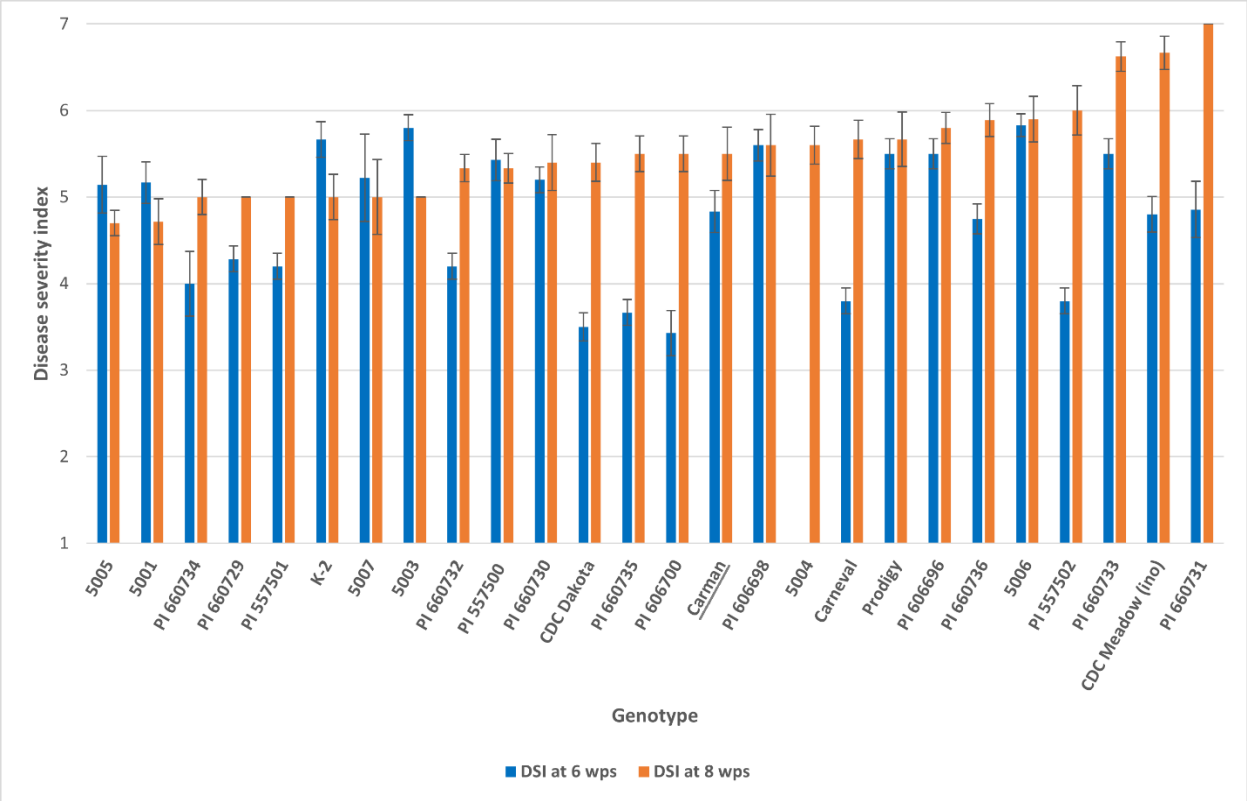
Supplementary Figure 2. Percentage Carman and CDC Meadow seed emergence following treatment with different concentrations (0, 1000, 5000, 10000 and 50000 macroconidia/ml) of three different *F. avenaceum* isolates; 1306.08 (Pea), Fa DAOM 2420768 (Wheat-76), and Fa DAOM 242378 (Wheat-387) and grown in vermiculite. Y axis represents the percentage emergence.



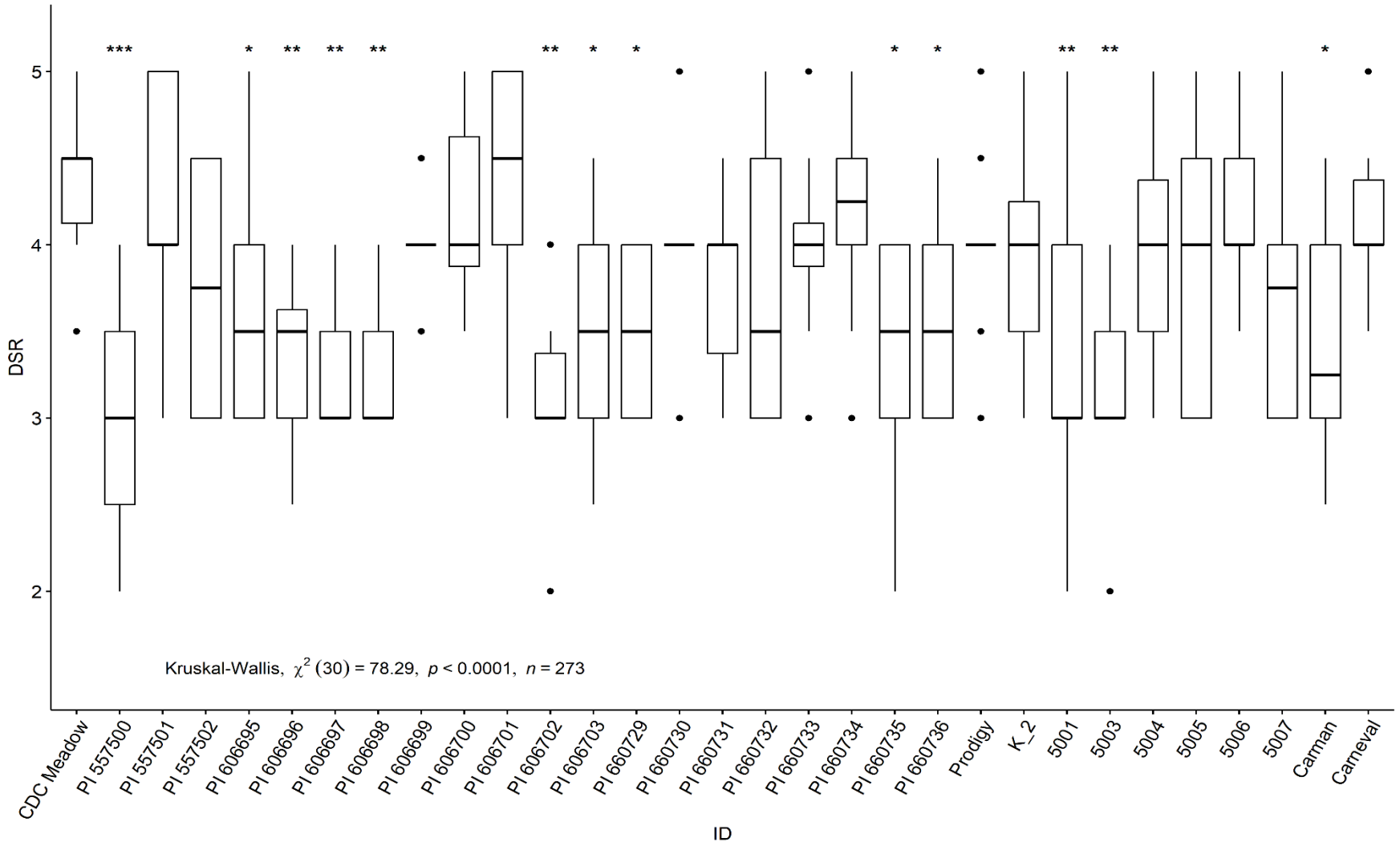
Supplementary Figure 3. Box plots showing disease severity rating of 14 genotypes following inoculation with *F. avenaceum* using seed soaking method (A) and corn meal layering method (B) of trial #2. Boxes with different letter groupings are significantly different. Each box represents the interquartile range of disease severity scores for a specific genotype, with the median indicated by the horizontal line within the box. Whiskers denote variability outside the upper and lower quartiles, and outliers are represented by individual points.



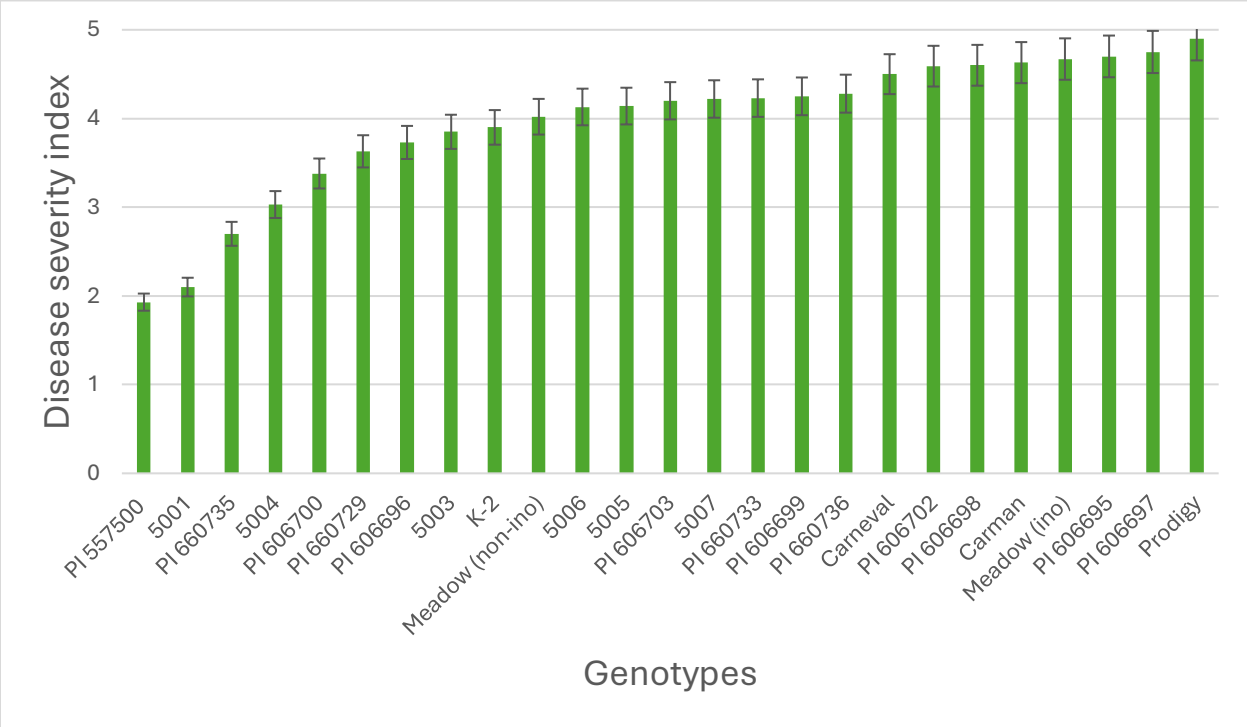
Supplementary Figure 4. Boxplot showing disease severity rating of genotype 'CDC Meadow' (A, non-pigmented seed coat) and 'CDC Dakota' (B, pigmented seed coat) following *Fave* inoculation using 4 inoculation methods. Results shown are from trial #2. Boxplots with different letter groupings are significantly different. Each box represents the interquartile range of disease severity scores for a specific genotype, with the median indicated by the horizontal line within the box. Whiskers denote variability outside the upper and lower quartiles, and outliers are represented by individual points.



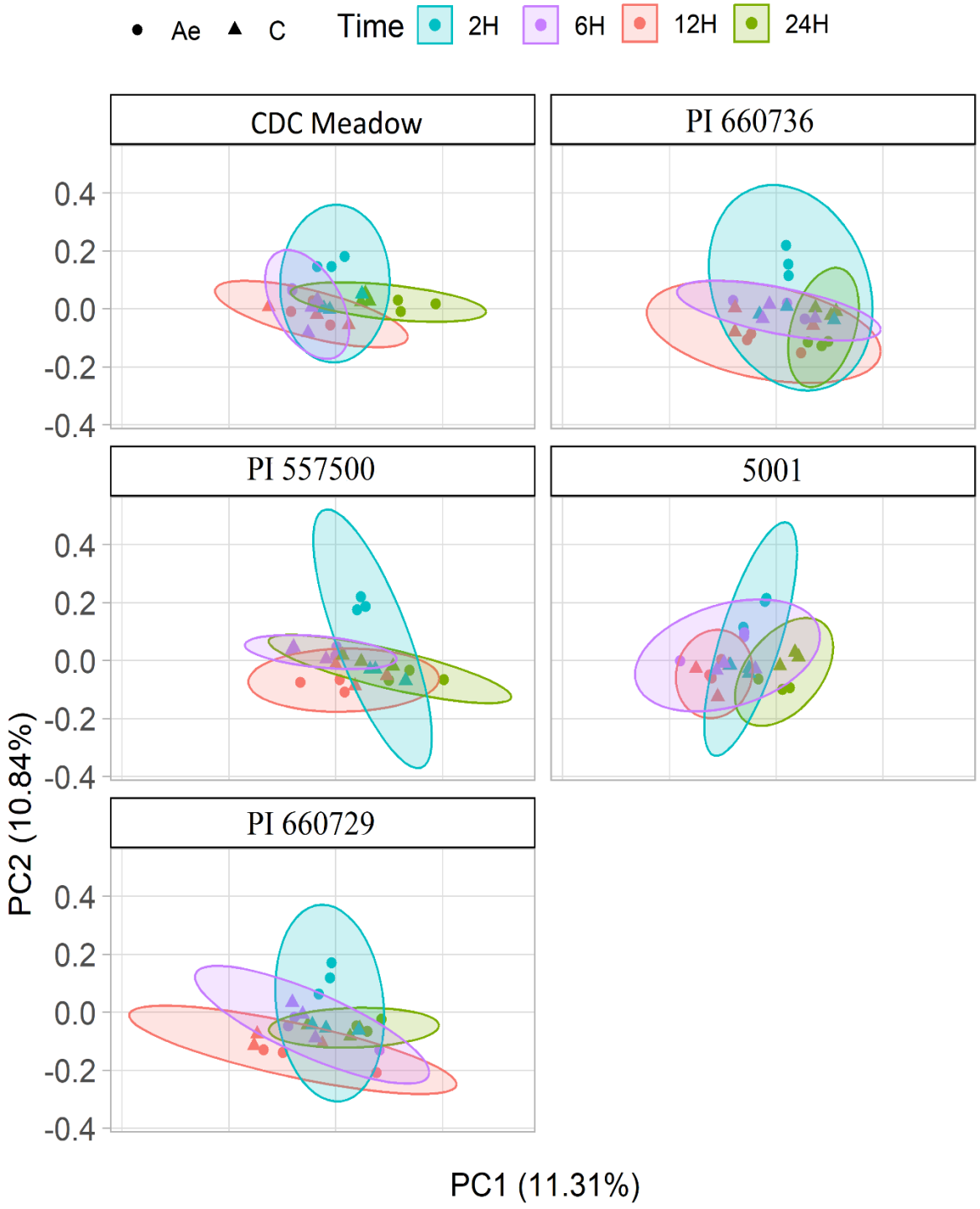
Supplementary Figure 5. Bar plots showing disease severity index of 26 genotypes screened in the field in the year 2020 at six and eight weeks post seeding (wps). Standard error was calculated from individual DSR values in each genotype, and other statistical analysis was not performed due to too many missing replicates.



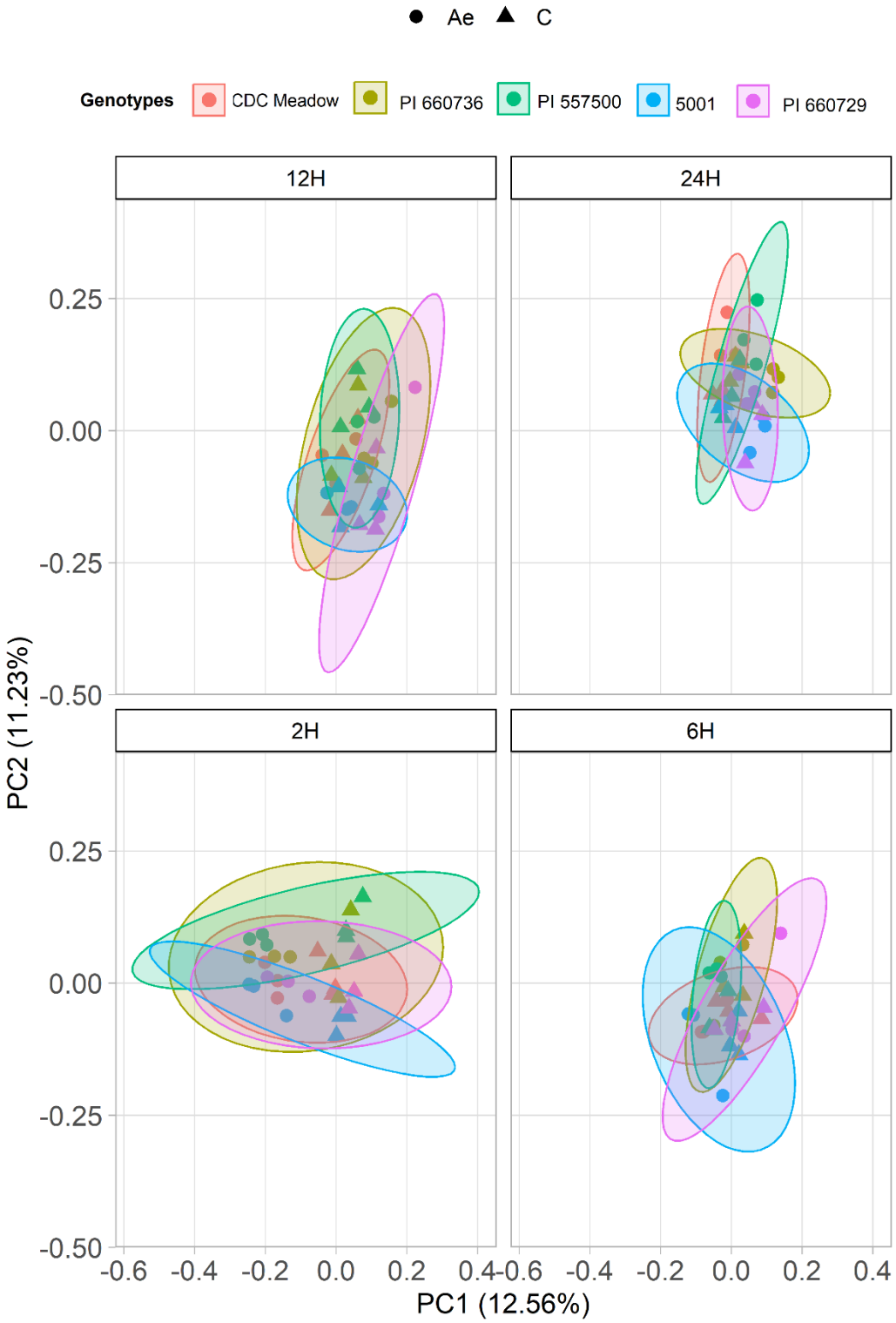
Supplementary Figure 6. Box and whisker plots showing Aphanomyces root rot (ARR) disease severity ratings on all genotypes screened in greenhouse conditions. The statistical significance was determined based on Kruskal Wallis test followed by Dunnet test to adjust for the multiple comparison effects. Symbols, * denotes significance level at p<0.05, ** denotes<0.005, *** denotes p<0.0005.



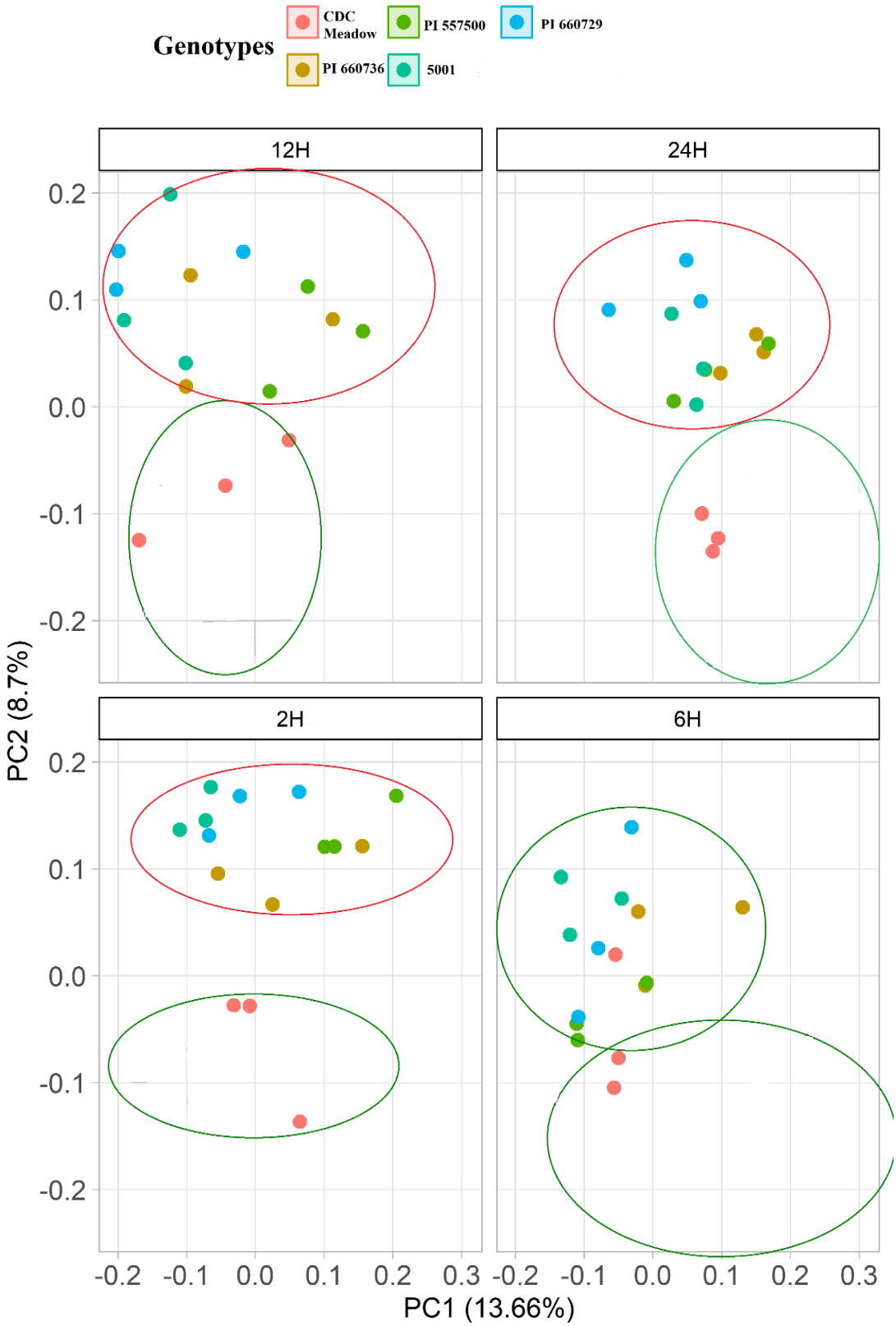
Supplementary Figure 7. Aphanomyces root rot (ARR) disease severity index of genotypes screened under field conditions in 2020. 'Non-ino' refers to non inoculated and 'ino' refers to inoculated genotype CDC Meadow genotype.



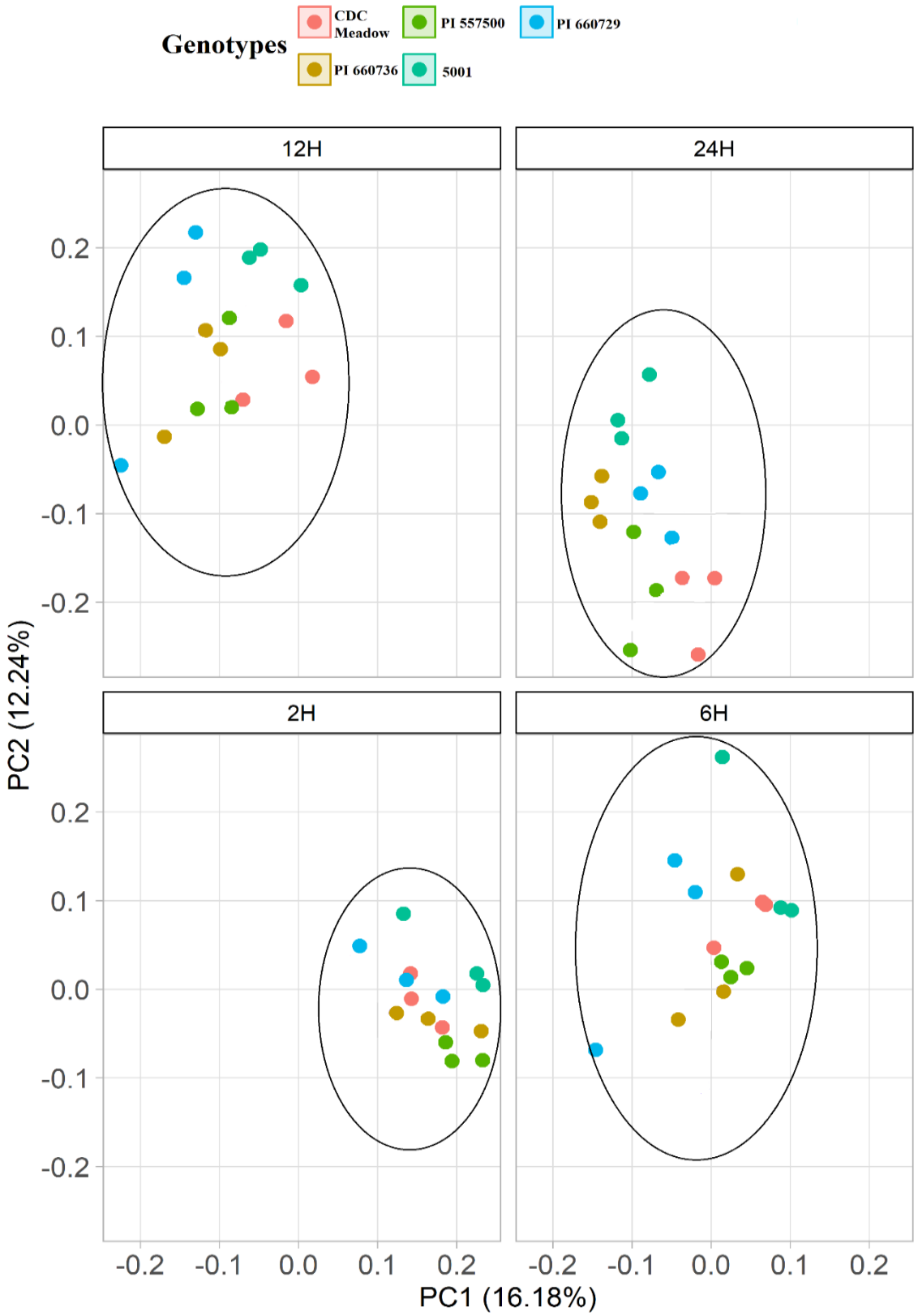
Supplementary Figure 8. Principal component analysis (PCA) of gene count matrix across both treatments (*A. euteiches* inoculated (circles) and mock-inoculated (triangles)) and all sampling times (2, 6, 12 and 24 hpi), for each genotype.



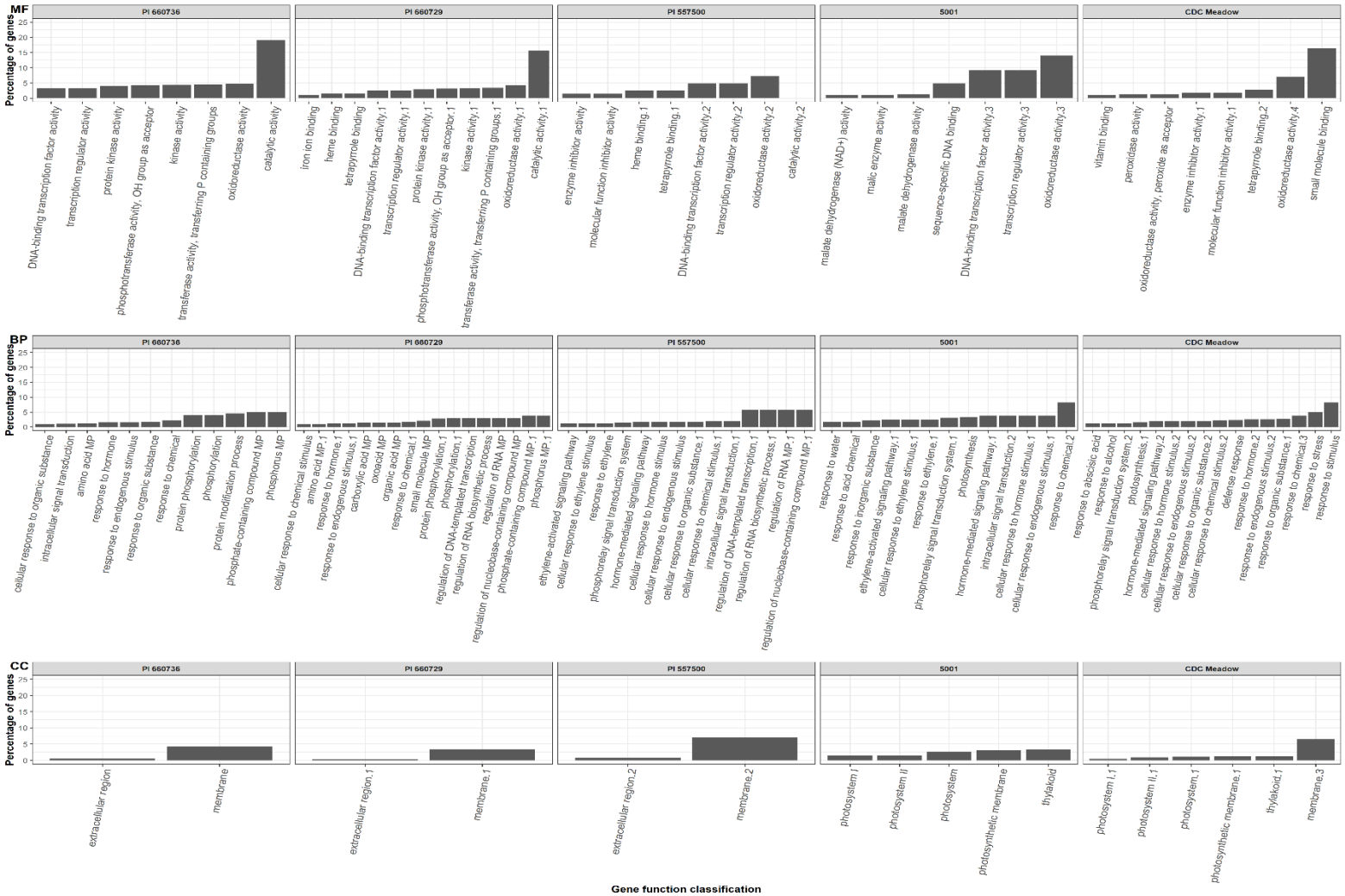
Supplementary Figure 9. PCA of gene count matrix of all genotypes in different time points. *A. euteiches* inoculated (*Ae*) and mock-inoculated (C)



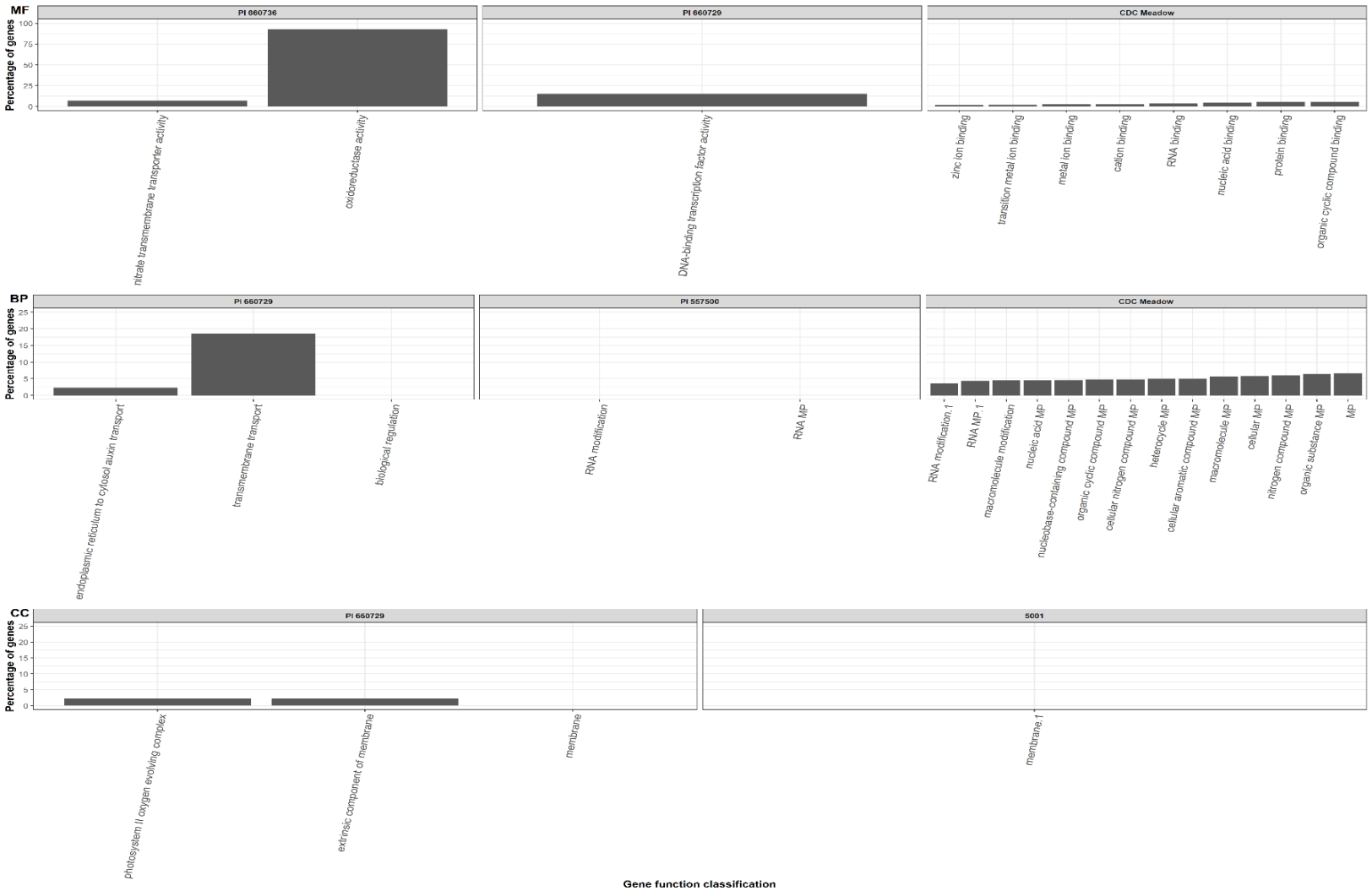
Supplementary Figure 10. PCA of mock-inoculated controls of all genotypes at all time points



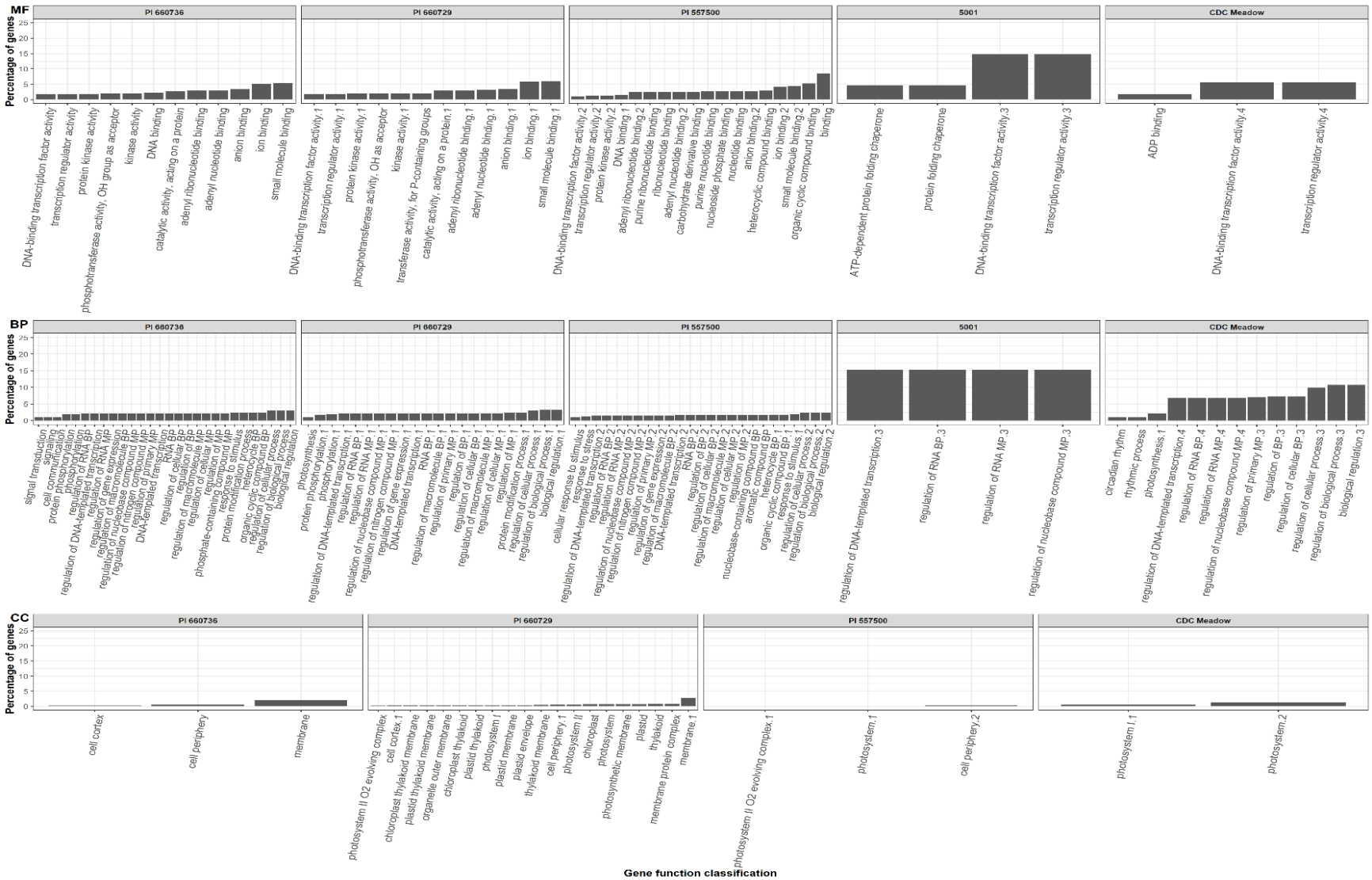
Supplementary Figure 11. PCA of inoculated all genotypes at all time points



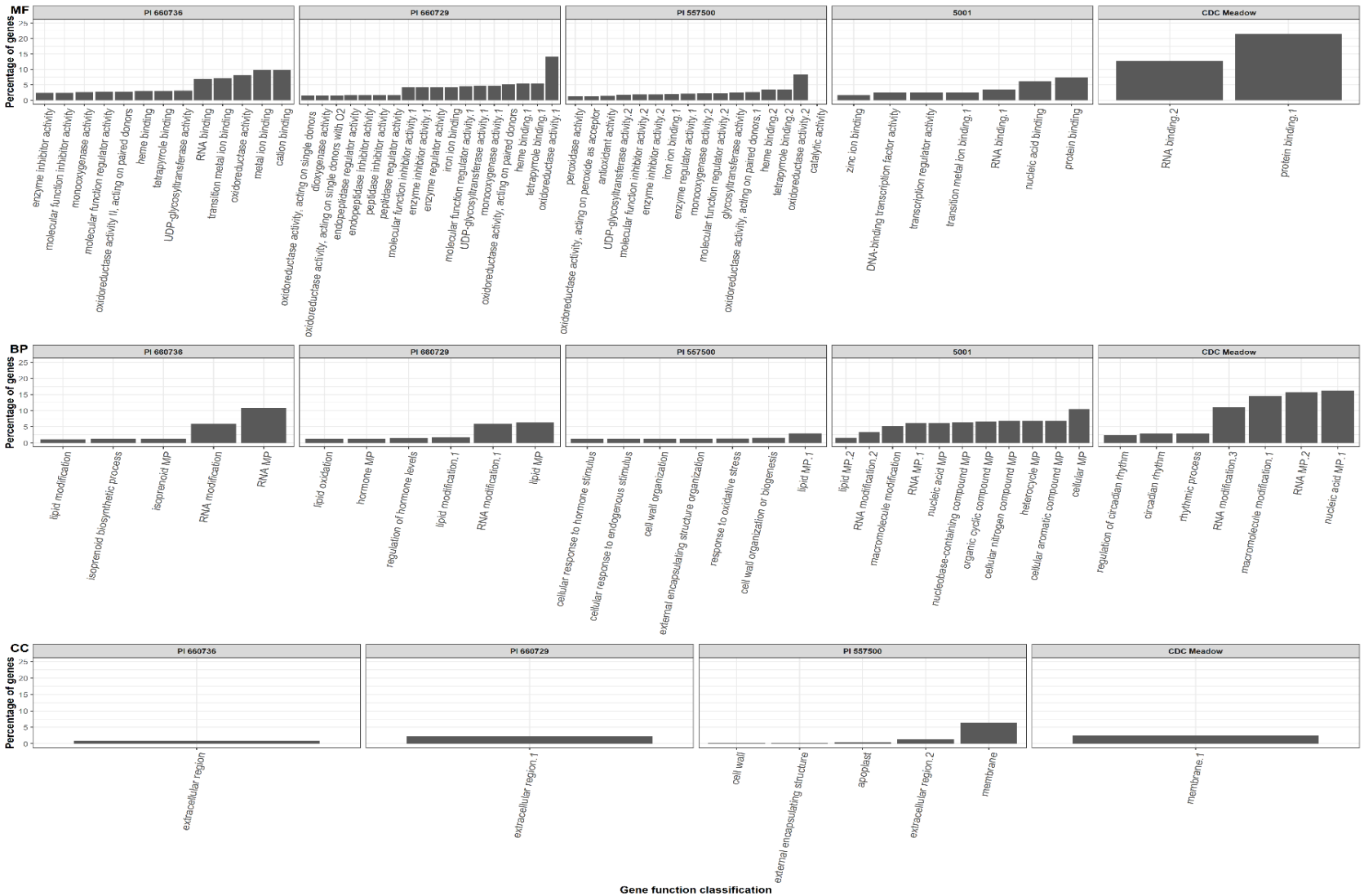
Supplementary Figure 12. Bar plots depicting a summary of the GO enrichment of upregulated genes in PR and S genotypes at 6 hpi. The x-axis shows the Gene function classification molecular function (MF), biological process (BP) and cellular compartment (CC) and the y-axis shows the percentage of genes enriched.



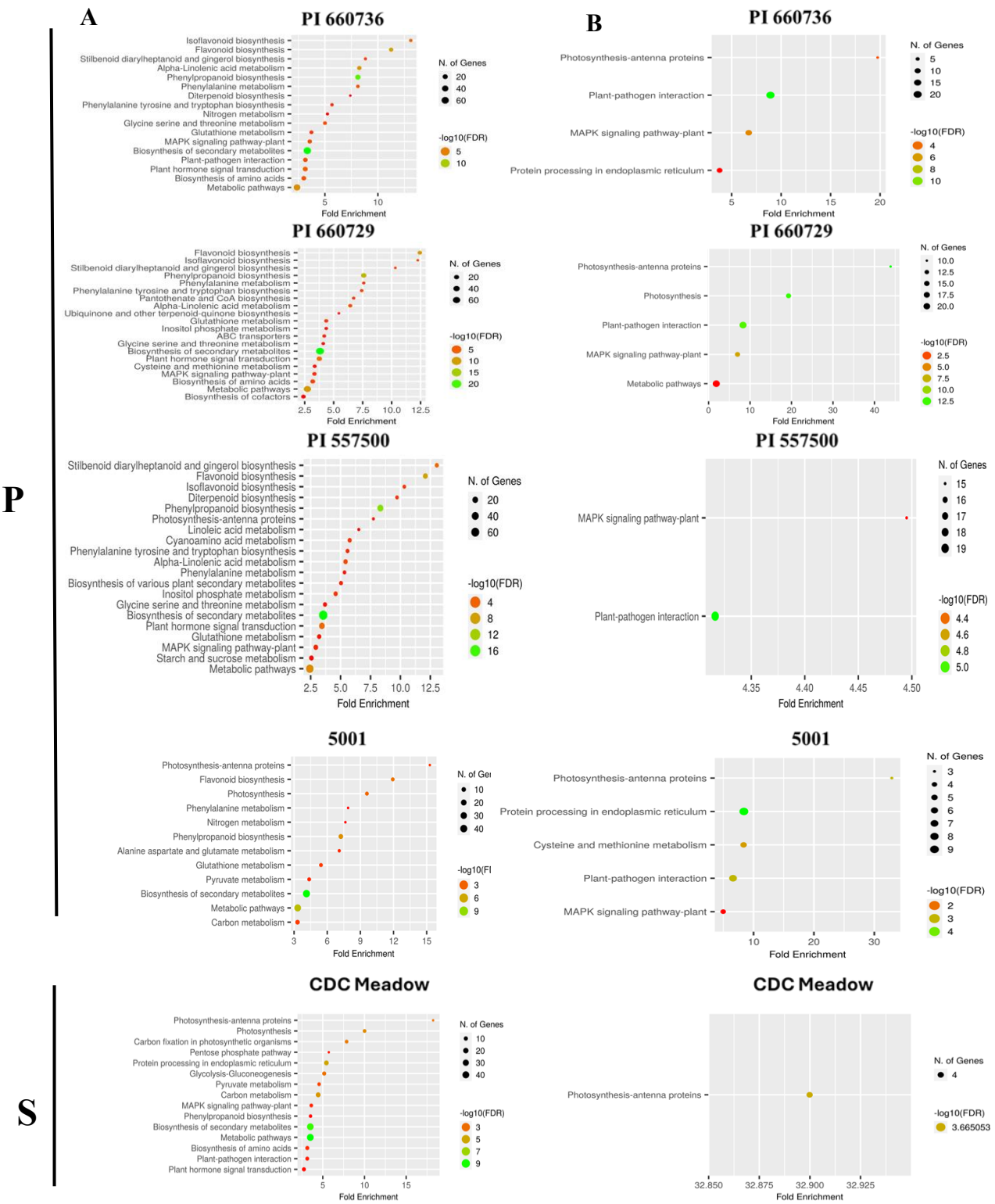
Supplementary Figure 13. Bar plots depicting a summary of the GO enrichment of downregulated genes in PR and S genotypes at 6 hpi. The x-axis shows the Gene function classification molecular function (MF), biological process (BP) and cellular compartment (CC) and the y-axis shows the percentage of genes enriched.



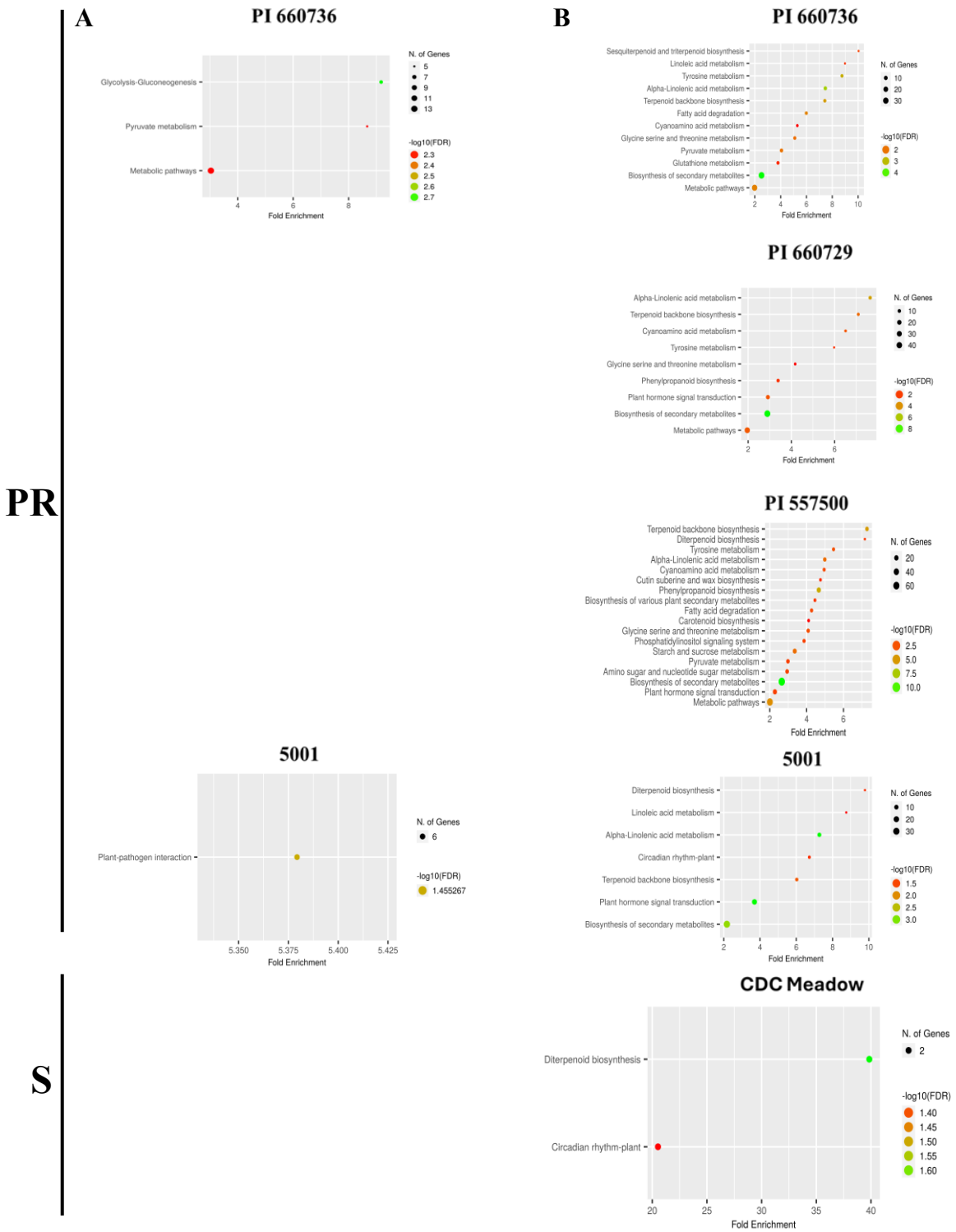
Supplementary Figure 14. Bar plots summarizing the GO enrichment of upregulated genes in PR and S genotypes at 24 hpi. The x-axis shows the Gene function classification molecular function (MF), biological process (BP) and cellular compartment (CC) and the y-axis shows the percentage of genes enriched.



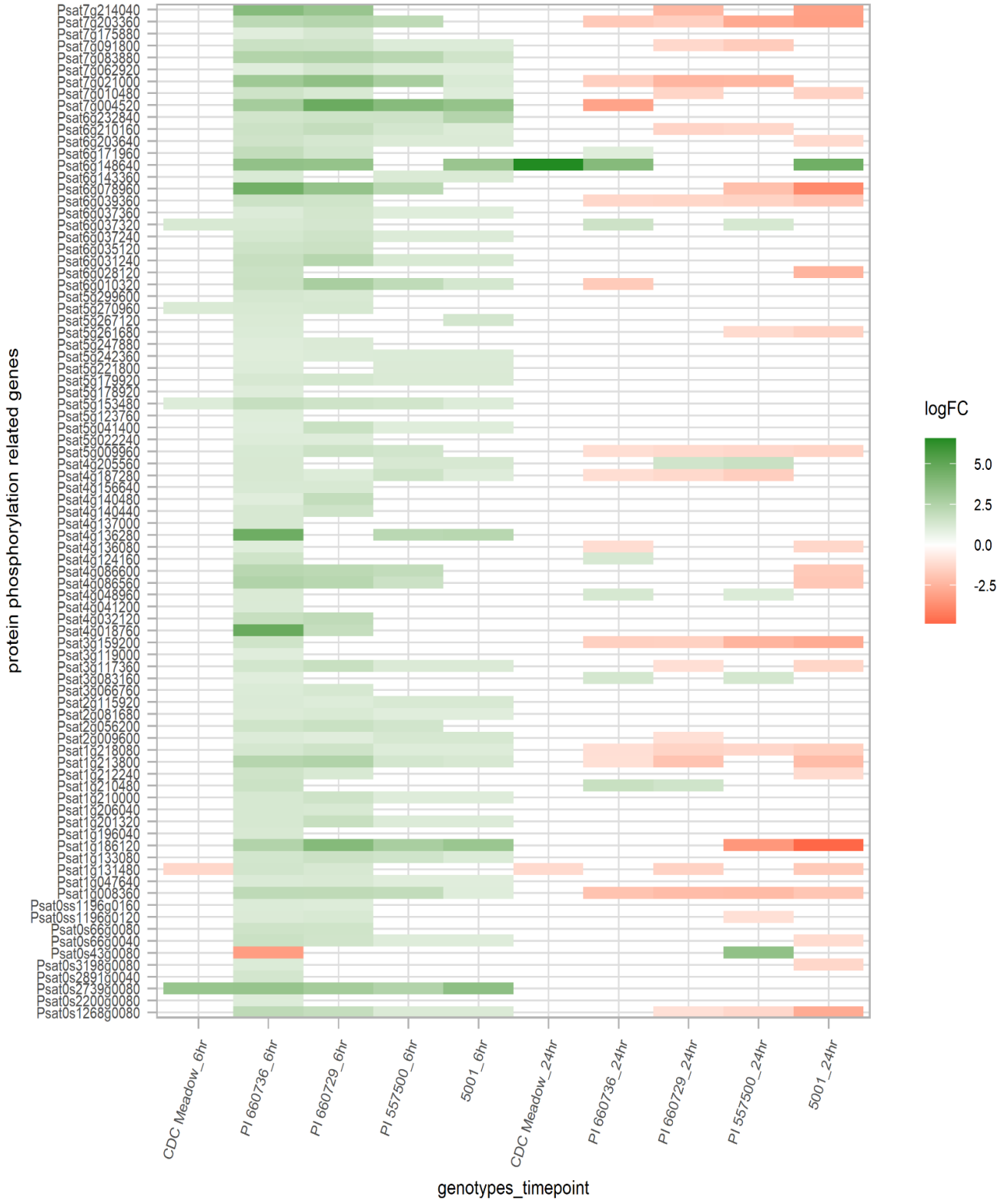
Supplementary Figure 15. Bar plots depicting a summary of the GO enrichment of downregulated genes in PR and S genotypes at 24 hpi. The x-axis shows the Gene function classification molecular function (MF), biological process (BP) and cellular compartment (CC) and the y-axis shows the percentage of genes enriched.



Supplementary Figure 16. Dot plots showing a summary of the enriched pathway network of upregulated genes in PR and S genotypes at 6 hpi (A) and 24 hpi (B). The x axis shows the fold enrichment and y axis shows the enriched pathway network.



Supplementary Figure 17. Dot plots summarizing the enriched pathway network of downregulated genes in PR and S genotypes at 6 hpi (A) and 24 hpi (B). The x axis shows the fold enrichment and y axis shows the enriched pathway network.



Supplementary Figure 18. Heatmap of phosphorylation-related genes expressed in different genotypes at 6 and 24 dpi.

Table S1. List of genotypes screened against *A. euteiches* in greenhouse and under field conditions.

ID	Genotype	Alternative	Remarks	References
PT1	PI 660736	RIL 847-68	screened in the greenhouse and field in 2020 and 2021	USDA GRIN database, McGee et al. 2012
PT2	PI 660735	RIL 847-53	screened in the greenhouse and field in 2020	USDA GRIN database, McGee et al. 2012
PT3	PI 660734	RIL 847-50	screened in the greenhouse	USDA GRIN database, McGee et al. 2012
PT4	PI 660733	RIL 847-45	screened in the greenhouse and field in 2020	USDA GRIN database, McGee et al. 2012
PT5	PI 660732	RIL 847-28	screened in the greenhouse	USDA GRIN database, McGee et al. 2012
PT6	PI 660731	RIL 847-22	screened in the greenhouse	USDA GRIN database, McGee et al. 2012
PT7	PI 660730	RIL 847-08	screened in the greenhouse	USDA GRIN database, McGee et al. 2012
PT8	PI 660729	RIL 846-07	screened in the greenhouse and field in 2020 and 2021	USDA GRIN database, McGee et al. 2012
PT9	PI 557501	90-2131	screened in the greenhouse	USDA GRIN database, Kraft et al. 1992
PT10	PI 557500	90-2079	screened in the greenhouse and field in 2020 and 2021	USDA GRIN database, Kraft et al. 1992
PT11	PI 606699	97-363	screened in the greenhouse and field in 2020	USDA GRIN database, Kraft et al. 1992
PT12	PI 606703	97-2154	screened in the greenhouse and field in 2020	USDA GRIN database
PT13	PI 606702	97-261	screened in the greenhouse and field in 2020	USDA GRIN database
PT14	PI 606701	97-2162	screened in the greenhouse	USDA GRIN database
PT15	PI 606700	97-2170	screened in the greenhouse and field in 2020	USDA GRIN database
PT16	PI 606698	96-2222	screened in the greenhouse and field in 2020	USDA GRIN database
PT17	PI 606697	96-2198	screened in the greenhouse and field in 2020	USDA GRIN database
PT18	PI 606696	96-2069	screened in the greenhouse and field in 2020	USDA GRIN database
PT19	PI 606695	96-2058	screened in the greenhouse and field in 2020	USDA GRIN database
PT20	PI 557502	90-2322	screened in the greenhouse	USDA GRIN database, Kraft et al. 1992
PT21	Carneval		screened in the greenhouse and field in 2020	R. Conner; unknown

PT22	Prodigy		screened in the greenhouse and field in 2020 and 2021	R. Conner; unknown
PT23	K2		screened in the greenhouse and field in 2020	L. Porter; Bodah et al. 2016
PT24	PI 669377	5007	screened in the greenhouse and field in 2020	L. Porter; Bodah et al. 2016
PT25	PI 669376	5006	screened in the greenhouse and field in 2020	L. Porter; Bodah et al. 2016
PT26	PI 669375	5005	screened in the greenhouse and field in 2020	L. Porter; Bodah et al. 2016
PT27	PI 669374	5004	screened in the greenhouse and field in 2020	L. Porter; Bodah et al. 2016
PT28	PI 669373	5003	screened in the greenhouse and field in 2020	L. Porter; Bodah et al. 2016
PT29	PI 669372	5001	screened in the greenhouse and field in 2020 and 2021	L. Porter; Bodah et al. 2016
PT30	Carman		screened in the greenhouse and field in 2020 and 2021	R. Conner; Feng et al. 2011
PS1	CDC Meadow		screened in the greenhouse and field in 2020 and 2021	Purchased commercial seed, Warkentin et al. 2007

Table S2. List of phosphorylation-related DEGs and their orthologues in *Medicago trunculata*.

Genes	Ortholog name	description
Psat4g018760	MTR_4g124990	LRR receptor-like kinase
Psat4g136280	MTR_8g465510	S-locus lectin kinase family protein
Psat6g078960	MTR_7g053200	Serine/Threonine-kinase OXI1-like protein
Psat7g214040	MTR_4g066660	calmodulin-domain kinase CDPK protein
Psat6g148640	MTR_1g082310	receptor-like cytosolic Serine/Threonine-kinase
Psat6g000920	MTR_1g026160	ACT-like tyrosine kinase family protein
Psat0s2739g0080	MTR_5g094380	tyrosine kinase family protein
Psat4g190480	N/A	N/A
Psat0s43g0080	MTR_4g105770	tyrosine kinase family protein
Psat7g021000	N/A	N/A
Psat7g004520	MTR_8g106100	LRR receptor-like kinase
Psat5g278880	MTR_2g094910	receptor-like Serine/Threonine-kinase NCRK protein
Psat4g086560	MTR_0154s0020	MAP3K-like kinase
Psat1g186120	MTR_2g020810	receptor-like cytosolic Serine/Threonine-kinase
Psat7g083880	N/A	N/A
Psat1g213800	N/A	N/A
Psat4g086600	MTR_0154s0020	MAP3K-like kinase
Psat7g203360	MTR_4g049730	MAP kinase kinase kinase
Psat0s1268g0080	MTR_7g081410	LRR receptor-like kinase family protein
Psat1g008360	MTR_2g072520	receptor-like kinase
Psat6g171960	MTR_1g090520	LRR receptor-like kinase family protein
Psat5g153480	MTR_3g031470	Serine/Threonine kinase, plant-type protein
Psat4g032120	MTR_4g117020	cysteine-rich RLK (receptor-like kinase) protein
Psat6g031240	MTR_1g010220	wall-associated receptor kinase-like protein
Psat6g010320	MTR_0341s0020	cysteine-rich receptor-kinase-like protein
Psat7g091800	MTR_4g094610	LRR receptor-like kinase family protein
Psat0s66g0040	MTR_1g021642	cysteine-rich receptor-kinase-like protein
Psat6g028120	MTR_5g024450	LRR receptor-like kinase family protein
Psat1g210480	MTR_2g011190	G-type lectin S-receptor-like Serine/Threonine-kinase
Psat6g210160	MTR_1g105585	cysteine-rich receptor-kinase-like protein
Psat6g039360	MTR_1g028890	LRR receptor-like kinase
Psat6g035120	MTR_1g055255	calcium-dependent kinase
Psat1g212240	MTR_2g010470	LRR receptor-like kinase family protein
Psat7g010480	MTR_8g104520	receptor-like kinase
Psat0s66g0080	MTR_1g021642	cysteine-rich receptor-kinase-like protein
Psat6g203640	MTR_1g102360	receptor-like Serine/Threonine-kinase plant
Psat4g124160	MTR_8g465410	S-locus lectin kinase family protein
Psat2g056200	MTR_5g071560	MAP kinase kinase kinase
Psat3g159200	MTR_7g446180	LRR receptor-like kinase
Psat6g232840	MTR_1g113960	ACT-like tyrosine kinase family protein
Psat4g130240	MTR_0070s0020	LRR receptor-like kinase family protein

Psat1g131480	MTR_2g040510	MAP kinase kinase
Psat3g117360	MTR_7g021570	LRR receptor-like kinase
Psat1g133080	MTR_2g041960	Serine/Threonine kinase family protein
Psat0s2891g0040	MTR_1g108910	cysteine-rich RLK (receptor-like kinase) protein
Psat6g037240	MTR_1g027030	stress-induced receptor-like kinase
Psat1g218080	MTR_2g008370	somatic embryogenesis receptor kinase
Psat5g299600	MTR_2g105680	LRR receptor-like kinase family protein
Psat1g206040	MTR_2g013210	receptor-like kinase
Psat1g201320	MTR_2g014920	LRR receptor-like kinase family protein, putative
Psat1g210000	MTR_2g011180	G-type lectin S-receptor-like Serine/Threonine-kinase
Psat5g179920	MTR_3g050780	receptor Serine/Threonine kinase
Psat4g205560	MTR_8g011780	Serine/Threonine-kinase OXI1-like protein
Psat4g137000	MTR_8g461040	MAP kinase-like protein
Psat4g187280	MTR_8g046290	receptor-like kinase
Psat4g140440	MTR_8g058250	LRR receptor-like kinase
Psat5g009960	MTR_3g115490	Serine/Threonine kinase family protein
Psat1g196040	MTR_2g016530	LRR receptor-like kinase
Psat6g037320	N/A	N/A
Psat5g270960	MTR_2g090405	cyclin-dependent kinase
Psat4g156640	MTR_8g028720	MAP kinase
Psat7g103040	MTR_4g093040	L-type lectin-domain receptor kinase
Psat6g143360	MTR_1g080020	phosphoenolpyruvate carboxylase-related kinase
Psat5g267120	MTR_0148s0080	S-locus lectin kinase family protein
Psat4g048960	MTR_4g109170	LRR receptor-like kinase family protein
Psat1g047640	MTR_6g088785	leucine-rich receptor-like kinase family protein
Psat4g041200	MTR_4g113710	receptor-like kinase
Psat2g115920	MTR_5g037410	tyrosine kinase family protein
Psat7g068280	N/A	N/A
Psat0ss1196g0120	MTR_4g123880	receptor-like kinase plant
Psat5g261680	MTR_2g088020	MAP kinase kinase kinase
Psat0ss1196g0160	MTR_4g123870	Pti1-like kinase
Psat3g066760	MTR_7g094100	LRR receptor-like Serine/Threonine-kinase RKF3, putative
Psat0s3198g0080	MTR_1g108910	cysteine-rich RLK (receptor-like kinase) protein
Psat2g081680	MTR_5g031870	receptor-like cytosolic Serine/Threonine-kinase
Psat2g009600	MTR_5g094390	Serine/Threonine-kinase WNK11-like protein
Psat1g213880	MTR_2g009670	wall associated kinase-like protein
Psat6g037360	MTR_1g027680	stress-induced receptor-like kinase
Psat6g037360	MTR_1g027990	stress-induced receptor-like kinase
Psat0s2200g0080	N/A	N/A
Psat5g022240	MTR_3g110180	casein kinase I-like protein
Psat5g221800	MTR_4g065080	Serine/Threonine kinase family protein
Psat5g178920	MTR_3g051770	calmodulin-domain kinase CDPK protein
Psat5g247880	MTR_2g081470	S-locus lectin kinase family protein

Psat5g123760	MTR_3g067795	tyrosine kinase family protein
Psat4g136080	MTR_8g465990	S-locus lectin kinase family protein
Psat5g242360	MTR_2g085210	ubiquitin ligase cop1, putative
Psat7g062920	MTR_8g088760	cysteine-rich RLK (receptor-like kinase) protein
Psat0s3723g0080	N/A	N/A
Psat5g041400	MTR_3g102400	Serine/Threonine kinase, plant-type protein
Psat4g140480	MTR_8g057660	receptor-like kinase
Psat7g175880	MTR_4g073140	adenine nucleotide alpha hydrolase-like domain kinase, putative
Psat3g119000	MTR_7g073230	receptor-like kinase plant-like protein
Psat3g083160	MTR_7g082280	tyrosine kinase family protein
Psat3g083160	MTR_7g082300	LRR kinase family protein
Psat3g070680	MTR_7g092430	LRR receptor-like kinase family protein

Table S3. Two-way ANOVA and TukeyHSD summary comparing relative pisatin levels in different genotypes (cultivars) between *A. euteiches* inoculated and mock-inoculated (treatments) at 2 h post-inoculation.

	Df	Sum sq	Mean sq	F value	Pr(>F)
cultivars	1	4.31E+12	4.31E+12	5.477	0.0474 *
treatments	1	1.29E+12	1.29E+12	1.634	0.237
cultivars:treatments	1	1.80E+12	1.80E+12	2.291	0.1685
Residuals	8	6.30E+12	7.87E+11		

TukeyHSD

cultivar	treatment	mean (Pk area)	sd	se	count	Cld
PI 660736	Infected	2070000	1730000	998816	3	a
PI 660736	Control	640000	340000	196299	3	a
CDC Meadow	Control	216666.67	189032.6	109138	3	a
CDC Meadow	Infected	96000	62193.25	35907.3	3	a

Table S4: Two-way ANOVA and TukeyHSD summary comparing relative pisatin levels in different genotypes (cultivars) between *A. euteiches* inoculated and mock-inoculated (treatments) at 24 h post-inoculation.

Two-way ANOVA

	Df	Sum sq	Mean sq	F value	Pr(>F)
cultivars	1	2.70E+12	2.70E+12	10.389	0.0122 *
treatments	1	1.85E+12	1.85E+12	7.119	0.0284 *
cultivars:treatment	1	1.48E+12	1.48E+12	5.71	0.0439 *
Residuals	8	2.08E+12	2.60E+11		

TukeyHSD

cultivars	treatment	mean (Pk area)	sd	se	count	Cld
PI 660736	Infected	1795000	1005000	580237	3	a
PI 660736	Control	306666.67	168621.86	97353.9	3	b
CDC Meadow	Infected	143333.33	11547.01	6666.67	3	b
CDC Meadow	Control	61333.33	23459.18	13544.2	3	b