Acta Universitatis Agriculturae Sueciae

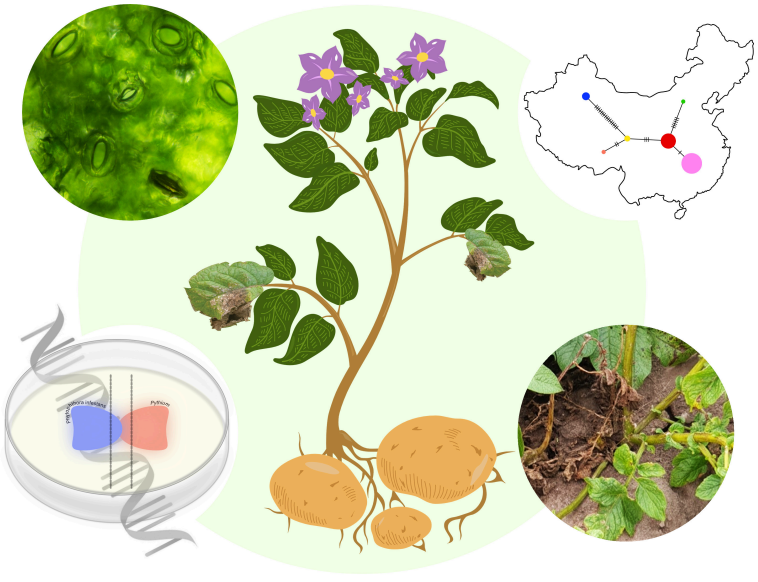
SLU

Doctoral Thesis No. 2025:7
Faculty of Landscape Architecture, Horticulture and Crop Production Science

From pathogen to prey: new insights into the role of effectors from *Phytophthora infestans*

Pathogenicity, population biology, and defence

Jenifer Seematti Sundar



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DOCTORAL THESIS

Alnarp 2025

Acta Universitatis Agriculturae Sueciae
2025:7

Cover: Illustration of *Phytophthora infestans* in potato and the focus areas of this thesis (design: Jenifer Seematti Sundar)

ISSN 1652-6880
ISBN (print version) 978-91-8046-442-0
ISBN (electronic version) 978-91-8046-492-5
https://doi.org/10.54612/a.658nio3jt9

Swedish University of Agricultural Sciences, Department of Plant Protection Biology, Alnarp, Sweden
Print: SLU Grafisk service, Alnarp 2025

From pathogen to prey: new insights into the role of effectors from *Phytophthora infestans* – Pathogenicity, population biology, and defence

Abstract

Late blight, caused by the highly adaptable oomycete *Phytophthora infestans*, is a global agricultural challenge. This thesis explores the role of effector proteins from *P. infestans* in pathogenicity, pathogen populations, and protection from mycoparasitic attack. Cell biology analyses reveal that *P. infestans* manipulates host metabolic pathways, including starch and lipid catabolism, to induce stomatal opening and facilitate sporulation. Effectors likely play a central role in bypassing host stomatal defences. To investigate effectors from a more environmental perspective two approaches were taken. Firstly, both the genetic diversity and local adaptation of *Avr2* across China were studied to investigate the maintenance of effectors in pathogen populations. Environmental factors like temperature and altitude shape the variability of this effector. These findings emphasise the need for region-specific strategies in resistance breeding and predicting pathogen evolution under climate change. Secondly, the responses of *P. infestans* to attack by mycoparasitic oomycetes were investigated using transcriptomics. Differentially expressed genes involved in sensing, signal transduction, gene regulation, detoxification, and defence, uncover components of oomycete innate immunity for the first time and suggest *P. infestans* is well equipped to recognise these biocontrol agents. Finally, this thesis reviews challenges in implementing biocontrol of *P. infestans* in Europe, emphasising the need to integrate molecular insights into practical, sustainable disease management approaches. Collectively, this work advances our understanding of *P. infestans* as a highly adaptable pathogen capable of both manipulating hosts and responding to antagonists, providing a foundation for developing more targeted and sustainable late blight control strategies.

Keywords: *Phytophthora infestans*, late blight, effectors, stomatal manipulation, mycoparasite, biological control, *Pythium*, transcriptomics, population genetics

Från patogen till byte: nya insikter om effektorers roll hos *Phytophthora infestans* – Patogenicitet, populationsbiologi och försvar

Abstract

Potatisbladmögel som orsakas av den anpassningsbara algsvampen *Phytophthora infestans*, utgör en global utmaning inom jordbruket. Denna avhandling undersöker effektorproteiners roll hos *P. infestans* i relation till patogenicitet, patogenpopulationer och försvar mot mykoparasiter. Cellbiologiska analyser visar att *P. infestans* manipulerar sin värdväxts metaboliska banor, inklusive stärkelse- och lipid-katabolism, för att öppna klyvöppningar och underlätta sporulering. Effektorer spelar i detta avseende sannolikt en central roll för att kringgå växtens försvar. För att undersöka effektorer ur ett mer miljömässigt perspektiv användes två olika tillvägagångssätt. För det första studerades både genetisk variation och local anpassning hos *Avr2* i Kina för att förstå hur effektorer kan bibehållas i patogenpopulationer. Miljömässiga faktorer som temperatur och altitud formar variationen hos denna effektor. Detta resultat betonar behovet av regionspecifika strategier för resistensförädling och för att förutspå patogenens evolution i samband med klimatförändringar. För det andra undersöktes *P. infestans* försvar mot mykoparasitiska algsvampar med hjälp av transkriptomik. Differentiellt uttryckta gener involverade i detektion, signaltransduktion, genreglering, detoxifiering och försvar ger en första inblick i en algsvamps medfödda immunitet som tyder på att *P. infestans* är rustad att känna igen och motverka angrepp från biokontrollorganismer. Slutligen granskar denna avhandling utmaningar med att implementera biokontroll av *P. infestans* i Europa och betonar behovet av att integrera molekylära insikter i praktiska, hållbara metoder för sjukdomsbekämpning. Sammantaget främjar detta arbete vår förståelse av *P. infestans* som en mycket anpassningsbar patogen som kan både manipulera sina värdar och motstå antagonister, vilket ger en grund för att utveckla mer målinriktade och hållbara strategier för kontroll av potatisbladmögel.

Keywords: *Phytophthora infestans*, potatisbladmögel, effektorer, manipulation av klyvöppning, mykoparasit, biokontroll, *Pythium*, transkriptomik, populationsgenetik

Dedication

To my parents, Caroline and Sundar, for their unwavering motivation.
To my daughter, Nova Innila, whose presence reignited my passion.

“Arise, awake, and stop not till the goal is reached.”
Swami Vivekananda

Contents

List of publications

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

I. Yang, L. N., Liu, H., Wang, Y. P., **Seematti, J.**, Grenville-Briggs, L. J., Wang, Z.*, & Zhan, J.* (2021). Pathogen-Mediated Stomatal Opening: A Previously Overlooked Pathogenicity Strategy in the Oomycete Pathogen *Phytophthora infestans*. Frontiers in plant science, 12, 668797. https://doi.org/10.3389/fpls.2021.668797

II. **Seematti, J.**, Wang, Y., He, M., Yang, L., Lankinen, Å, Zhan, & J., Grenville-Briggs, L. J.*. Population genetics and local adaptation of *Avr2* in *Phytophthora infestans* populations from China. (manuscript)

III. **Seematti, J**.¤, Rocher, F.¤, Vetukuri, R. R., & Grenville-Briggs, L. J.*. When the pathogen becomes the prey: Defence responses of *Phytophthora infestans* to mycoparasitic attack by *Pythium oligandrum* and *Pythium periplocum*. (manuscript)

IV. Hashemi, M., Tabet, D., Sandroni, M., Benavent-Celma, C., **Seematti, J.**, Andersen, C. B.*, & Grenville-Briggs, L. J.* (2022). The hunt for sustainable biocontrol of oomycete plant pathogens, a case study of *Phytophthora infestans*. Fungal Biology Reviews, 40, 53-69. https://doi.org/10.1016/j.fbr.2021.11.003

All published papers are published open access.

¤ Equally contributing authors

* Corresponding author

The contribution of Jenifer Seematti Sundar to the papers included in this thesis was as follows:

I. Planned and performed the infection and sample collection. Performed molecular work for RT-qPCR. Performed microscopy to confirm the results of the first author. Analysed these results and gave input on the writing of the paper.

II. Performed multiple sequence alignment and all the population genetic analyses. Interpreted the results, and wrote the manuscript with the help of the co-authors.

III. Performed *in silico* analyses along with the other first author. Wrote the manuscript along with the co-authors.

IV. Performed literature searches and gave input on writing the review.

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Abbreviations

ABA	Abscisic Acid
ABC	ATP-Binding Cassette
AGC	Protein Kinase A, G, and C family
AGC-Pl	AGC kinases specific to Plants
AMOVA	Analysis of Molecular Variance
AMT	Annual Mean Temperature
ANOVA	Analysis Of Variance
ATP	Adenosine Triphosphate
Avr	Avirulence
BCA	Biological Control Agent
bZIP	Basic Leucine Zipper
C2H2	Cysteine-2 Histidine-2 Zinc Finger Protein
CAMK	Calcium/Calmodulin-dependent Protein Kinase
CAMK_CDPK	Calcium-dependent Protein Kinase in the CAMK family
CAZyme	Carbohydrate-Active Enzyme
cDNA	Complementary DNA
CDR	Central Double Crop Region
CRN	Crinkling and Necrosis

DE	Differential Expression
DEG	Differentially Expressed Gene
DMT	Drug/Metabolite Transporter
dN/dS	Nonsynonymous to Synonymous Substitution Rate Ratio
dpi	Days Post-Inoculation
ETI	Effector-Triggered Immunity
ETS	Effector-Triggered Susceptibility
FC	Fold Change
GH	Glycosyl Hydrolase
GPCR	G Protein-Coupled Receptor
Hd	Haplotype Diversity
hpi	Hours Post-Infection
hp-inoculation	Hours Post-Inoculation
HR	Hypersensitive Response
INPP	Inositol Polyphosphate Phosphatase
IPCC	Intergovernmental Panel on Climate Change
IPM	Integrated Pest Management
LD	Lipid Droplet
MBCA	Microbial Biological Control Agent
MFS	Major Facilitator Superfamily
MOP	Multidrug/Oligosaccharidyl-lipid/Polysaccharide
MYB	Myeloblastosis Protein Family
NCBI	National Center for Biotechnology Information
NLP	Necrosis- and Ethylene-inducing peptide 1-like proteins
NLR	Nucleotide-binding Leucine-rich Repeat
NOD	Nucleotide-binding Oligomerization Domain

NSR	Northern Single Crop Region
P. infestans	*Phytophthora infestans*
PAMP	Pathogen-Associated Molecular Pattern
Pfam	Protein Family Database
Ph. infestans	*Phytophthora infestans*
PHI-base	Pathogen-Host Interactions database
Pi	*Phytophthora infestans*
PIPK	Phosphatidylinositol Phosphate Kinase
PITG	*Phytophthora infestans* T30-4 Gene
Po	*Pythium oligandrum*
Pp	*Pythium periplocum*
PRR	Pattern Recognition Receptor
PTI	PAMP-Triggered Immunity
Py. oligandrum	*Pythium oligandrum*
Py. periplocum	*Pythium periplocum*
R protein	Resistance protein
RLK	Receptor-Like Kinase
RLP	Receptor-Like Protein
RNS	Reactive Nitrogen Species
ROS	Reactive Oxygen Species
RT-qPCR	Real-Time quantitative Polymerase Chain Reaction
RxLR	Arginine-X-Leucine-Arginine
SAR	Stramenopiles, Alveolates, and Rhizaria
SMR	Southwestern Mixed Crop Region
SNP	Single Nucleotide Polymorphism
SWR	Winter Crop Region

TAG	Triacylglycerol
TCBD	Transporter Classification Database
TF	Transcription Factor
TKL	Tyrosine Kinase-Like
TKL-Cr-3	TKL *C. reinhardtii*-specific 3 family
TKL-Pl-4	TKL Plant specific 4 family
TSAR	Telonemids, Stramenopiles, Alveolates, and Rhizaria
USDA	United States Department of Agriculture
UV	Ultra Violet
WP	Wettable Powder
ΔΔCt	Delta Delta Cycle Threshold
π	Nucleotide Diversity
ω	dN/dS ratio

1. Introduction

1.1 From symbiosis to strife: the complex world of plant-microbe interactions

Microorganisms are omnipresent and include bacteria, fungi, oomycetes, viruses, archaea, and protozoa that interact with one another in their ecosystems. These organisms do not exist solely as single cells but form communities of single or mixed species (Davey & O'toole, 2000). Interactions occur between the same species, with different species, or even among entirely different genera, families, or even kingdoms, and are also dependent on exposure to various environmental conditions and hosts in some cases. These interactions can be beneficial, antagonistic, or neutral, and include competition, parasitism, mutualism, synergism, commensalism, amensalism, and antagonism (as defined below) (Barton & Northup, 2011).

Among these myriad interactions, the interplay between plants and microbes is a complex, dynamic and continuous process, integral to the maintenance of terrestrial ecosystems. Over millions of years, plants and microbes have co-evolved, forming complex associations that integrate host and non-host species into a unified ecological entity known as the holobiont (Sánchez-Cañizares et al., 2017). One of the main drivers of plant-microbe interactions are plant exudates which microbes identify in the soil (Braga et al., 2016). This can also be where the plant selects specific microbial communities based on the biotic and abiotic conditions (Berg et al., 2015). In both natural and agricultural ecosystems, plants frequently encounter a range of microbial invaders, including beneficial and pathogenic organisms, predominantly bacteria and fungi. Beneficial microbes support plants through various mechanisms, namely commensalism and mutualism.

Commensalism is a symbiotic relationship where one organism benefits while the other is unaffected. For instance, microbes feed on nutrients secreted by plants and, in doing so, indirectly suppress harmful pathogens by outcompeting them for space and resources, potentially protecting the plant (Le et al., 2021). In contrast, mutualism benefits both organisms, as seen with rhizobia, which fix nitrogen for plant growth while receiving carbohydrates and a suitable environment from the plant in return (Beijerinck, 1888; Frank, 1889). Specifically for plants, a mutualistic relationship can also benefit them by the microbes promoting plant growth by producing phytohormones (Bělonožníková et al., 2022; Egamberdieva, 2009; García de Salamone et al., 2001), suppressing plant pathogens through antagonistic interactions (Le et al., 2021), and aiding plants cope with environmental stresses (Egamberdieva, 2009; Yasmin et al., 2021). Studying beneficial microbes can lead to novel strategies to enhance plant health and productivity.

While beneficial microbes support plant health, pathogenic microbes employ antagonistic strategies that challenge plant survival, leading to significant agricultural and biodiversity losses. The interplay between plants and their pathogenic microbes is a dynamic evolutionary arms race (Flor, 1942; Jones & Dangl, 2006), which is a key focus of the field of plant pathology. Pathogens have evolved diverse strategies to invade, colonise, and exploit their hosts while evading or suppressing plant immune responses. These strategies are closely linked to their modes of nutrition and relationships with host plants. Based on these characteristics, plant pathogens can be broadly classified into biotrophs, necrotrophs and hemibiotrophs (Grenville-Briggs & Van West, 2005; Lewis, 1973; Thrower, 1966). Biotrophs, such as powdery mildews, rely on living host tissues and establish long-term interactions without killing the host. In contrast, necrotrophs, like *Botrytis cinerea*, actively kill host cells and feed on the dead tissue. Hemibiotrophs, including *Phytophthora* species, exhibit a dual lifestyle, starting as biotrophs before switching to necrotrophy. This flexibility in lifestyle poses significant challenges for disease management, as it allows pathogens to evade and exploit various plant defences at different stages of infection. Each strategy represents an evolutionary adaptation to overcome host immunity, making it essential to study these mechanisms comprehensively. These pathogenic lifestyles contrast with the beneficial associations plants establish with other microorganisms, revealing the dual nature of plant-microbe interactions. Harnessing the knowledge of plant-

microbe interactions is pivotal in shaping innovative and sustainable approaches to plant disease management, addressing both challenges and opportunities in agriculture.

Plant disease management relies on monitoring, crop rotation, deployment of genetic resistance, maintenance of healthy soils, sanitation, application of chemical fungicides, and exploitation of biological control agents (BCAs). Breeding for resistance remains a cornerstone of sustainable agriculture, yet resistance genes often fail to provide long-term protection due to the rapid evolution of pathogens (McDonald, 2009). Chemical fungicides, while effective, face increasing scrutiny due to environmental and health concerns, as well as the emergence of fungicide-resistant pathogen strains (Berger et al., 2017; Geiger et al., 2010). BCAs, which include beneficial microbes and natural antagonists, offer a promising alternative by suppressing pathogens and can reduce reliance on synthetic chemicals. However, inconsistent performance across environments limits their adoption, urging further research into the factors governing their efficacy.

Integrated pest management (IPM) is currently the most recommended strategy for plant protection (Vilvert et al., 2022). IPM combines the approaches mentioned above, including preventive measures like disease outbreak prediction, good cultural practices such as using healthy seeds, resistant cultivars, crop rotation, and biological control, along with the targeted application of pesticides based on disease monitoring or forecasting (Adolf et al., 2019; W. Fry, 2008). The success of IPM depends on its adaptability to diverse agricultural systems and its integration with emerging technologies such as precision agriculture, remote sensing, and machine learning for more accurate disease monitoring and forecasting. Additionally, fostering collaboration among researchers, farmers, and policymakers is crucial to refining IPM practices and ensuring their widespread adoption. Ultimately, a holistic approach that balances innovation, sustainability, and practicality is essential to address the complex challenges posed by plant diseases in a changing global landscape.

With these management strategies in mind, it is important to focus on specific pathogens, such as oomycetes, that pose significant threats to plant health. The oomycete *Phytophthora infestans* represents a major challenge globally and is a model organism for the study of plant disease dynamics.

1.2 The relentless oomycete

In the evolutionary tree of life, TSAR is one of the Eukaryote supergroups and is comprised of telonemids (T), stramenopiles (S), alveolates (A), and Rhizaria (R) (Burki et al., 2020). Of these, the latter three groups form SAR, accounting for approximately half of all eukaryotic species diversity (Del Campo et al., 2014). SAR includes microbial algae such as diatoms and dinoflagellates, large seaweeds like kelps, free-living protozoa such as ciliates, as well as extensively studied protozoan parasites like apicomplexans and oomycetes (Figure 1; Grattepanche et al., 2018). Oomycetes, also known as water molds, are filamentous heterotrophic microorganisms belonging to the *Stramenopila* group within the kingdom *Chromista*, which were previously mistakenly thought to be true fungi. Despite their classification, they exhibit fungus-like characteristics, such as filamentous growth in the vegetative stage, absorptive nutrition, and spore formation for asexual and sexual reproduction (Latijnhouwers et al., 2003). Oomycetes inhabit diverse environments and can be either pathogenic or non-pathogenic. Many species, such as those from the genera *Phytophthora* and *Pythium*, are economically important plant pathogens. Others cause diseases like saprolegniasis in fish (*Saprolegnia parasitica*) or infections in mammals, as seen with *Lagenidium giganteum* and *Pythium insidiosum*.

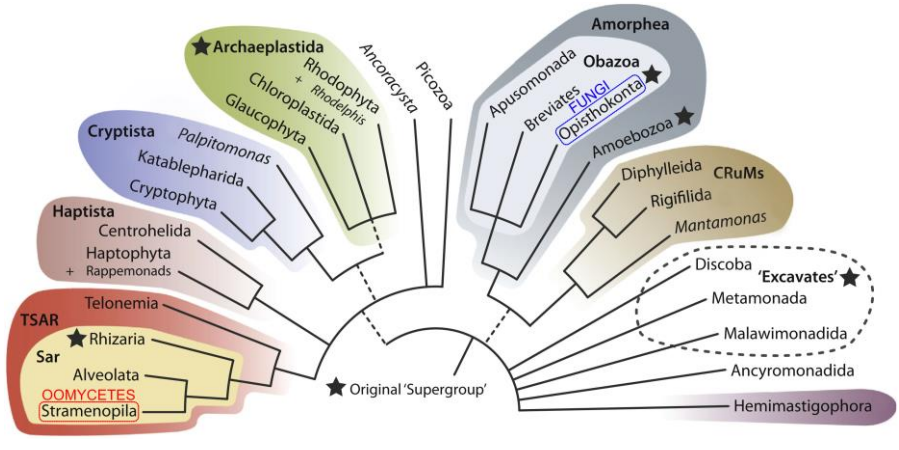


Figure 1. The new tree of Eukaryotes with updated supergroups. The colour-coded groupings represent the current supergroups. Dashed lines indicate areas of uncertainty regarding the monophyly of specific groups. The star symbol corresponds to groups that were considered supergroups in early versions. Oomycetes of *Stramenopila* and true Fungi of *Opisthokonta* are marked in red and blue, respectively. Image adapted from Burki et al., 2020.

Among the diverse oomycetes, *Phytophthora* species, the plant-destroyers, stand out due to their ability to infect a wide range of hosts, from wild plants in natural ecosystems to economically critical crops. The timeline of biological milestones in the genus *Phytophthora*, starting from the first potato blight in Europe in 1845, has been tabulated by Brasier et al. (2022). The impact of this pathogenic genus extends far beyond agriculture, influencing biodiversity and ecosystem stability. As of 2021, this genus contains 200 described species that are all pathogens (Brasier et al., 2022; Chen et al., 2022; Scanu et al., 2021). Of particular significance is *Phytophthora infestans*, a notorious pathogen that has shaped both agricultural practices and historical events through its devastating effects.

Phytophthora infestans is a pathogen of *Solanaceae* plants, causing late blight mainly in potato and tomato. *Phytophthora infestans* (Mont.) de Bary originated in the Andean region (Gómez-Alpizar et al., 2007), and the first recorded instance of late blight in potato crops was in 1843. In 1845, the disease crossed the Atlantic Ocean from the United States to Europe (Reader, 2008). Upon reaching Ireland, a country strongly dependent on potatoes as a main food source, the pathogen resulted in a near-complete destruction of the crop along with the death of a million people and displacement and emigration of another million (Ross, 2002; Vanhaute et al., 2007). After the Irish Potato Famine of the 1840s, by the beginning of the 20th century, late blight had spread worldwide resulting in the global destruction of potatoes and tomatoes. In the late 1970s, the second migration brought the A2 mating type of *P. infestans* to Europe, enabling sexual reproduction and increasing genetic diversity within late blight populations (Fry et al., 1992; Spielman et al., 1991; Yuen & Andersson, 2013). Sexual reproduction generates new genotypes, facilitating rapid evolutionary adaptation, especially in novel environments (Smith, 1971). Today, yield loss and control costs of potato late blight alone amount to around € 6.1 billion annually (Bubolz et al., 2022). As described above, even though IPM is the most recommended management strategy in plant protection (Vilvert et al., 2022) including the growth of resistant cultivars, the prevailing strategy for managing diseases remains the application of synthetic fungicides and pesticides, which is also the case with late blight.

P. infestans is diploid with a fully sequenced 240 Mb genome. It was one of the first oomycetes to have its genome sequenced, and this genomic information has enabled the study of host-pathogen interactions, genetic

evolution, and the mechanisms of infection. Due to its capability for sexual reproduction and the biotic and abiotic selection pressures it encounters, it is capable of rapid evolution, leading to the introduction of new and fitter genotypes. The pathogen and disease have continually re-emerged several times worldwide, further intriguing researchers (Fry et al., 2015). Hence, several molecular tools and resources, like gene-editing techniques, transcriptomics, and proteomics, were developed for *P. infestans*, facilitating functional studies in oomycetes. The reproductive strategies of *P. infestans* and near-global distribution provide it with high adaptability. Thus, it is now considered a model species for oomycete studies. Even though extensive research is focused for the most part on its parasitic behaviour, interaction with plant hosts, and development of better disease control strategies, this pathogen can be a valuable tool for studying microbe-microbe interactions in a wider ecological context.

To fully grasp the adaptability and pathogenic success of *P. infestans*, a key starting point lies in exploring its intricate lifecycle, which serves as the foundation for its interactions with hosts and the environment. Advances in genomic and transcriptomic studies of this pathogen could pave the way for precision agriculture, where targeted interventions can disrupt its life cycle without harming non-target organisms.

1.3 The infection journey of *Phytophthora infestans*

Phytophthora infestans follows a complex and highly specialised infection process that involves multiple stages of interaction with the host plant. It is hemibiotrophic, starting as a biotroph feeding off living plant cells without causing immediate harm before switching to necrotrophy, where it kills host cells to facilitate further colonisation. During the biotrophic phase, the pathogen uses key molecular strategies to manipulate host cell functions and suppress the plant immune response to establish a successful infection and facilitate nutrient uptake. Necrosis is necessary for the pathogen to absorb nutrients from dead tissue, ensuring its continued growth and dissemination. This dynamic between biotrophy and necrotrophy is a hallmark of the pathogenic strategy of *P. infestans*. Additionally, it can also reproduce both sexually and asexually, but the preferred method differs between geographical regions.

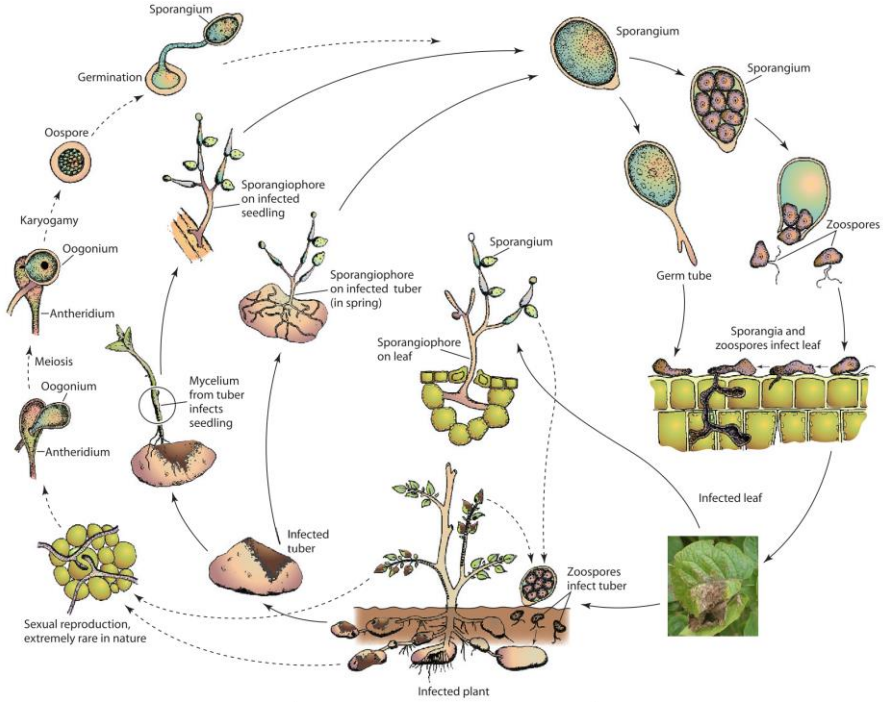


Figure 2. Disease cycle of late blight caused by *Phytophthora infestans* based on an image from Agrios, 2005.

P. infestans transitions through several specialised cellular stages throughout its infection cycle and can infect host leaves, stems and tubers (Figure 2). Multi-nucleate spores called sporangia are released from infected tissues. These asexual sporangia are dispersed to new plants through rain or are spread over long distances by wind. Sporangia can germinate directly or indirectly. Under wet and cool conditions, sporangia undergo cleavage, leading to the formation of multiple zoosporangia (also called zoospores). Each sporangium releases six to eight uninucleate motile wall-less zoospores that can swim for several hours. Upon contact with a host leaf surface, these biflagellate chemotactic zoospores lose their flagella, undergo encystment, and form a cyst with a cell wall that subsequently germinates. To penetrate the leaf surface, the germ tube originating from a sporangium or cyst develops a specialised structure traditionally referred to as an appressorium. High turgor pressure and this structure's thick cell wall were previously thought to generate sufficient force for the penetration peg to breach the plant epidermis, aided by the secretion of cell wall-degrading enzymes.

However, recent findings challenge this concept. Instead of true appressoria like those observed in fungi, *P. infestans* produces appressoria-like hyphal swellings. At the penetration site, only 10 – 20% of the maximal turgor is converted into invasion pressure (Bronkhorst et al., 2021; MacDonald et al., 2002; Müller & Scheuring, 2024), suggesting a mechanism that relies less on brute than is the case with fungal appressoria. Recently, Bronkhorst et al. (2021) identified an alternative mode of entry termed “naifu invasion”, to explain how the appressorium-like hyphal swellings facilitate penetration. Bronkhorst et al. (2021) showed that these structures apply actin-mediated polar forces at an oblique angle, slicing through the plant's cuticle and cell walls like a knife. An intact cellulose-rich cell wall, strengthened by the action of transglutaminase enzymes has also been shown to be essential for the correct formation of these hyphal swellings and pathogenicity (Brus-Szkalej et al., 2024; Grenville-Briggs et al., 2008). To reflect these insights, it has been proposed that these specialised structures be termed "naifu-appressoria" (Brus-Szkalej et al., 2024).

During the biotrophic stage, the germ tubes extend and develop into branching hyphae which grow into the intercellular space. Feeding structures called haustoria invaginate the host cell plasma membrane allowing for nutrient acquisition and the secretion of effectors by the pathogen. Once the nutrients get depleted, the necrotrophic stage begins with plant cell death, leading to the appearance of necrotic lesions on the infected tissues. Hyphae exit through the stomata and produce asexual sporangiophores, which release sporangia that are dispersed through water and wind, leading to the continuation of the cycle. Under optimal conditions, this cycle can be completed in just four days, ending with billions of spores during one growing season (Hardham, 2007; Judelson, 1997; Kamoun et al., 2015).

P. infestans is heterothallic with both A1 and A2 mating types necessary for sexual reproduction (Galindo & Gallegly, 1960; Smoot et al., 1958). For sexual reproduction to occur within a population, both mating types must coexist in the same field and infect the same plant. A1 and A2 secrete α1 and α2 hormones (Ko, 1988), respectively, which are significant for partner detection through pheromone receptors. This triggers the development of sexual structures, namely antheridia in the A1 mating type and oogonia in the A2 mating type. The antheridium attaches to the oogonium, and a single male nucleus from the antheridium migrates into the haploid oogonium to fertilize it. The fertilized oogonium develops into a diploid oospore, a thick-

walled, diploid resting spore. These oospores are highly durable resting structures that can survive in the soil or plant debris for extended periods under harsh conditions. Through overwintering, when conditions become favourable, the oospores germinate either directly, or via the production of sporangia, in soil or decaying plant material, which initiates a new infection cycle (Medina & Platt, 1999). The diploid oospores share genetic material from both parents, resulting in high genetic diversity. There can be one or several populations in a single region which complicates disease management. Despite being heterothallic, *P. infestans* is mostly clonal in several regions around the world, to the point that Agrios (2005) states that "sexual reproduction is rare in nature" for this pathogen. It is to be noted that even post asexual reproduction, *P. infestans* has a high degree of diversity (Chizhik & Martynov, 2017; Kamoun, 2003).

The genetic diversity arising from both asexual and sexual reproduction enhances the adaptability of *P. infestans* and contributes to its persistence as a major plant pathogen. A critical aspect of its pathogenicity lies in the secretion of effector proteins, which play a central role in modulating host immune responses and facilitating infection.

1.4 Molecular chess: strategic roles of effectors in pathogen success

As described by the disease triangle (Figure 3), disease development requires the interplay of a virulent pathogen, a susceptible host, and a favourable environment (Moore et al., 2011; Scholthof, 2007). These components create conditions for the pathogen to infect and proliferate successfully. Central to this process is the recognition between the plant and pathogen, which initiates a cascade of plant immune responses. Initially, the pathogen must overcome the plant's passive defences, which are constantly present to impede entry. These include barriers such as the waxy cuticle, closed stomata, bark, and sticky resin. If the pathogen manages to breach these defences, active immune responses are triggered.

Plants have evolved an intricate, dual-layered innate immune system to detect and counteract pathogenic threats (Cook et al., 2015; Thomma et al., 2011). The first layer of plant immunity is PAMP-triggered immunity (PTI), activated when membrane-bound host pattern recognition receptors (PRRs) recognise conserved molecular structures called pathogen-associated

molecular patterns (PAMPs) (Couto & Zipfel, 2016; Jones & Dangl, 2006). So far, all the known PRRs belong to the receptor-like kinase (RLK) or receptor-like protein (RLP) families (Boutrot & Zipfel, 2017). Some well-characterised PRRs include FLS2 (Flagellin-Sensing 2), EFR (EF-Tu Receptor), and CERK1 (Chitin Elicitor Receptor Kinase 1). First identified and extensively studied in *Arabidopsis thaliana*, FLS2 recognises a conserved peptide sequence (flg22) derived from bacterial flagellin, a major component of bacterial motility structures (Chinchilla et al., 2006). Specific to *Brassicaceae* species like *Arabidopsis thaliana*, EFR detects a conserved peptide (elf18) from bacterial elongation factor Tu (EF-Tu), a crucial bacterial protein involved in protein synthesis (Zipfel et al., 2006). Widely studied in *Arabidopsis thaliana* and *Oryza sativa*, CERK1 detects chitin, a major component of fungal cell walls (Miya et al., 2007).

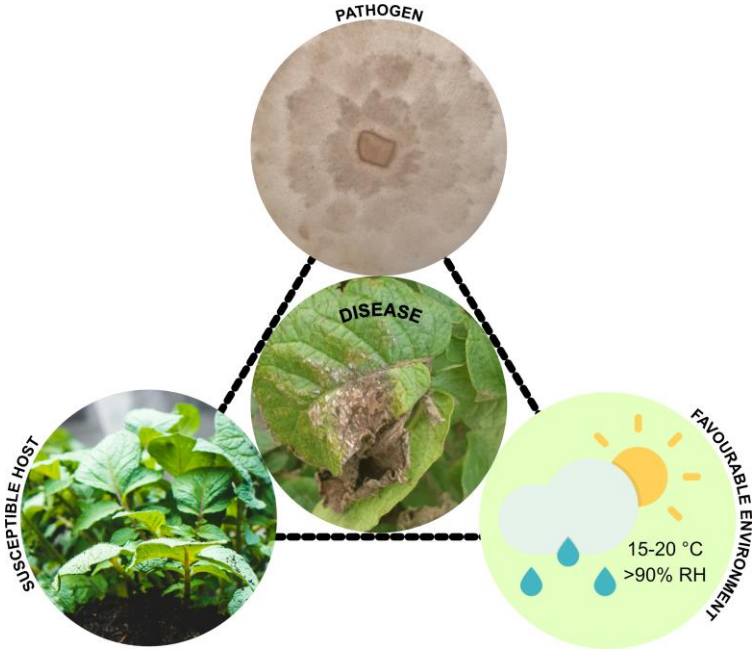


Figure 3. Disease triangle of phytopathology depicting late blight of potato caused by *Phytophthora infestans*. The favourable environmental conditions detail the optimum temperature and relative humidity required for the pathogen.

In response to PTI, effective pathogens deploy small virulence proteins known as effectors to suppress PTI, resulting in effector-triggered

susceptibility (ETS) (Chisholm et al., 2006; Jones & Dangl, 2006). In filamentous plant pathogens such as *Phytophthora* spp. and *Pythium* spp., genes are distributed in a bipartite manner as per the two-speed genome concept (Dong et al., 2015). Essential and widely conserved housekeeping genes are situated in gene-dense and repeat-poor regions, while effector genes are located in gene-sparse and repeat-rich regions enriched with transposable elements and repetitive sequences which enables their rapid evolution.

Effectors are broadly divided into two major classes: apoplastic effectors and cytoplasmic effectors. Apoplastic effectors, such as necrosis and ethylene-inducing peptide-like proteins (NLPs), function in the extracellular space; cytoplasmic effectors, including the RxLR and CRN (crinkling and necrosis) families, the two most abundant classes in *P. infestans* (Dou & Zhou, 2012; Wawra et al., 2012), are translocated into host cells (Fawke et al., 2015). The genome sequence of *P. infestans* reveals 563 RxLR and 196 CRN candidate effector genes (Haas et al., 2009; Lacaze et al., 2023).

RxLR effectors are characterized by an N-terminal signal peptide, followed by a conserved Arg-X-Leu-Arg (RxLR) motif, often associated with an Asp-Asp-Arg (EER) motif (Bhattacharjee et al., 2006; Rehmany et al., 2005; Saraiva et al., 2023; Whisson et al., 2007). Downstream of the RxLR/EER motif, many RxLR effectors feature a WY domain, predicted to be present in nearly half of the RxLR effectors from *P. infestans* (Wood et al., 2020). The RxLR motif facilitates host cell entry, while the highly diverse C-terminal domains determine specific functions, such as suppressing host immunity or promoting susceptibility (Y. Wang et al., 2022; Whisson et al., 2016). CRN effectors, on the other hand, contain an N-terminal signal peptide, an LXLFLAK motif, and a highly conserved "HVLVxxP" motif that separates the N- and C-terminal regions (McGowan & Fitzpatrick, 2017; Stam et al., 2013). Named for their crinkling and necrosis-inducing activity, CRNs play a pivotal role in suppressing plant defences and inducing cell death, as exemplified by PiCRN8 (The PLOS Pathogens Staff, 2015; van Damme et al., 2012).

While PTI represents the first line of defence in plants, cytoplasmic effectors are critical in activating the second layer of plant immunity, known as effector-triggered immunity (ETI). ETI is initiated when plant resistance (R) proteins recognise specific avirulence (AVR) effector proteins, often resulting in a hypersensitive response (HR), a localised cell death that limits

pathogen spread (Birch et al., 2009). Recent studies indicate that PTI and ETI are not mutually exclusive processes but interact synergistically to enhance plant resistance (Ngou et al., 2021; Yuan et al., 2021).

The interaction between R and AVR proteins follows a "gene-for-gene" model (Day, 1974). For example, the fungal *AvrLm1* – *Rlm1* pair in *Leptosphaeria maculans-Brassica napus* (Ansan-Melayah et al., 1998) and the oomycete *Avr1b* – *Rps1b* pair in *Phytophthora sojae-Glycine max* (Shan et al., 2007) exemplify this specificity. These interactions underscore the molecular co-evolutionary arms race between pathogens and their hosts, with effectors often serving as critical targets of mutation and evolutionary responses to selection. Pathogen effectors undergo rapid evolution to evade host recognition, frequently through mutations such as amino acid substitutions, gene duplication, or horizontal gene transfer. These modifications allow pathogens to overcome plant resistance mechanisms, while plants simultaneously evolve to detect these newly adapted effectors. Extensive cloning and characterisation of *R* and *Avr* genes from diverse pathosystems have significantly advanced our understanding of these dynamic molecular interactions (Dangl & Jones, 2001; Ellis et al., 2000; Gabriel, 1999; White et al., 2000). Such studies reveal the sophisticated strategies employed by pathogens to evade detection and by plants to detect intruders, forming the basis for resistance breeding. Insights into R and AVR interactions have been pivotal in developing crops with durable resistance for decades, as R genes are harnessed to recognise pathogen effectors and trigger robust immune responses.

As described above, effectors actively manipulate host cellular machinery to facilitate immune suppression. For example, AVR3a stabilises the host protein CMPG1 and suppresses the cell death pathways that are used to activate HR (Bos et al., 2010), while some other RxLR effectors modulate host transcription to reprogram cellular processes in favour of the pathogen (Wang et al., 2023). Another example is how effectors influence stomatal dynamics. Guard cells in plant stomata regulate the aperture, acting as barriers to prevent the entry of microbes like bacteria. Some effectors, such as HopZ1a and HopX1 secreted by the bacterial pathogen *Pseudomonas syringae*, specifically target guard cells to induce stomatal opening, creating an entry point for the pathogen (Gimenez-Ibanez et al., 2014; Jiang et al., 2013; Zhou et al., 2015). This strategy enables pathogens to bypass physical barriers like the cuticle, which serve as the plant’s first line of defence. In

this thesis, several effectors from *P. infestans* have been identified that manipulate stomatal opening to facilitate the release of secondary inoculum (Paper I; Yang et al., 2021). Moreover, effectors contribute to nutrient acquisition and tissue colonisation (Todd et al., 2022). By altering hormone pathways such as those mediated by jasmonic acid (H. Wang et al., 2024) or salicylic acid (Z. Wang et al., 2023), *P. infestans* effectors can skew the physiological state of the plant to favour pathogen growth. These multifaceted roles of effectors – suppressing immune responses, manipulating host cellular machinery, and overcoming physical barriers – are critical to the success of *P. infestans* as a plant pathogen.

Some well-characterized effectors in *P. infestans* known to be detected by R genes are AVR1 (van der Lee et al., 2001), AVR2 (Gilroy et al., 2011), AVR3a (Armstrong et al., 2005), AVR4 (Van Poppel et al., 2008), and AVR-blb1 (Vleeshouwers et al., 2008). AVR2 accumulates at the haustorium formation site, and enhanced susceptibility of potato plants to *P. infestans* is observed during its overexpression (Gilroy et al., 2011), contributing to the virulence of the pathogen. AVR2 is recognized by the R2 resistance protein in certain *Solanum* species, following a classical gene-for-gene interaction model (Gilroy et al., 2011).

Like other effectors, the *Avr2* gene is located within a rapidly evolving region of the *P. infestans* genome, characterized by a high density of transposable elements and extensive sequence variation (Haas et al., 2009). Hence, *P. infestans* populations frequently carry diverse allelic variants of *Avr2*, many of which result in loss of recognition by R2 while retaining their virulence functions. This genomic plasticity of *Avr2* reflects its evolutionary flexibility and underscores its critical role as both a molecular weapon for host manipulation and a target in the co-evolutionary arms race between *P. infestans* and its hosts.

Ultimately, the molecular chess game played by *P. infestans* showcases its strategic deployment of effectors, such as AVR2, not just to evade detection but to exploit the host environment fully. This nuanced interaction between effector activity and host immunity offers critical insights into the mechanisms of late blight and holds promise for advancing resistance breeding strategies in crops. However, as environmental pressures and resistance breeding evolve, *P. infestans* must continually adapt, refining its effector arsenal to sustain its survival and virulence. Understanding how

these effectors evolve in response to environmental factors is essential for devising more effective control measures and improving crop resilience.

1.5 From genes to geography

Scientific evidence and reports from the Intergovernmental Panel on Climate Change (IPCC) forecast drastic climate changes in the coming decades due to human intervention (Altizer et al., 2013; IPCC, 2023; Sills et al., 2010; Wu et al., 2019). These changes will impact ecosystems, influencing the survival, reproduction, and spread of pathogens. According to the disease triangle, pathogen evolution is shaped largely by the selection pressures of geographical location and the specific hosts present in a locality (Qiao et al., 2013). Environmental factors such as light, temperature, humidity, carbon dioxide concentration, and altitude play a critical role in shaping pathogen evolution by influencing fitness, virulence, reproduction, and survival (Juroszek et al., 2020). However, environmental considerations are often overlooked in studies of pathogen effectors

Temperature is a major abiotic factor that influences all ecological communities and interactions (Cossins & Bowler, 1987). In host-pathogen interactions, temperature can strongly affect epidemic development by acting on critical stages of the pathogen life cycle (Sharma et al., 2011; Tooley et al., 2009). For every plant-pathogen interaction, there is an optimal temperature range at which disease develops. For instance, the optimal temperatures for late blight epidemics caused by *P. infestans* are 16 – 23 °C in most temperate climate zones (Hyre, 1954; Maziero et al., 2009; Rotem et al., 1971). Deviations from this "disease optimum" reduce disease severity. In China, *P. infestans* populations originating from cooler regions exhibit higher virulence frequency and race complexity compared to those from warmer areas (Wu et al., 2016). Altitude acts as a proxy for temperature (Nakato et al., 2023) because higher altitudes experience lower air pressure and humidity, higher heat radiation absorption, and thus lower temperatures (MeteoSwiss, n.d.). Therefore, it also significantly affects pathogen fitness and pathogenicity.

Living organisms, including pathogens, evolve to adapt to environmental changes through mechanisms such as acclimation (Ghalambor et al., 2007). In pathogens, adaptation to local conditions can occur via the accumulation of beneficial mutations (Desai & Fisher, 2007) or the specific regulation of

gene transcription in response to genotype-environment interactions (Linder et al., 2008). The evolutionary success of *P. infestans* in adapting to diverse environmental conditions underpins its ability to thrive in varied agroecosystems (Wu et al., 2020). This has been documented in countries with distinct environmental pressures, including Mexico (Shakya et al., 2018), Poland (Chmielarz et al., 2014), and the Nordic regions (Brurberg et al., 2011). Adaptations to temperature fluctuations and local UV radiation (Wu et al., 2019) enable *P. infestans* to persist across regions with widely differing climates. Such adaptability also allows the pathogen to occupy new ecological niches, potentially expanding its geographic range and agricultural impact as global temperatures rise. Phenotypic plasticity, alongside genetic diversity, further enables *P. infestans* to thrive under unfavourable conditions (Lurwanu et al., 2020).

In addition to host resistance and pathogen biology, environmental conditions significantly influence the evolution of effector genes, as demonstrated for effectors such as Pi02860 (Yang et al., 2022), *Avr3a* (Yang et al., 2018), and *Avr4* (Waheed et al., 2021). Effector genes, often located in dynamic genomic regions, undergo rapid genetic changes with minimal fitness penalties (Dong et al., 2015; Haas et al., 2009). Environmental pressures can influence both genetic variation and expression of effectors (Banta et al., 1998; Martin, 2000; Mboup et al., 2012; Menna et al., 2015), enabling pathogens like *P. infestans* to adapt to the pressures of changing agricultural landscapes. Understanding how the environment drives avirulence (*Avr*) effector diversity is especially crucial for agriculture since these are important in resistance breeding. For example, effectors such as *Avr2* may undergo rapid mutations, enabling selection, to evade recognition in environments dominated by specific host resistance genes, highlighting the evolutionary flexibility of this pathogen. This genetic diversity is not merely a consequence of genetic drift caused by geographic separation but a strategic adaptation that enhances the ability of the pathogen to exploit hosts under varied environmental conditions.

Population genetic studies provide valuable insights into how geographic and environmental factors shape *P. infestans* populations. For instance, changes in local populations often reflect the selection of genotypes better adapted to new climatic conditions (Hoffmann & Sgró, 2011), leading to shifts in the gene pool. Investigations of *P. infestans* populations in Bangladesh (Wharton et al., 2023), North-West Russia (Runno-Paurson et

al., 2022), and Estonia (Runno-Paurson et al., 2016) reveal the importance of geographic pressures on population structure. Effector-specific studies, such as those on *Avr1* (Shen et al., 2021), *Avr3a* (Yang et al., 2018), and *Avr2* (Yang et al., 2020), further demonstrate how local cropping practices and environmental conditions influence effector diversity. These studies complement organism-wide analyses, providing a comprehensive understanding of population adaptation of pathogens.

The *P. infestans* population in China is largely clonal, with the A1 mating type predominating, making the sexual cycle rare (Li et al., 2013; Ludwiczewska et al., 2024; Zhu et al., 2015). This will be a relatively stable population that provides an ideal system to study local adaptation, which is driven by natural selection acting on mutations. These mutations provide the genetic material upon which selection can act, enabling the population to adapt to local environmental pressures. Additionally, China's diverse agricultural practices for potatoes and climatic conditions, including varying temperatures and altitudes, make it a valuable context for investigating the population genetics of effectors. This study, which analyses approximately four times more samples and includes several additional geographic regions and collection years compared to previous research (Yang et al., 2020), provides valuable insights that enhance our understanding of *P. infestans* evolution and guide strategies for managing late blight in the context of a changing climate. However, effective disease management goes beyond understanding pathogen evolution and effector dynamics. It requires a multifaceted approach that incorporates both biological and environmental strategies to mitigate losses.

1.6 Mycoparasitism and the promise of *Pythium*

Key strategies to combat plant diseases include crop rotation, proper irrigation, chemical treatments, and physical controls like soil sterilisation and temperature management. While synthetic pesticides often target specific pathogens, they risk resistance development and may be costly for small-scale farmers (Anaduaka et al., 2023; Haq et al., 2024; Schütte et al., 2017). Adapting irrigation practices to limit excessive moisture is also crucial in disease prevention (Saeed et al., 2021). In recent years, the search for eco-friendly and sustainable alternatives to synthetic chemical pesticides has led to the urgent need for practical biological control solutions. These

methods use natural enemies such as predators, parasites, antagonistic microbes or other beneficial organisms to manage harmful organisms. There are three primary types of biological control: classical, augmentative, and conservation (Collinge et al., 2022; Haq et al., 2024; Unruh, 1993). Classical biocontrol involves introducing natural enemies from a different region to control an invasive species, while augmentative biocontrol entails releasing mass numbers of natural enemies/antagonists to suppress pests or pathogens. Conservation biocontrol focuses on enhancing the habitat or conditions for the natural enemies and beneficial microbes already present to improve their effectiveness.

Microbial biocontrol agents (MBCAs) interact with their targets through several mechanisms, including hyperparasitism (of which mycoparasitism involves fungi or oomycetes), the production of antimicrobial compounds (antibiosis), competition for resources, and the induction of host resistance mechanisms (Paper IV - Hashemi et al., 2022; Köhl et al., 2019). To sustainably manage potato late blight, our recent review highlighted nearly 100 studies specifically addressing the use of biological control agents against *P. infestans* either *in vitro* or *in planta* (Hashemi et al., 2022). A particularly promising form of biocontrol is mycoparasitism. Mycoparasites are fungi or oomycetes that parasitize other fungi or oomycetes, often using specialized mechanisms to kill or suppress their host. In the case of *P. infestans*, several species of *Pythium* have been explored for their potential as mycoparasitic MBCAs.

With at least 355 species of soil-borne pathogens (Rodriguez et al., 2024), *Pythium* species are often parasitic oomycetes in terrestrial and aquatic habitats. As well as affecting mammals (Phillips et al., 2008), fish (Sati, 1991; Veldhuis Kroeze et al., 2023) and algae (Kawamura et al., 2005), most species are phytopathogens, but a few are mycoparasites. One of the most researched mycoparasitic species is *Py. oligandrum*, which forms a physical association by coiling around the hyphae (Horner et al., 2012) of a diverse range of oomycetes and fungi (Gerbore et al., 2014), such as *Fusarium oxysporum* (Benhamou et al., 1999), *Phytophthora parasitica* (Picard et al., 2000), *B. cinerea* (Paul, 1999), and other *Pythium* species (Berry et al., 1993; Deacon & Henry, 1978), followed by the secretion of lytic enzymes that help the MBCA to absorb the released nutrients. Apart from disease suppression, it is also beneficial to the plant by promoting plant growth (Le Floch et al., 2003) through short-term colonisation of the root rhizosphere of various crop

plants (Martin & Hancock, 1987). During the colonisation of *P. infestans*, beginning with the sensing of host-specific signals, *Py. oligandrum* secretes putative effectors and cell wall degrading enzymes to antagonise it (Hashemi et al., 2022; Horner et al., 2012; Liang et al., 2020). This BCA has been developed into two wettable powder formulations: Polyversum® has multiple targets including *Alternaria* species, *B. cinerea*, and *Rhizoctonia solani* (*Polyversum | Biopreparaty*, n.d.), and Polygandron WP® specifically targets *P. infestans* (*Polygandron WP | Biopreparaty*, n.d.). Collinge et al. (2022) provide a comprehensive list of various BCAs and the commercial biocontrol products derived from them.

Another mycoparasite under scrutiny is *Py. periplocum* which was initially identified during a survey of mycoparasitic *Pythium* species from cultivated soils of California (Ribeiro & Butler, 1995). Though not as well-studied as *Py. oligandrum*, Ribeiro & Butler identified *Py. periplocum* to severely suppress a broad spectrum of fungi and *Pythium* species. It has also been confirmed as an aggressive mycoparasite of *B. cinerea* (Paul, 1999). After this, there is no contemporary research on this mycoparasite. However, it holds promise as a potential new MBCA. Apart from its historical importance as a mycoparasite, *Py. periplocum* is also a spiny oogonial species like *Py. oligandrum* and coils around host hyphae. Several fungal hosts have similar or sometimes higher susceptibility to it than the latter (Ribeiro & Butler, 1995). Out of the five *Phytophthora* species included by Ribeiro & Butler (1995), four were slightly to moderately susceptible, leading to the possibility that *P. infestans* could be similar or maybe more susceptible.

Much research has typically focused on testing these mycoparasites for their ability to parasitize diverse fungal or oomycete prey. However, less attention has been given to understanding how the prey species respond to mycoparasitic attacks. While these prey species are typically well-studied plant pathogens, little is known about their ability to counteract these antagonists or if and how they mount defence responses against such mycoparasitic attacks. For instance, transcriptome analysis of *Trichoderma harzianum* T4 has identified mycoparasitism-related genes against *B. cinerea* (Y. Wang et al., 2023), and *Py. oligandrum* was found to trigger shifts in the transcriptome of the trunk pathogenic fungus *Phaeomoniella chlamydospora* while inducing grapevine resistance (Yacoub et al., 2020). However, there is a lack of knowledge regarding the specific responses of *B. cinerea* or *P.*

chlamydospora to these mycoparasitic challenges. The few examples that focus on both the mycoparasite and its prey include the dual transcriptomic analysis of the interaction between *Py. oligandrum* and *Py. myriotylum* that causes the soft-rot of ginger (Daly et al., 2021) and the rhizobacterium *Lysobacter capsici AZ78 – Ph. infestans* interaction (Tomada et al., 2017).

2. Thesis aims

The overarching aim of this thesis is to unravel the complex roles of *P. infestans* effectors in pathogenicity, adaptation, and defence mechanisms against antagonistic challenges while exploring sustainable biocontrol strategies to mitigate the impact of potato late blight in agriculture. This research focuses on three specific objectives:

I) New roles for effectors in pathogenicity: Stomatal manipulation (Paper I)

Investigate *P. infestans*-mediated stomatal manipulation, an underexplored mechanism contributing to pathogenicity, and identify if effectors play a role in facilitating this process.

II) The maintenance of effectors in pathogen populations: Genetic diversity of the *Avr2* effector gene (Paper II)

Examine the genetic diversity and adaptive potential of the *Avr2* effector gene in populations of *P. infestans* from different geographical locations in China. Analyse the association between genetic variation and environmental pressures, providing insights into local adaptation and effector evolution.

III) The involvement of effectors in counter-attack and defence against biological antagonists (Papers III & IV)

Evaluate advancements in biocontrol strategies targeting *P. infestans* and study the response of this phytopathogen to antagonism by two mycoparasitic species, *Pythium oligandrum* and *Py. periplocum*. These investigations aim to elucidate pathogen-mycoparasite interactions and identify potential vulnerabilities for sustainable biocontrol.

This thesis integrates molecular, ecological, and biocontrol perspectives to address key questions surrounding *P. infestans* biology and its management,

contributing to a broader understanding of plant-pathogen dynamics and sustainable agricultural practices.

The specific objectives of the research manuscripts in this thesis were:

1) The aim of Paper I was to investigate how *P. infestans* manipulates guard cell processes to bypass stomatal closure and induce stomatal opening for sporangiophore release. In addition, we wanted to identify the biochemical and effector-mediated pathways involved in this process.
2) Paper II aimed to investigate the genetic diversity of the *Avr2* effector gene among populations of *P. infestans* across the major potato-cropping regions in China. Additionally, the study sought to uncover the genetic mechanisms driving variation in *Avr2*, identify signs of selection acting on the gene, and evaluate the influence of environmental factors such as annual mean temperature and altitude at collection sites on its spatial distribution.
3) The aim of Paper III was to investigate the molecular strategies of *P. infestans* in response to mycoparasitic attack by *Py. oligandrum* and *Py. periplocum*. This study investigated the nature of the responses and if they were species-specific. We focused on determining if *P. infestans* could sense specific antagonists and initiate a response through signalling molecules, transcription factors, effectors, and secondary metabolites. Insights from this work on oomycete-oomycete interactions highlight the roles of effectors that can be potential molecular targets for biocontrol. It also advances the understanding of pathogen resilience and immunity in hostile microbial environments.

3. Methodology

3.1 Growth and maintenance of potato and oomycetes

Solanum tuberosum cv Desirée plants were grown at 19 °C, 60% humidity, and 16 h light (120 – 150 µmol/m²/s) and the leaves were taken from 5 – 6-week-old plants. *Pythium oligandrum* (CBS 530.74) and *Py. periplocum* (CBS 532.74) were grown on V8-$CaCO_3$ media at 18 °C in the dark (Horner et al., 2012; Kushwaha et al., 2017a, 2017b). *P. infestans* strain A21b (Paper I) and strain 88069 (Papers I & III) were maintained on rye sucrose agar at 18 °C in the dark (Grenville-Briggs et al., 2008).

For the detached leaf assays, leaves were sprayed on the abaxial side with a *P. infestans* sporangial suspension (~80,000 sporangia/ml) and sealed in boxes at 18 °C in the dark (Paper I). Before confrontation, *Pythium* species were maintained in liquid V8-$CaCO_3$, and *P. infestans* in liquid pea broth, both at 20 °C in the dark (Paper III).

3.2 Nucleic acid extraction and sequencing

For qPCR to quantify effector gene expression, RNA was extracted following Resjö et al. (2017) from a time-course of potato cv Desirée leaves inoculated with *P. infestans* strain 88069 or from in vitro pre-infection structures (Grenville-Briggs et al., 2008). RNA purity and integrity were assessed, and cDNA was synthesised (Paper I). Total DNA was extracted from mycelium collected from lesions on potato leaves sampled across China (Paper II) and genomic DNA was stored at -20 °C. Approximately 100 µg of RNA was extracted from samples from oomycete confrontation assays (Paper III).

Avr2 (PITG_22870) was amplified from the *P. infestans* genome using primers AVR2F2/R2 (F2:5´-GACCAAACGGCGTACTTCAT-3´ and R2:5´-CGCGAGCTCTTAACTCCT-3´) (Gilroy et al., 2011), cloned (L.-N. Yang et al., 2020), and sequenced (Paper II). RNA sequencing (Liang et al., 2020) was performed for further analysis of *P. infestans* responses when antagonised by *Pythium* (Paper III).

3.3 Microscopy

Potato leaves were kept at 18 °C in the dark for 2 h to ensure stomatal closure. Detached leaves were mounted on slides and photographed at 20× magnification (Nikon Ni-U), and epidermal strips were peeled at 12 and 24 hpi. Strips were incubated in KCl/MES buffer with various treatments at 18 °C for 2 h, then imaged at 20× magnification. Stomatal apertures were measured using ImageJ 1.50, and experiments were repeated thrice with three leaves per treatment (Paper I).

In order to determine the possible mechanism behind *P. infestans*-mediated stomatal opening, biochemical assays were performed to determine the levels of the starch, lipid droplets, hydrogen peroxide and nitric oxide in the guard cells. After specific treatments for each case, Nikon (Ni–U) fluorescence microscope was used at different excitation wavelengths along with ImageJ 1.50 to quantify the contents. The accumulation of H_2O_2 in mesophyll cells was measured using a digital camera (Paper I).

3.4 Effector selection and qPCR

Pathogens secrete effectors to manipulate host mechanisms for pathogenicity and colonisation. To investigate if this occurs in *P. infestans*-mediated stomatal opening, apoplastic fluids from potato leaves infected with *P. infestans* A21b were infiltrated into healthy potato leaves (Paper I). Observing the expected phenotype, extracellular proteomes were sequenced using four liquid media as described by Meijer et al. (2014). Proteins were filtered based on phosphorylation and glycosylation predictions to identify stable secreted candidates. Among these, PITG_15152, a cytoplasmic effector homologous to PSR2 of *P. sojae* (Xiong et al., 2014), and PITG_11755, an apoplastic effector, were selected for further study. RT-qPCR quantified PITG_11755 and PITG_15152 expression at 6, 12, 24, and

48 hpi, and in pre-infection stages (non-sporulating mycelium, sporangia, zoospores, germinating cysts, and appressoria). Results were analysed using the ΔΔCt method to determine relative gene expression changes.

AVR2, a key effector in the avirulence phenotype (Gilroy et al., 2011; T. van der Lee et al., 2001), coevolves with *Solanum* R gene families R2 and Rpi-mcq1 (Saunders et al., 2012), highlighting its role in the host-pathogen arms race. Overexpression of AVR2 increases potato susceptibility to *P. infestans* (Turnbull et al., 2017), making it a good candidate for population genetics studies (Paper II).

3.5 Population genetics and environment association analysis

For Paper II, the *Avr2* sequences from the field samples were aligned with the reference *Avr2* sequence PITG_22870 (Sequence: XM_002902940.1) using MUSCLE (Edgar, 2004) in MEGA-X v.10.2.2 (Kumar et al., 2018), excluding sequences without start/stop codons. Nucleotide haplotypes were reconstructed with PHASE in DnaSP v.6.12.03 (Rozas et al., 2017) to determine the diversity metrics (haplotype/nucleotide diversity, polymorphic sites). A median-joining nucleotide haplotype network was constructed in PopArt v.1.7 (Leigh & Bryant, 2015) to visualise the relationship among the reconstructed haplotypes. Tajima's D tested selective neutrality (Tajima, 1989). A hierarchical AMOVA was used to analyse the genetic variation in *Avr2* among cropping regions, among populations within regions, and within populations using the package poppr 2.9.6 (Kamvar et al., 2014) in R 4.3.0 (*R: The R Project for Statistical Computing*, n.d.). Mutations and selection were assessed using dN/dS ratios in the HyPhy package (Kosakovsky Pond et al., 2005) with the Nei-Gojobori model (Nei & Gojobori, 1986), while amino acid diversity was calculated manually. To note, temporal variations were not tested since the primary focus was on spatial genetic diversity across regions.

The pathogen requires a thermal profile to enable infection and multiplication year-round, both during infection in the potato host and in the absence of the host. Therefore, annual mean temperature (AMT) is a more comprehensive indicator of thermal adaptation of the pathogen instead of seasonal growing temperatures. The temperature data was obtained from World Climate (http://www.worldclimate.com/). Altitude was additionally

chosen as another environmental factor in the study due to its negative association with AMT. The relationship between diversity and these environmental factors was examined with Pearson's product-moment and Spearman's rank correlation tests.

3.6 Mycoparasite-prey confrontation assay

For confrontation assays (Paper III), ~5 cm^3 mycelium of *Py. oligandrum* or *Py. periplocum* and *P. infestans* were placed on V8 agar with a polycarbonate membrane (Liang et al., 2020). Interaction zone (1 cm) samples were collected at 12 hpi, snap-frozen for RNA extraction, and sequenced. Controls included *P. infestans* interacting with itself. Five replicates were prepared per interaction.

3.7 RNA-Seq analysis and gene annotation

Raw RNA reads from an ongoing project in our laboratory (NCBI accession number PRJNA637834; Liang et al., 2020) were used in Paper III. Trimming and pre-processing of the reads were performed using Atria v3.2.2 (Chuan et al., 2021) and Salmon v1.10.1 (Patro et al., 2017), respectively. For this study, only the expression data of *P. infestans* was considered. Differential expression (DE) analysis was conducted using both edgeR (Robinson et al., 2010) and DESeq2 (Love et al., 2014) pipelines, each employing their respective normalisation methods. Differentially expressed genes (DEGs) included in this analysis were those identified as differentially expressed (over- or under-expressed) by both methods.

With the control being *P. infestans* interacting with itself (PiPi), three specific contrasts were analysed:

1. *P. infestans* interacting with *Py. oligandrum* compared to the control (PiPo_PiPi)
2. *P. infestans* interacting with *Py. periplocum* compared to the control (PiPp_PiPi)
3. *P. infestans* interacting with *Py. oligandrum* compared to *Py. periplocum* (PiPo_PiPp)

Functional annotation of genes was conducted using predefined biological frameworks and tools (Figure 4). Annotations were based on

the proteome and genome files of the *P. infestans* T30-4 strain (Haas et al., 2009).

- G-protein coupled receptors (GPCRs): Identified using a Pfam list of known characteristics of this gene family
- NOD-like receptors (NLR): Identified using Pfam characteristics representing NLR N-terminal, C-terminal, and nucleotide oligomerization domain (NOD) domains
- Kinases: The *P. infestans* proteome was screened with HMMER v3.3.2 (Eddy, 1998), followed by screening for kinase domains. Receptor-like kinases (RLKs) and receptor-like proteins (RLPs) were annotated using iTAK v1.8 (Zheng et al., 2016).
- Transcription factors (TFs): Annotated using iTAK v1.8 and DeepTFactor (Kim et al., 2021)
- Transporters: Protein datasets were downloaded from the Transporter Classification Database (TCDB) (Saier et al., 2021) on September 9, 2024. The analysis focused on four transporter families: ATP-binding cassette (ABC) superfamily (TC# 3.A.1), Major facilitator superfamily (MFS; TC# 2.A.1), Drug/metabolite transporter (DMT) superfamily (TC# 2.A.7), and Multidrug/oligosaccharidyl-lipid/polysaccharide (MOP) flippase superfamily (TC# 2.A.66).
- Secondary metabolites: Annotated using antiSMASH fungal version 7.1.0 (Blin et al., 2023) on the *P. infestans* genome.
- Effectors: Prediction and characterization were performed using the following tools:
    - EffectR (Tabima & Grünwald, 2019)
    - EffectorP 3.0 (Sperschneider & Dodds, 2022)
    - SignalP 6.0 (Teufel et al., 2022)
    - SignalP 3.0 (Bendtsen et al., 2004)
    - ApoplastP 1.0.2 (Sperschneider et al., 2018)
    - blast against the Pathogen-Host Interactions database (PHI-base) (Urban et al., 2022)

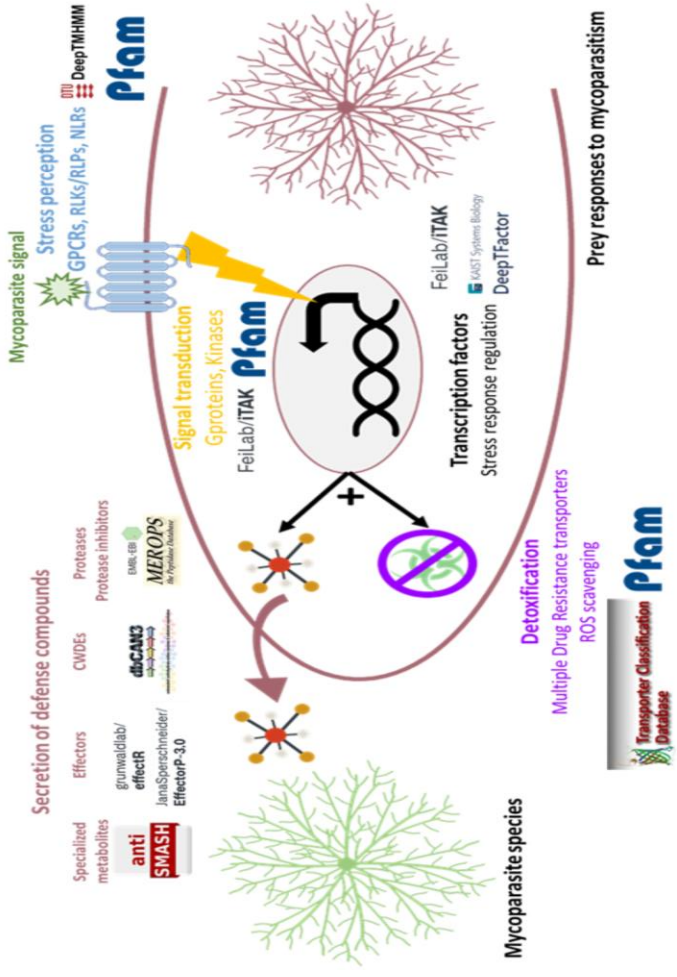


Figure 4. Gene annotation pipeline. Specific databases were used to annotate the differentially expressed genes of *P. infestans* during its response to mycoparasitic attacks by *Py. oligandrum* and *Py. periplocum* at 12 hpi.

3.8 Statistical analyses

ANOVA for stomatal aperture, starch, lipids, H_2O_2, and NO in guard cells was performed in SAS 9.4 (SAS Institute Inc., 2021) with Duncan tests for significant treatment differences (Paper I). Statistical analyses for Papers II and III were conducted in R 4.3.0, with Monte Carlo simulation methods employed for significance testing in Paper II.

4. Results and discussion

4.1 Pathogen-mediated stomatal opening by *P. infestans*: an overlooked pathogenicity strategy

Prokaryotic microbes depend on stomata, which are open under light conditions and closed under the dark, for entry and eventual colonisation of plants (Melotto et al., 2006). So far, pathogen-induced stomatal dynamics and related defence mechanisms predominantly originated from studies on systems involving bacterial pathogens and fungi (Melotto et al., 2006, 2017; Ye et al., 2020). *P. infestans* can penetrate plant epidermal cells via appressoria, bypassing stomata during initial colonization. However, stomata play a crucial role in the pathogen's secondary inoculum phase, serving as the exit points for sporangia discharge (Farrell et al., 1969; Grenville-Briggs et al., 2008). While Wang et al. (2021) recently reported that stomatal defences might play a role in potato immunity to the oomycete *P. infestans*, their specific contributions to immunity remain unclear.

In response to infection, the plant immune response typically forces the guard cells surrounding the stomatal pores to close the stomata and die if required (Mukhtar et al., 2016), for example in response to rust fungal invasion (Ye et al., 2020). When we tried verifying this in the potato-*P. infestans* interaction, in dark conditions when the stomata are the least open, nearly half of the guard cells at infection sites exhibited hypersensitive-like responses, including cell browning and death (Paper I). This response, observed as early as 8 hours post-infection (hpi) during early infection, resulted in permanently closed stomata that prevented sporangiophore emergence (Paper I). This closure will affect pathogen colonisation. This phenomenon was consistent across potato cultivars, suggesting it represents a universal defence mechanism of potato to *P. infestans* infection. However,

the remaining stomata gradually reopened, reaching maximum apertures by 48 hpi (Figures 5A & 5B). This pathogen-induced opening, which suppressed guard cell death, enabled sporangiophore emergence and sporangia release (Figure 5C), highlighting the dual flow between potato defences and *P. infestans* manipulation of its host to enhance pathogen fitness.

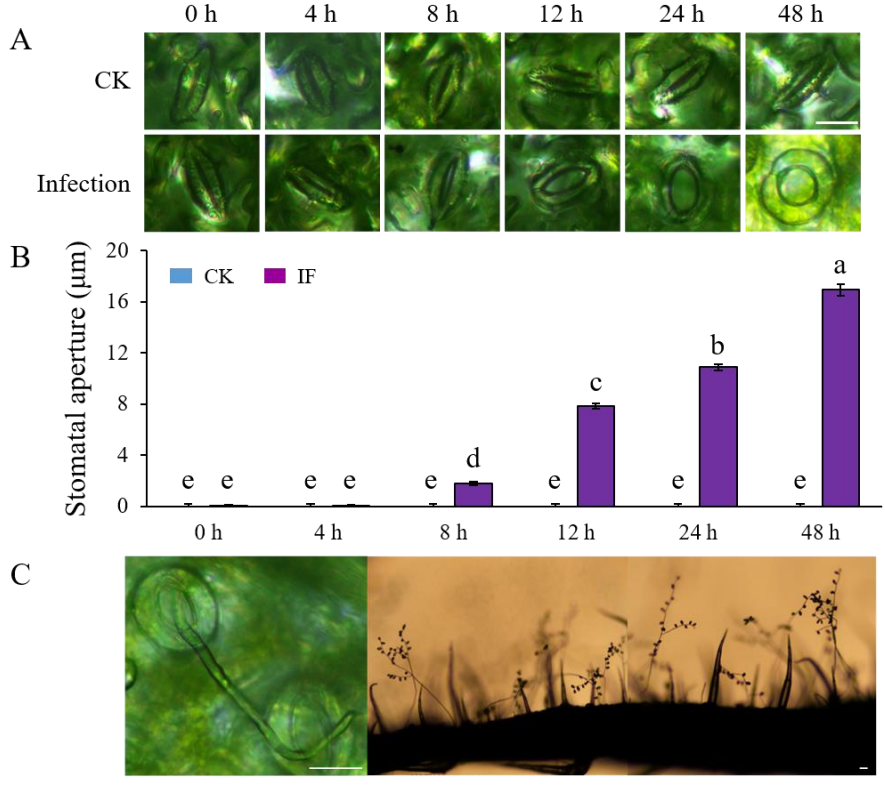


Figure 5. Stomatal aperture significantly increased after *P. infestans* inoculation under dark conditions. (A) Stomatal aperture of potato leaves after infection; (B) Quantification of stomatal aperture after infection; (C) Left – Emerging *P. infestans* sporangiophore through open stomata at 72 hpi, Right – Several sporangia (lemon-shaped spots on the right image) seen on potato leaf after 4 – 5 days post-inoculation. Scale bar = 20 μm. Within a panel = photos from a representative of one replicate; among panels = photos pooled from several replicates. CK and IF indicate control and infection, respectively. Image adapted from Yang et al. (2021).

Starch, lipids, and oxidative radicals like hydrogen peroxide (H_2O_2) are critical components of signal transduction pathways that regulate stomatal

dynamics (Horrer et al., 2016; McLachlan et al., 2016; Shimazaki et al., 2007). Pathogens may exploit these molecules and manipulate them to bypass stomata-based defences, thereby complicating the plant's defensive strategies.

Starch, a ubiquitous carbohydrate in plants (Pallas, 1964), serves as the primary energy reserve in higher plants, including potatoes (Zeeman et al., 2010). Its breakdown is associated with light-induced stomatal opening (Horrer et al., 2016; McLachlan et al., 2016) (Figure 6A). To confirm this link, we quantified the starch content in *Solanum tuberosum* cv. Desirée leaves under light and dark conditions (Figure 6B). In the dark, starch degradation began at 12 hpi and left only trace amounts by 24 hpi (Paper I).

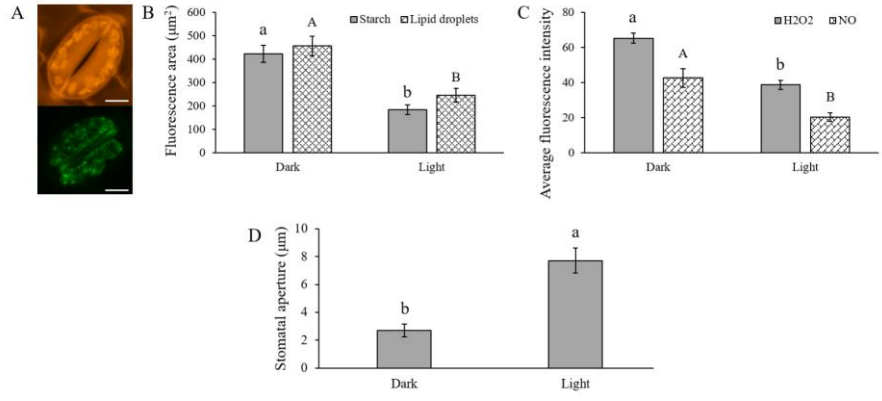


Figure 6. Light-induced stomatal opening in potato is linked to the metabolism of triacylglycerols (TAGs), starches, H_2O_2, and NO in guard cells. A) guard cells in potato leaf stomata containing significant amounts of starch (top) and TAGs (bottom) – Scale bar = 20 µm; B) the quantities of starch and TAGs in guard cells were notably reduced under light (100 mmol/m^2/s for 6 hours), compared to the dark; C) levels of H_2O_2 and NO in guard cells were significantly lower in light than in dark; D) stomatal aperture size was considerably larger in light compared to dark. Within a panel = photos from a representative of one replicate; among panels = photos pooled from several replicates. Image adapted from Yang et al. (2021).

The breakdown of starch produces smaller sugars like glucose and malate, which are metabolized during stomatal opening (De Angeli et al., 2013; Santelia & Lawson, 2016). At high concentrations, these sugars elevate guard cell turgor pressure, facilitating stomatal opening (Flütsch et al., 2020; Santelia & Lawson, 2016). Our analysis showed a decline in sugar concentrations corresponding to stomatal closure (and starch levels) over

time after infection. However, despite reduced sugar levels during stomatal closure, the aperture size did not match that at 48 hpi when only starch remnants were present. This indicates that glucose and malate play at least a partial role in this pathogenicity strategy.

Like starch, lipids are abundant in plants (Sakaki et al., 1995). Triacylglycerol (TAG), a major lipid energy reserve, is stored as lipid droplets (LDs) within guard cells. TAG contributes to stomatal opening by generating ATP and activating proton pumps such as H^+-ATPases. In the presence of light, TAG levels in guard cells significantly decline (McLachlan et al., 2016). Our observations of TAG dynamics (Figure 6A) revealed that its breakdown in guard cells began before 8 hpi, with LDs nearly depleted by 12 hpi, coinciding with the onset of starch degradation (Paper I). These findings suggest that TAG catabolism is a critical pathway initiating stomatal opening.

Reactive oxygen species (ROS), such as hydrogen peroxide (H_2O_2), and reactive nitrogen species (RNS), such as nitric oxide (NO), are central regulators of stomatal movement. These free radicals accumulate under dark (Desikan et al., 2004; Zhang et al., 2017) or ABA-induced (Jannat et al., 2011; Rodrigues et al., 2017) conditions, promoting stomatal closure, but decrease during stomatal opening under light (Desikan et al., 2004; She et al., 2004) (Figure 6C). In our study, ROS and RNS levels in guard cells dropped significantly from 8 hpi onward, concurrent with the start of stomatal opening (Paper I). This reduction parallels TAG breakdown, highlighting the potential involvement of ROS and RNS in *P. infestans*-mediated stomatal opening.

Our observations revealed that the levels of free radicals in guard cells decreased between ~4 and 8 hours post-inoculation (hp-inoculation), preceding lipid droplet (LD) breakdown, which began around 8 hpi. This lipid catabolism, in turn, occurred before starch degradation, which started at 12 hpi. This sequential process suggests that upon sensing *P. infestans*, guard cells likely rely on ROS and RNS to trigger lipid breakdown.

The energy generated through lipid catabolism likely activates proton pumps, such as H^+-ATPases, to initiate stomatal opening (McLachlan et al., 2016). Subsequently, starch degradation amplifies this process by increasing guard cell turgor pressure, maximising stomatal aperture (De Angeli et al., 2013).

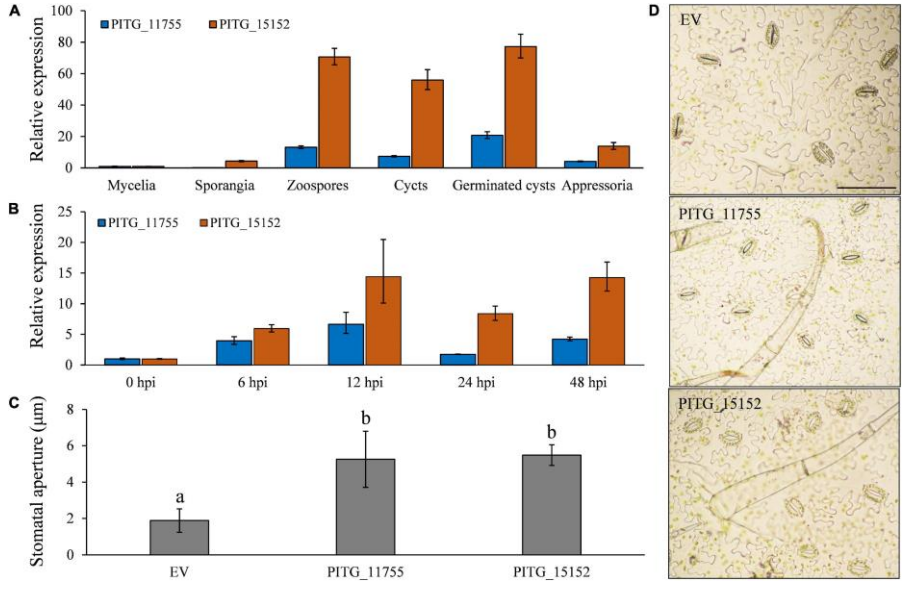


Figure 7. An apoplastic and a cytoplasmic effector are involved in *P. infestans*-mediated stomatal opening in potato leaves. A) Relative expression levels in the different life stages of *P. infestans*; B) Relative expression levels during the time course of infection of potato leaves; C) Stomatal aperture after overexpression of the effectors; D) microscopy images showing stomatal aperture after overexpression of the effectors. Scale bar = 20 µm. Within a panel = photos from a representative of one replicate; among panels = photos pooled from several replicates. EV denotes the empty vector. Image adapted from Yang et al. (2021).

When healthy potato leaves were infiltrated with apoplastic fluids (AFs) containing a mixture of secreted *P. infestans* effectors, stomatal opening was observed, with a significant increase in the stomatal aperture (Paper I). By 15 hp-inoculation, TAG levels decreased markedly, accompanied by reductions in H_2O_2 and NO in most guard cells (Paper I). However, starch levels remained unchanged at 15 hp-inoculation and showed a decline in only a few guard cells after leaf senescence at 24 hp-inoculation (Paper I). These findings indicate that effectors play a crucial role in inducing stomatal opening.

Among the potential effector candidates, the apoplastic PITG_11755 and the cytoplasmic PITG_15152 showed high expression during the pre-infection stages of *P. infestans* (Figure 7A). In the infection process of potato leaves, both effectors exhibited significant upregulation early at 6 hp-inoculation, peaking at 12 hp-inoculation and diminishing at 24 hp-

inoculation before rising again at 48 hp-inoculation (Figure 7B). This expression pattern aligns with their potential roles in initiating stomatal opening (~8 hpi in our study) and in later stages when sporangiophores begin to emerge through stomata. Overexpression studies confirmed that both PITG_11755 and PITG_15152 significantly increased stomatal apertures, validating their involvement in infection-mediated stomatal opening (Figures 7C & 7D). Thus, these apoplastic and cytoplasmic effectors likely coordinate to regulate stomatal dynamics during *P. infestans* infection in potato leaves.

4.2 Local adaptation and genetic diversity of the *Avr2* effector in Chinese *P. infestans* populations

Pathogen effectors are critical players in host-pathogen interactions, shaping disease dynamics in natural and agricultural ecosystems. These proteins, such as AVR2 in *P. infestans*, are key to understanding how pathogens adapt and thrive under different conditions. This study focuses on the population genetics of AVR2 across diverse regions of China, aiming to uncover how regional environmental factors shape its evolutionary dynamics. By examining genetic variation of this effector, the research seeks to explain the contributions of specific effectors to pathogen adaptation and the interplay between environmental pressures and pathogen evolution.

Since the focus was on nucleotide sequences that can be translated to proteins, *Avr2* nucleotide sequences with modified start codons or no stop codons were removed resulting in 415 sequences. Of these, 7 sequences were identical to the reference *Avr2* sequence.

Haplotype diversity (Hd) in the 17 populations ranged from 0.46 (Inner Mongolia) to 0.97 (Fuqing), with an average of 0.78 (Table 1). Nucleotide diversity (π) ranged from 0.0014 (Enshi) to 0.0393 (Fuzhou), with an average of 0.0290 (Table 1). The Winter crop region (SWR) accounts for only about 10% of the total potato production among China's four major potato-cropping regions. In contrast, Northern single crop region (NSR) and Southwestern mixed crop region (SMR) produce 50% and 35%, respectively (USDA Foreign Agricultural Service, 2022) and also have mainly reliable seed production systems. Despite such low potato production, SWR still demonstrates the highest levels of genetic diversity in *Avr2*. This is likely a result of the importation of seed potatoes into SWR from various regions of

China. Such human activities increase the chances of introducing new genetic variations to the area, potentially altering the local genetic pool.

A total of 146 polymorphic sites were identified, most of which were single nucleotide polymorphisms (SNPs). Central double crop region (CDR) and SWR had the highest number of polymorphic sites, 110 and 77, respectively, compared to the other cropping regions (Table 1). Notably, CDR and SWR account for only about 10% and 5% of the total potato-growing area in China (USDA Foreign Agricultural Service, 2022). This suggests that *Avr2* in these regions may have undergone more evolutionary changes, such as mutations or recombination events, over time. Supporting this, Yang et al. (2020) identified an intragenic recombinant among isolates from SWR, further highlighting the genetic diversity in these areas.

Table 1. Population diversity parameters of the *Avr2* effector in *P. infestans* for the different cropping regions and fields across China. Hd and π denote the haplotype and nucleotide diversities, respectively.

Cropping region	Potato growing area	Population	Sample size	Number of haplotypes	Polymorphic sites	Hd	π
NSR	50 %	Gansu	29	3	2	0.56	0.0018
		Heilongjiang	17	3	2	0.52	0.0016
		Inner Mongolia	26	4	26	0.46	0.0296
		Ningxia	28	3	2	0.55	0.0017
		Total	100	5	26	0.68	0.0238
CDR	10%	Enshi	32	2	1	0.48	0.0014
		Henan	23	8	31	0.67	0.0330
		Suizhou	10	4	26	0.64	0.0388
		Wuhan	18	4	104	0.63	0.0344
		Total	83	10	110	0.73	0.0361
SMR	35%	Anshun	41	3	2	0.51	0.0018
		Bijie	24	2	1	0.52	0.0015
		Shizhu	27	3	2	0.64	0.0022
		Wanzhou	14	3	2	0.60	0.0019
		Yunnan	35	17	18	0.85	0.0057
		Total	141	18	18	0.67	0.0030
SWR	5%	Fuqing	21	16	10	0.97	0.0097
		Fuzhou	28	13	52	0.85	0.0393
		Guangdong	4	1	0	0	0
		Guangxi	38	10	59	0.75	0.0375
		Total	91	35	77	0.88	0.0438
		Combined	415	53	146	0.78	0.0290

The nucleotide haplotype network showed four haplotypes were shared by most of the potato fields, namely H4 (35.18 %), H16 (25.30 %), H18 (15.66

%), and H2 (8.67 %) (Figure 8). Hence, these haplotypes are not localised but dominate across regions.

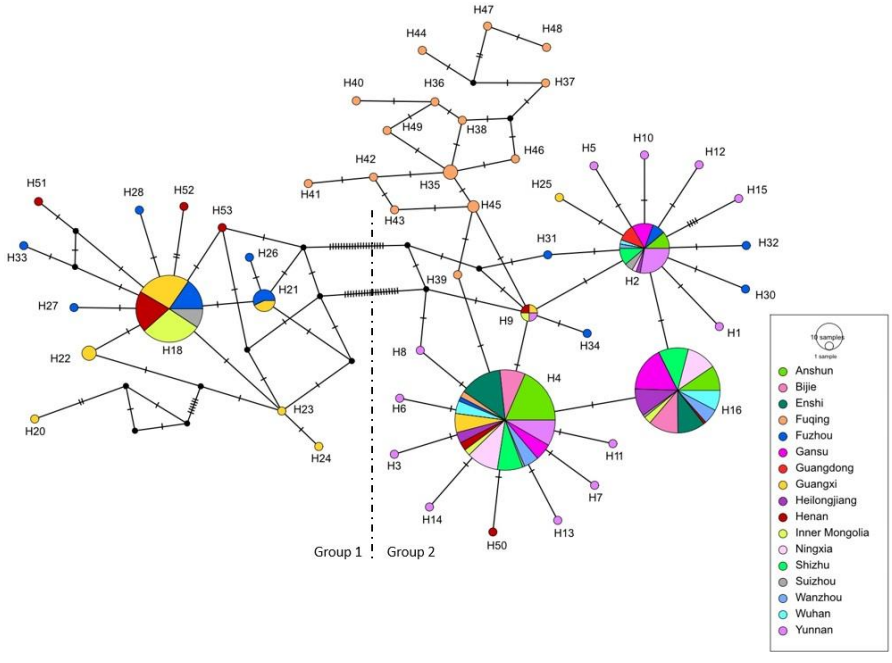


Figure 8. Nucleotide haplotype network of *Avr2*. It is based on the median-joining approach. Each circle is a distinctive haplotype. Haplotypes are denoted by the letter H followed by a corresponding number. Circle size expresses the number of isolate sequences contained in a haplotype. Mutations are shown as hatch marks on the branches.

All the 7 sequences that were identical to the reference belonged to H21 (Fuzhou and Guangxi). Maintenance of this exact copy of *Avr2* may reflect a specific functional importance in interactions between *P. infestans* and the environment, including specific host cultivars in these regions. Both Fuzhou and Guangxi are located in SWR suggesting the environmental conditions there might favour this variant.

Indels separated the haplotype network into two groups (Figure 8). Even though this study included more samples from several more regions and years, a similar separation into two groups was observed by Yang et al. (2020).

Apart from H9 and H21, the remaining haplotypes were unique to their locations. Fuqing (15) and Yunnan (12) had the highest number of individual

haplotypes (Table 1). Except for H4, the other 16 haplotypes detected in Fuqing were clustered. In the case of several other regions, most isolates diverged from the dominant haplotype by a single mutation. Many of these dominant haplotypes also differed from each other by 1 or 2 mutations. This sort of close-knit network with a very low number of mutations between the isolates indicates that there is significant genetic similarity or homogeneity. Additionally, as can be seen in Figure 8 and confirmed from Table 1, Fuqing had the most diverse population with almost all of its haplotypes differing by a single mutation. On the other hand, Inner Mongolia had the least diverse population with almost all of its samples included in the major haplotypes. This genetic homogeneity could be due to gene flow from infected plant material or a predominating clonal population (which is true for China).

Table 2. Hierarchical AMOVA among cropping regions, among populations within regions, and within populations. 'Populations within regions' and 'Within populations' indicate the 17 fields and 415 samples, respectively.

Source of variation	df	Sum of squares	Variance	% variation	p-value
Among regions	3	772.1	1.25	12.4	0.138
Among populations within regions	13	1449	4.58	45.4	0.001 ***
Within populations	398	1691	4.25	42.2	0.001 ***
Total	414	3912	10.1	100.00	

Only 12% of the total genetic variation (non-significant) is attributed to differences among cropping regions (Table 2). This suggests that populations within different cropping regions are relatively similar genetically, thereby supporting the observations from the haplotype network. It indicates either gene flow among the cropping regions (e.g., due to seed or pathogen movement) or similar selective pressures across regions maintaining homogeneity. In contrast, a substantial 45% of the variation exists between fields within cropping regions, suggesting that fields have distinct genetic compositions, potentially due to localised factors like management practices, microclimate, selective pressures, or limited gene flow between fields. A similar proportion of variation (42%) is found among isolates within fields. This indicates high diversity at the isolate level. This is either likely due to

the mutations that we observed or field-specific gene flow or local adaptation.

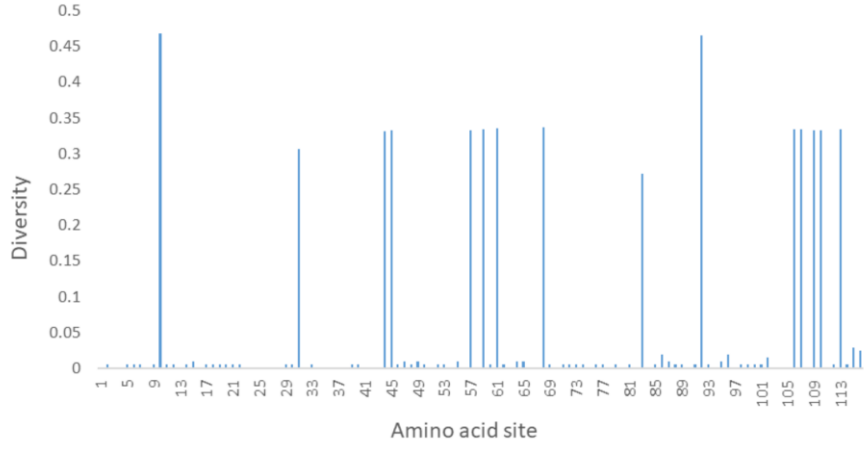


Figure 9. Amino acid diversity of the AVR2 effector

Suizhou had balancing selection of *Avr2*. Wuhan and Yunnan indicated an excess of low-frequency polymorphisms compared to what would be expected under neutral evolution (Paper 2). The other *Avr2* populations did not have strong evidence of an evolutionary response to selection (Paper 2). This result is reinforced by the 1.68 overall nonsynonymous (dN = 5.74) to synonymous substitution (dS = 3.41) rate ratio (ω) in the combined population of the 17 fields. $\omega > 1$ suggests positive (diversifying) selection, implying natural selection favours changes in the protein sequence, potentially due to adaptation to new environments or functional diversification. This is confirmed by the overall Tajima's D which was negative. These results suggest a complex pattern of selection pressures highlighting the intricate interplay of genetic diversity and selection forces. Since effectors are integral to the host-pathogen arms race and tend to have sequences with high variation, this is reasonable for *Avr2*.

Despite the high genetic variation at the nucleotide level, only 13% (15 positions) of amino acids showed elevated diversity (Figure 9). Notably, all 15 positions align with those identified by Yang et al. (2020). Observing the same diverse sites across a larger sample size, spanning different geographical regions and multiple years, suggests these variations are likely occurring at relatively high frequencies within the population. This

consistency implies that the amino acid changes are well tolerated and may be essential for the structure, function, or molecular interactions of AVR2.

A strong positive correlation was observed between the annual mean temperature (AMT) and diversities (Figures 10B & 10D). Consequently, temperature had a direct proportional relationship with both population diversity parameters, with the association being slightly more pronounced in the case of Hd. In contrast, altitude showed a negative impact, albeit significantly so only with π (Figures 10A & 10C). This indicates that AMT and altitude are a part of the selection pressures responsible for the genetic diversity in *Avr2* among the major potato-cropping regions and fields.

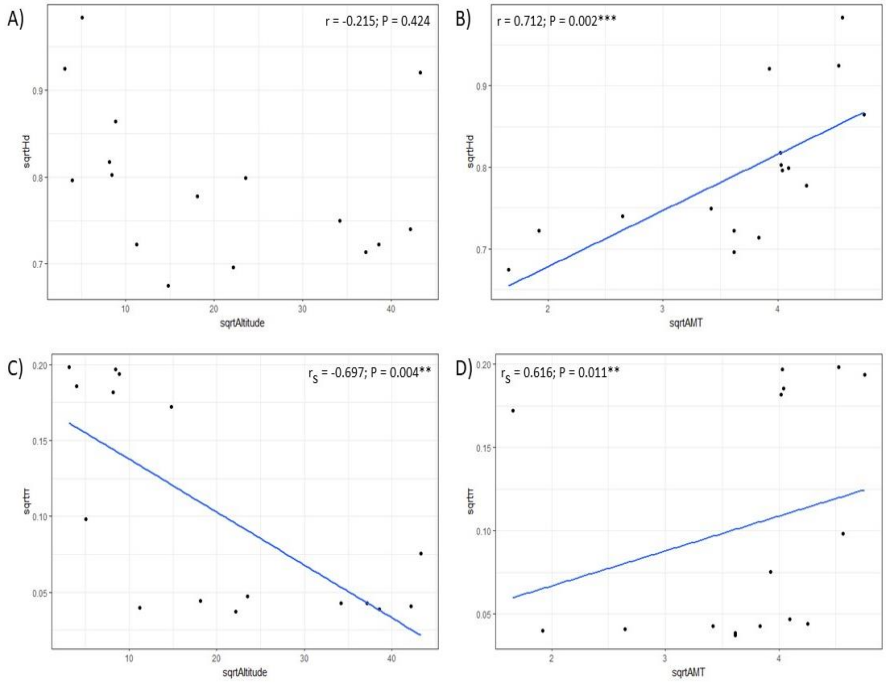
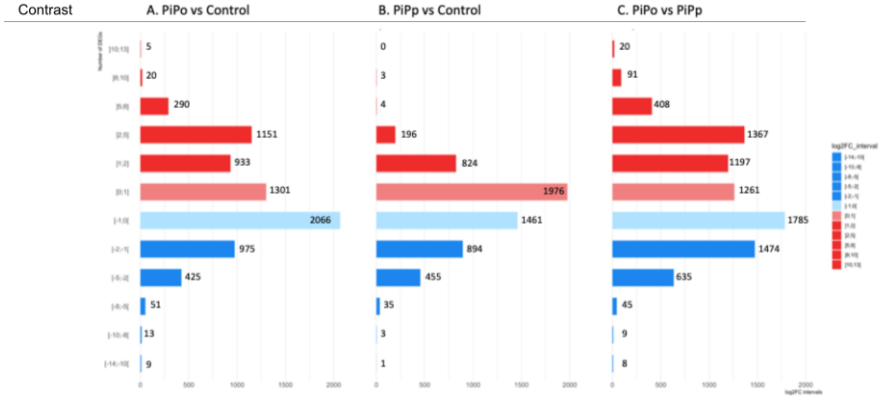


Figure 10. Relationship between the haplotype (Hd) and nucleotide (π) diversities and the annual mean temperature (AMT) and altitude of the location of the fields. r and rs denote Pearson correlation coefficient and Spearman's rank correlation coefficient, respectively.

4.3 Transcriptomic responses of *P. infestans* to mycoparasitic attack by two *Pythium* BCAs

In this study, we investigated the molecular interactions between *P. infestans* and the mycoparasites *Py. oligandrum* and *Py. periplocum* at 12 hours post-interaction to understand the defence responses of *P. infestans* under antagonistic stress. Transcriptomic analysis revealed differentially expressed genes in *P. infestans* that responded specifically to each *Pythium* species. Interestingly, a subset of upregulated genes exhibited conserved responses in both interactions, indicating their potential role as core components in the defence strategy of *P. infestans*.



Contrast	A. PiPo vs Control	B. PiPp vs Control	C. PiPo vs PiPp
Number of DEGs	7239	5852	8300
Number of DEGs log2FC>0	3700	3003	4344
Number of DEGs log2FC<0	3539	2849	3956

Figure 11. Barplot of the intensity of expression changes in *P. infestans* genes in response to mycoparasitism. The plot represents the number of DEGs across log2FC intervals for each contrast. Over- and under-expressed genes in the confrontations compared to the control are in red and blue, respectively. The number of DEGs for each contrast and the number of DEGs with specific log2FC values are displayed below the respective plots. Abbreviations: Pi (*P. infestans*), Po (*Py. oligandrum*), and Pp (*Py. periplocum*).

Across all tested contrasts, 9,904 genes were differentially expressed (DE) in at least one comparison (Figure 11). In response to *Py. oligandrum* and *Py. periplocum*, 58.7% and 47.5% of tested genes were DE, respectively (Figure 11). Additionally, 67.4% of DEGs showed important differences in the responses to *Py. oligandrum* (Po) and *Py. periplocum* (Pp) in the PiPo-

PiPp contrast (Figure 11), indicating *P. infestans* can sense and respond uniquely to each mycoparasite. Gene expression differences with a more than two-fold increase (log2FC > 1), compared to the controls, accounted for 64.8% of overexpressed DEGs in response to *Py. oligandrum* but only 34.2% in response to *Py. periplocum* (Figures 11A and 11B).

Of the 9,330 genes DE in at least one *Pythium* confrontation vs. control contrasts, 7,557 (81%) showed specific responses, either unique to one contrast or with opposite expression patterns across contrasts i.e. overexpressed in one contrast while under-expressed in the other one (Figure 12, green bars). Without analysing multiple time points, we cannot be sure whether this type of expression pattern is characteristic of a specific overall response or due to a delay in the timing of the response due to the different speeds of attack by the different mycoparasites. In contrast, 1,773 genes (19%) were DE in both of the confrontation vs control contrasts with the same behaviour, including 728 overexpressed (Figure 12, red bars) and 1,045 under-expressed genes (Figure 12, blue bars) in response to mycoparasitism by both *Pythium* species.

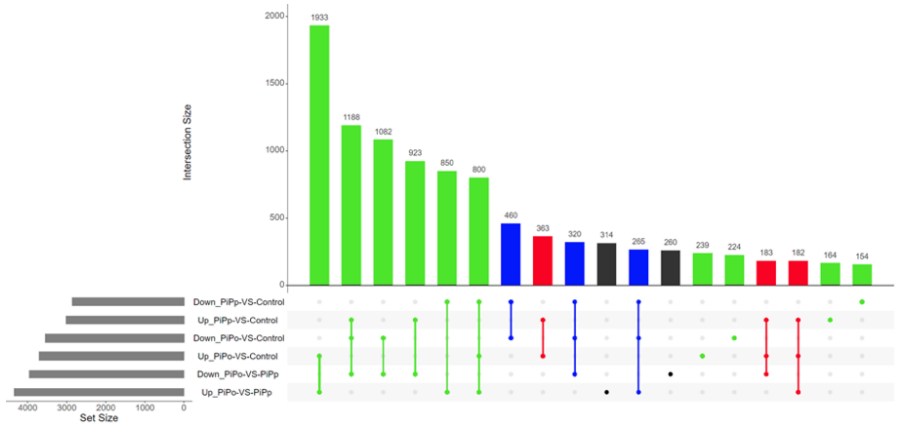


Figure 12. Upset plot illustrating the responses of *P. infestans* (Pi) to the mycoparasites *Pythium oligandrum* (Po) and *Py. periplocum* (Pp). The plot shows the number of over- and under-expressed DEGs across the three contrasts, categorized as either conserved or specific to the mycoparasites. Horizontal bars indicate the total number of DEGs for each contrast–behaviour category, while vertical bars represent the intersection size between these categories. Red bars represent conserved overexpressed DEGs in confrontation conditions compared to control; Blue bars represent conserved under-expressed DEGs in confrontation conditions compared to control; Green bars represent specific responses to at least one of the mycoparasites compared to control, regardless of expression behaviour.

Stress perception receptors were the first category looked at during annotation of the DEGs, in order to identify if *P. infestans* can sense and respond to mycoparasitic attack. A total of 53 genes encoding putative stress perception receptors were DE in at least one confrontation vs. control contrast. Of these, 10 showed the same expression pattern in response to mycoparasitism by both *Pythium* species, including 8 genes (4 GPCRs, 3 RLKs, and 1 NLR) that were overexpressed compared to controls (Table 3). While being overexpressed in the confrontation conditions compared to the control, 7 of these receptor genes were highly expressed in all conditions including the controls (Figure 13). Hence, it is possible they can be constitutively expressed in *P. infestans* or maybe also involved in self-recognition (in the case of the control). However, since the control was performed with two plugs of the same isolate of *P. infestans* from the same plate, the former case is more likely.

Table 3. log2 fold changes of the conserved DEGs encoding putative stress sensing receptors for each confrontation versus control contrasts.

Gene ID	Function	log2FC PiPo vs Control	log2FC PiPp vs Control
PITG_00315	GPCR	0.4293973	1.0161851
PITG_00896	GPCR	1.0876099	2.2095652
PITG_17353	GPCR	1.2053903	0.8958801
PITG_19640	GPCR	1.2404941	0.8077708
PITG_06733	GPCR	-1.28794	-1.24597
PITG_03457	NLR	0.3074422	0.6518098
PITG_09665	RLK	0.4625873	0.8969073
PITG_23090	RLK	5.2274937	4.0161790
PITG_23143	RLK	2.9049448	3.0519972
PITG_03388	RLP	-0.77132	-0.47811

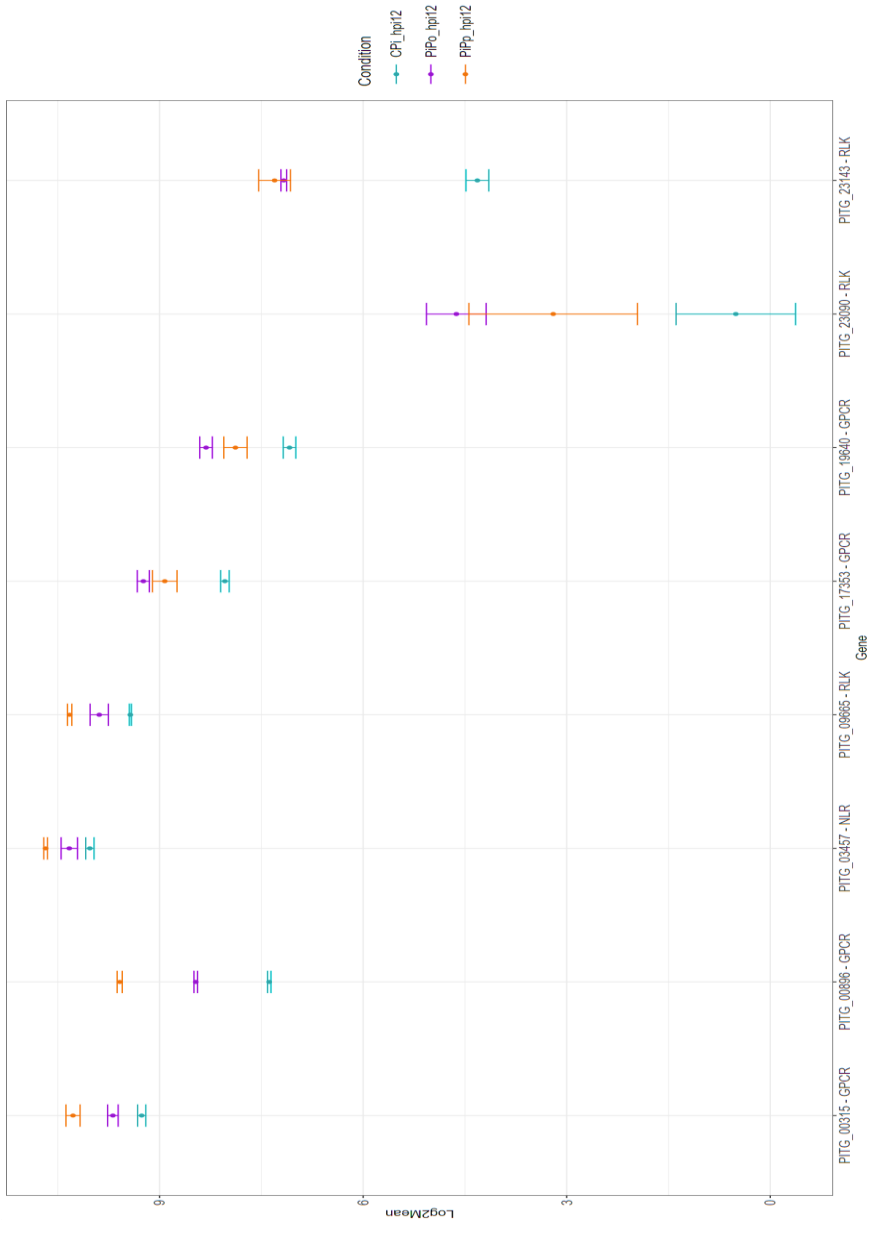


Figure 13. Expression profiles of the conserved DEGs coding for putative stress sensing receptors that are overexpressed in the confrontation conditions in comparison to controls. The dot plot represents the log2 of the mean of normalized expression values of each gene. Values are color-coded based on the condition: grey-blue for control (Cpi), purple for PiPo, and orange for PiPp. Standard deviations are indicated by horizontal bars.

The presence of a GPCR domain in *P. infestans* indicates a direct connection between environmental sensing and intracellular signalling. In our study, these pathways likely help *P. infestans* sense and evade its antagonists. Several GPCR-bigrams were identified. GPCR-bigrams indicate the presence of alternative G-protein signalling pathways which are essential for plant pathogens since they rely on cellular signalling to respond to environmental cues and identify suitable hosts (van den Hoogen et al., 2018). PITG_00896 is a protein containing a GPCR-Phosphatidylinositol phosphate kinase domain (PIPK) and PITG_00315 and PITG_17353 are proteins with a GPCR-Inositol polyphosphate phosphatase domain (INPP). GPCR-PIPK and GPCR-INPP are involved in the phosphoinositide pathway, a crucial process for cell signalling, membrane trafficking, and cytoskeletal dynamics (Qin & Wei, 2021). PIPK activity produces PIP2, and INPPs can dephosphorylate PIP2 (van den Hoogen et al., 2018), suggesting that GPCR-PIPKs and GPCR-INPPs in oomycetes may function together in a phosphorylation-dephosphorylation cycle. In our findings, the log2FC of these genes varied between PiPo and PiPp versus the control. This response difference may reflect a tailored defence strategy by *P. infestans*, depending on the perceived threat level of each mycoparasite. PITG_23094, encoding a putative RLK, is less expressed in the control condition with log2 mean value of 0.51 while being expressed 37 times more when facing *Py. oligandrum* (log2FC=5.23) and 16 times more when facing *Py. periplocum* (log2FC=4.016). This RLK contains a CGC motif suggesting a specialized function possibly linked to calcium signalling. Its expression in response to *Py. oligandrum* being over twice that in response to *Py. periplocum* might mean that *P. infestans* is perceiving *Py. oligandrum* as a greater threat upon sensing this antagonist, thereby leading to a stronger stress response. However, the difference in these responses may also reflect subtle differences in the timing of the secretion of mycoparasitic determinants by each of these antagonists, which could lead to a differential response by *P. infestans*.

Understanding the role of kinases can provide insights into the stress response and signalling networks of *P. infestans*. Interaction with the *Pythium* spp. involves an extensive reprogramming of the genes coding for kinases with a total of 268 kinase encoding genes DE for at least one of the confrontation versus control contrasts, among which, 48 (17.9%) were DE for both contrasts. These belonged to 56 different kinase families with an

over-representation (55%) of the tyrosine kinase-like plant specific 4 family (TKL-Pl-4), the calcium-dependent protein kinase family (CAMK_CDPK), the plant specific family of the AGC group (AGC-Pl) and the tyrosine kinase-like *C. reinhardtii*-specific 3 family (TKL-Cr-3). When *P. infestans* is antagonised, the possible roles of these categories are:

- AGC: stress response and activation of defence mechanisms
- CAMK: defence signalling by modulating calcium which is required in high levels for stress response
- TKL: Defence responses, membrane trafficking, cell death and cell wall strengthening

Within the conserved set of kinases, the expression changes were more drastic in response to *Py. oligandrum* than to *Py. periplocum*. For the kinase gene set specifically responding to *Py. oligandrum*, the number of over and under-expressed DEGs was balanced with 87 overexpressed and 89 under-expressed genes. However, for the kinase genes responding specifically to *Py. periplocum*, this was not the case with 87 of the 112 specific DE kinase genes overexpressed in the confrontation. This again displays the tailored responses of *P. infestans* to each *Pythium* spp. To note, a protein kinase may initially be overexpressed to activate defence pathways against mycoparasitism, followed by under-expression to prevent prolonged responses that could harm *P. infestans*.

Transcription factors (TFs) and other regulators of gene expression are essential for the adaptability and response mechanisms of *P. infestans*. A total of 460 genes predicted to code for transcription regulators displayed significant expression changes in response to at least one of the *Pythium* confrontations in comparison to the control, of which 84 responded to both *Py. oligandrum* and *Py. periplocum* with the same behaviour in comparison to the control. A total of 39 TF families were involved in the response to mycoparasitism, and the MYB and MYB-related (44 DE responses) family was one of the families representing a major proportion of the DE results with an iTAK annotation. In *P. infestans*, MYB was demonstrated to regulate important processes in growth and development (Xiang & Judelson, 2010) while some MYBs act as regulators of sporulation (Xiang & Judelson, 2014). Hence, it is possible this family could help maintain the structural integrity, adjust the metabolism, and potentially enhance the reproductive capacity of *P. infestans* to counteract the stress induced by the mycoparasites. Notably, a cluster of 7 DEGs, including 1 bZIP, 1 C2H2, and 1 SET, was particularly

highly under-expressed in response to *Py. oligandrum* when compared to *Py. periplocum*. In *P. infestans*, bZIP was previously found to be involved in defence against oxidative stress (Gamboa-Meléndez et al., 2013). C2H2 is a zinc-finger factor which is involved in various regulatory factors. SET might be involved in epigenetic regulation in *P. infestans* during mycoparasitic attack, since epigenetic mechanisms may be able to respond faster to this kind of stress than other regulatory tools.

To identify genes involved in detoxification, the study focused on four transporter families known for their roles in detoxification and stress responses: ABC (Transporter Classification Database (3.A.1), n.d.), MFS (Transporter Classification Database (2.A.1), n.d.), DMT (Transporter Classification Database (2.A.7), n.d.), and MOP (Transporter Classification Database (2.A.66), n.d.). A total of 192 genes encoding these transporters were differentially expressed in at least one confrontation vs. control contrast, with 53 (27.6%) showing consistent DE behaviour in response to both *Py. oligandrum* and *Py. periplocum*. Among these conserved DEGs, 19 were over-expressed, and 34 were under-expressed compared to controls. In the response specific to *Py. oligandrum*, 49 genes were over-expressed, and 58 were under-expressed, while in the response specific to *Py. periplocum*, 52 genes were over-expressed, and 29 were under-expressed. The ABC transporter family dominated the DE results, with 107 DE responses. Among the conserved overexpressed DEGs, PITG_13554 and PITG_07134, both coding for ABC transporters, were notably characterised by very intense gene expression increase especially in response to *Py. oligandrum* with log2FC of 6.9 and 4.2, respectively, in comparison to the control.

Effectors are well-known as virulence factors, and the *P. infestans* genome contains hundreds of genes encoding these proteins. Interestingly, this number far exceeds what would be necessary solely for infecting *Solanum* species. This observation led us to hypothesise that effectors might play a broader role in environmental interactions, including competition with other microbes in the ecosystem. Specifically, we propose that the overexpression of effector genes during confrontation with mycoparasites, compared to the control, might indicate their involvement in an active counterattack response. Hence, we mined our data for differentially overexpressed effectors (Figure 14). Among the conserved effector DEGs, 30 were overexpressed and 76 were under-expressed in response to both *Py. oligandrum* and *Py. periplocum* compared to the control. In the response

specific to *Py. oligandrum*, 146 DEGs were overexpressed and 142 were under-expressed, while in the response specific to *Py. periplocum*, 108 DEGs were overexpressed and 86 were under-expressed compared to the control. The large number of overexpressed effector genes suggests that effectors are important in the response to mycoparasitic attack. Since most of these are specific, this suggests that *P. infestans* may deploy specific effectors to attack or compete with individual microbes, including antagonists, which it meets in the environment.

Among the effector genes with oomycete-specific motifs, 2 contain the classic RxLR-EER motif (Whisson et al., 2007), 1 has an RxLR-dEER motif (Ai et al., 2020), 3 have a YxSL motif, 1 a QxLR motif, and 1 a CGC motif. Of the effectors predicted by EffectorP 3.0, 12 were classified as apoplastic, 6 as cytoplasmic, and 6 as potentially localizing to both compartments. There were 2 protease inhibitors (PITG_12138 and PITG_12131). PITG_18999 coding for a serine protease is one the RxLR-ERR effectors. There were 4 CAZymes – PITG_11942, PITG_15377, PITG_20868, and PITG_06788. The gene PITG_11942 encodes a YxSL effector from the Auxiliary Activity Family 17, corresponding to copper-dependent lytic polysaccharide monooxygenases (Carbohydrate-active enzymes Database, n.d.). PITG_15377 encodes a YxSL effector from the GlycosylTransferase Family 60. PITG_20868 contains a QxLR motif and encodes a protein from the Glycoside Hydrolase Family 16 subfamily 2, while PITG_06788 encodes a protein from the Glycoside Hydrolase Family 7.

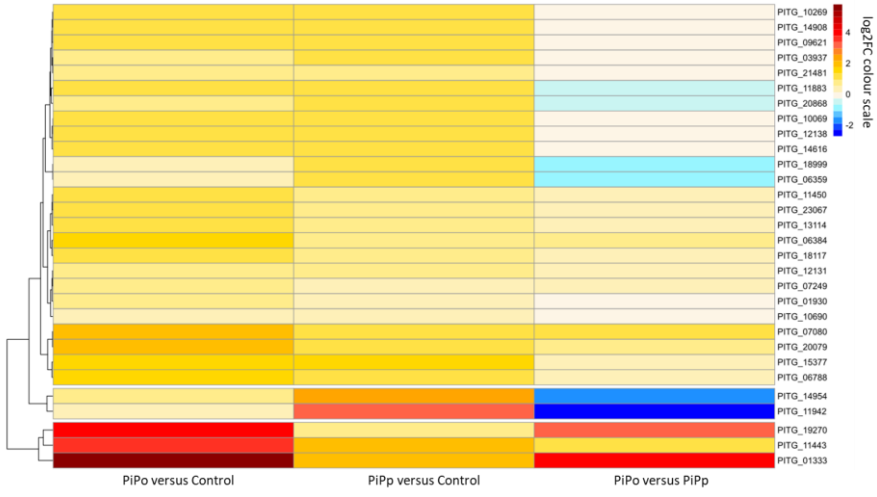


Figure 14. Expression changes of the conserved overexpressed effector-encoding DEGs. The heatmap represents the log2FC of the effector DEGs.

Most DEGs identified as effectors appear involved in host manipulation, stress response, and maintaining cellular integrity. Some, however, may be expressed to defend *P. infestans* by counteracting or disrupting the *Pythium* species. One notable example of effector versatility is PITG_18117, which operates differently depending on location and the interacting antagonist/host. In the *Solanum lycopersicum* – *P. infestans* pathosystem, PITG_18117 functions in the cytoplasm (Hoyo, 2017), while in our study with the mycoparasitic *Pythium* species, it acts in the apoplast. Its cytoplasmic domains likely support signalling or interaction with host proteins to modulate responses, whereas its apoplastic activity may modify the extracellular environment by interacting with cell wall components or host defence compounds to influence host responses.

Table 4. Gene annotations of the conserved overexpressed effector-encoding DEGs, including the effectors with similarities from PHI-base

Gene ID	Annotation	UniProt
PITG_01333	Sushi repeat (SCR repeat)	Complement control module protein, putative
PITG_01930	-	Fibronectin type-III domain-containing protein
PITG_03937	Ring finger domain	RING-type domain-containing protein
PITG_06359	-	Uncharacterized protein

PITG_06384	EGF-like domain	EGF-like domain-containing protein
PITG_06788	Glycosyl hydrolase family 7	cellulose 1,4-beta-cellobiosidase (non-reducing end)
PITG_07080	-	Uncharacterized protein
PITG_07249	-	EGF-like domain-containing protein
PITG_08943	-	Avr2 family secreted RxLR effector peptide protein, putative
PITG_09621	-	Protein kinase
PITG_10069	-	Protein phosphatase
PITG_10204	Glycosyl hydrolase family 7	cellulose 1,4-beta-cellobiosidase (non-reducing end)
PITG_10269	-	Uncharacterized protein
PITG_10690	-	Uncharacterized protein
PITG_11443	-	WRKY transcription factor 19
PITG_11450	-	Cysteine-rich protein
PITG_11883	-	Uncharacterized protein
PITG_11942	-	Uncharacterized protein
PITG_12131	Kazal-type serine protease inhibitor domain//Kazal-type serine protease inhibitor domain//Kazal-type serine protease inhibitor domain	Protease inhibitor Epi4
PITG_12138	Kazal-type serine protease inhibitor domain//Kazal-type serine protease inhibitor domain	Kazal-type serine protease inhibitor, putative
PITG_12551	Elicitin	Elicitin
PITG_12561	Elicitin	Elicitin
PITG_13114	RNA recognition motif. (a.k.a. RRM, RBD, or RNP domain)	Elicitin-like protein
PITG_14616	-	Uncharacterized protein
PITG_14908	-	Uncharacterized protein
PITG_14954	-	Secreted RxLR effector peptide protein, putative (other genes encoding same protein are PITG_14959, PITG_14961, PITG_14962)
PITG_15377	Glycosyltransferase (GlcNAc)	GlcNac transferase
PITG_16866	Necrosis inducing protein	Necrosis-inducing protein NPP1
PITG_18117	Calcineurin-like phosphoesterase	Calcineurin-like phosphoesterase domain-containing protein
PITG_18999	Subtilase family	subtilisin
PITG_19270	-	Uncharacterized protein
PITG_20079	-	Uncharacterized protein
PITG_20868	Beta-glucan synthesis-associated protein SKN1/KRE6/Sbg1//Beta-glucan synthesis-associated protein SKN1/KRE6/Sbg1	Beta-glucan synthesis-associated protein, putative

PITG_21481	-	Calcineurin-like phosphoesterase domain-containing protein
PITG_22547	RXLR phytopathogen effector protein	RxLR effector protein
PITG_23067	-	Dynein heavy chain

Some key effectors related to defence and counterattack include proteins with EGF-like domain (PITG_06384, PITG_07249), glycosyl hydrolase family 7 enzymes (PITG_06788), putative RxLRs (PITG_14954, PITG_22547), WRKY transcription factor 19 (PITG_11443), and Kazal-type protease inhibitors (PITG_12131, PITG_12138) (Table 4). EGF-like domain-containing proteins may aid cyst adhesion to the host, allowing cyst germination and initial tissue penetration, followed by other functions like competing for resources and modulating the extracellular environment (Engel, 1992; Hohenester & Engel, 2002; Liu et al., 2006; Savidor et al., 2008). Similarly, glycosyl hydrolase (GH) enzymes play crucial roles in carbohydrate breakdown. As observed in plant infections (Ospina-Giraldo et al., 2010), GH7 enzymes act as pathogenicity factors, targeting carbohydrates within plant cell walls. PITG_14954, induced during non-host interactions with pepper (Lee et al., 2014), may contribute to a flexible defence strategy and confer resilience against initial mycoparasitic antagonisms. Serine protease inhibitors like the Kazal-type were upregulated, potentially counteracting proteolytic damage inflicted by antagonists. Daly et al. (2021) noted a similar response in *Py. myriotylum* during interaction with *Py. oligandrum*, suggesting that Kazal-type protease inhibitors might be conserved in oomycetes during mycoparasitic interactions with *Pythium* spp. All available knowledge of the conserved and overexpressed effector encoding genes are in Table 5.

Table 5. Conserved and overexpressed effector-encoding DEGs identified in this study that were previously reported in other studies

Gene ID	Available knowledge
PITG_01333	It is one of the top 50 transcripts of *P. infestans* upregulated at 12 hpi & 24 hpi during the infection of leaves of *Solanum tuberosum* by the *P. infestans* transformant 88069tdT10 l. (Kandel, 2014)
PITG_06788	(Ah-Fong et al., 2017) - Belongs to glycosyl hydrolase7 (GH7) enzyme category

	- Specific increased expression during early infection of potato tubers leads to 10-fold higher total cellulase CPM in *P. infestans* than *Py. ultimum*
	Orthologous to PrG_72061 (*P. ramorum*) and PsG_108891 (*P. sojae*) (Haas et al., 2009)
	Exoglucanase 1 which is a component of the germinating cyst and appressorium cell wall proteome (Grenville-Briggs et al., 2010)
	(Resjö et al., 2017) - Linked to cell wall biosynthesis and remodelling which are highly important for the correct formation and function of germinating cysts and appressoria - Transcript levels were higher at 6 hpi when compared to the levels in mycelium. Around 6-8 hpi, the appressorium production and penetration of the leaf surface are at the highest rates → critical function during the initial steps of infection
PITG_07080	Protein shared by oospores and nonsporulating hyphae in *P. infestans* (Niu, 2010)
PITG_07249	(Qian et al., 2018) - Pectinacetylesterase-associated protein in *P. infestans* - Functions in catalytic activities
PITG_08943	- PHI-base entry: PHI:5088; PHI:10633 - Similar to PiAvr2 (PITG_22870) from *P. infestans* T30-4
PITG_10204	- PHI-base entry: PHI:10659 - Similar to PsGH7a from *P. sojae* P6497
PITG_10269	*Phytophthora sojae* putative lectin (Gonzalez-Tobon et al., 2022)
PITG_11450	(Carballo, 2022) - Uncharacterized protein - Found in the tomato apoplastic proteome upon *P. infestans* infection
PITG_11883	(Cano Mogrovejo, 2011) - Secreted protein - Shows an extended gene induction period of 2 & 3 dpi on potato in *P. infestans* 06_3928A isolate
PITG_12131	(Cano Mogrovejo, 2011) - epi4 gene - 3 Kazal-like domains - Secreted - Serine protease inhibitor in *P. infestans* - Transcriptionally induced during pre-infection stages (germinated cyst) and early stages of infection of potato, thereby suggesting a role during host colonisation - Gene induction peaks @ 16 hpi and during biotrophy @ 2 dpi; declines during the necrotrophic phase (4-5 dpi) – ONLY potato; no gene induction on tomato

PITG_12138	(Cano Mogrovejo, 2011) - epi17 gene - 2 Kazal-like domains - Secreted - Serine protease inhibitor in *P. infestans* - Transcriptionally induced during pre-infection stages (germinated cyst) and early stages of infection of potato, therefore suggesting a role during host colonisation - Gene induction peaks @ 6-16 hpi and during biotrophy @ 2dpi; declines during the necrotrophic phase (4-5 dpi) – on potato - Gene induction on tomato @ 2-3 dpi
PITG_12551	- PHI-base entry: PHI:111; PHI:760 - Similar to INF1 from *P. infestans* NL-88069
PITG_12561	- PHI-base entry: PHI:762 - Similar to INF2A from *P. infestans* NL-88069
PITG_14954	(Yin et al., 2017) - RxLR effector gene - Consensus sequence to PITG_14959, PITG_14961, PITG_14962
	(Lee et al., 2014) - PexRD14 effector gene - Expressed during non-host interaction of *P. infestans* with pepper @ 12hpi
PITG_16866	- PHI-base entry: PHI:666 - Similar to NPP1 from *P. nicotianae* 1828
PITG_18117	It interacts with *Solanum lycopersicum* in the cytoplasm. (Hoyo, 2017)
	(Cano Mogrovejo, 2011) - Enzyme hydrolase - Secreted protein - Shows an extended gene induction period of 2 & 3 dpi on potato in *P. infestans* 06_3928A isolate
PITG_19270	(Meijer et al., 2014) - Part of the secretome of *P. infestans*
PITG_20079	- Single transmembrane protein (trans-membrane domain-containing protein)
PITG_22547	- PHI-base entry: PHI:10641 - Similar to Avrvnt1 (PITG_16294) from *P. infestans* T30-4

Of the 146 DEGs specifically overexpressed in response to *Py. oligandrum*, 115 exhibited a two-fold increase compared to the control. Among these, 19 had an RxLR-like pattern, 3 a CGC-like pattern, and 2 a CRN-like pattern. Additionally, 5 DEGs – PITG_08943, PITG_10204, PITG_12561,

PITG_12551, and PITG_16866 – showed strong matches to validated effectors in PHI-base (Tables 4 & 5). This highly overexpressed set also included 3 protease inhibitors, 1 serine protease, and 9 CAZymes. Among the 108 DEGs specifically overexpressed in response to *Py. periplocum*, 42 displayed a two-fold increase in comparison to the control. Of those, 13 had an RxLR-like pattern, including PITG_22547, and 2 a CGC-like pattern. There were also 1 protease inhibitor and 3 CAZymes.

As observed, there are several protease inhibitors and CAZymes that were overexpressed, be it conserved responses or specific. Protease inhibitors potentially help *P. infestans* counteract mycoparasite proteases that degrade host proteins, protecting its cellular integrity. CAZymes may reinforce the cell wall against degradation or disrupt the mycoparasite's structures, highlighting their roles in defence and counterattack during mycoparasitism. Finally, we investigated secondary metabolites, which are important for the competitive advantage of *P. infestans* against other microbes. A total of 49 DEGs predicted to be associated with specialized metabolism were identified, with 8 responding to both *Py. oligandrum* and *Py. periplocum*. Among these, 3 were overexpressed in response to both mycoparasites. 18 and 6 DEGs were overexpressed in response to *Py. oligandrum* and *Py. periplocum* compared to the control, respectively, while 13 and 10 were under-expressed. There are no tools specific for oomycetes, and the antiSMASH tool used was built for fungi, which might explain the low number of secondary metabolite DEGs seen. Alternatively, *P. infestans* may prioritize energy-efficient responses in these interactions, activating only the secondary metabolites most relevant to countering specific pressures imposed by the *Pythium* species. It could also indicate that the other defence mechanisms we discussed so far are more effective or primary in response to these mycoparasites. The secondary metabolite response may therefore be only a small, complementary part of the broader defence strategy.

5. Conclusions

This thesis explored the plant pathogenic oomycete *Phytophthora infestans*, the causal agent of late blight, from multiple perspectives and highlighted potential new roles for effectors in diverse *P. infestans*-environmental interactions. While many bacterial and fungal plant pathogens rely on stomata for entry and colonisation, *P. infestans* bypasses stomata during initial infection by penetrating epidermal cells via naifu-appressoria. Stomata serve mainly as exit points for sporangia discharge. Paper I demonstrated that potato responded by closing nearly half of its stomata to prevent colonisation, a race-independent defence mechanism in this host plant. However, *P. infestans* counteracted this by suppressing guard cell death and fully reopening stomata. The study identified a series of chemical processes in *Solanum tuberosum* cv. Desirée leaves, including starch degradation, increased sugar levels, lipid breakdown, and a reduction in free radicals like H_2O_2 and NO. From our observations, the possible mechanism could likely be

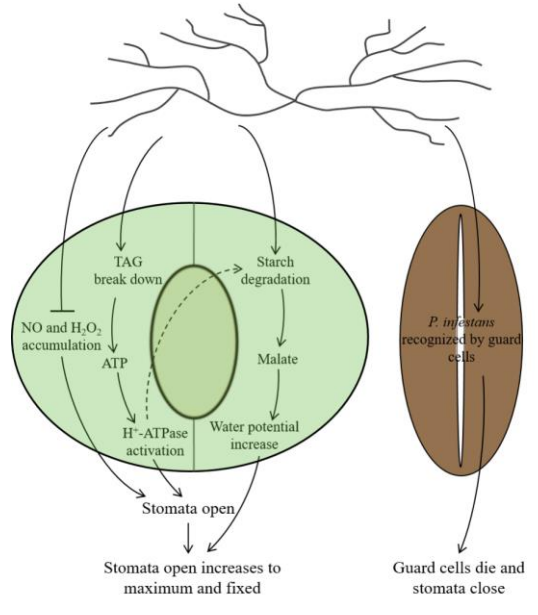


Figure 15. Proposed model for *P. infestans*-mediated stomatal opening. Image adapted from Yang et al. (2021).

that *P. infestans* manipulates pathways related to stomatal defences leading to a reduction in the amount of free radicals in guard cells, followed by lipid catabolism, which in turn generates energy to degrade starch, ultimately increasing guard cell turgor pressure and leading to stomatal opening (Figure 15). Infiltration of healthy plants with apoplastic fluids from infected plants highlighted the involvement of effectors secreted by *P. infestans* in this process. We identified two effectors that played significant roles in initiating stomatal opening.

While Paper I focused on the role of effectors in pathogenicity, the genetic diversity and environmental adaptation of the avirulence effector AVR2 provide further insight into how *P. infestans* populations evolve and thrive under different conditions. Such effectors influence disease dynamics in both natural and agricultural ecosystems. Genetic variation in *Avr2* was observed across several locations in China, with regions of low potato cultivation, such as the Central Double Cropping Region (CDR) and Winter Cropping Region (SWR), exhibiting more mutations. Human activities, particularly the importation of seed potatoes, likely contributed to this high genetic diversity. Four dominant haplotypes, shared across multiple locations, indicated significant genetic homogeneity. Two distinct groups were formed in the haplotype network by indels (Figure 16). In Yang et al. (2020), the group 1 proteins were avirulent, had significantly different physiochemical parameters, and had a lower overall percentage of disorder tendency. Hence, it is highly likely that the groups in our study possess similar traits.

Despite genetic similarities between different cropping regions, high diversity at the isolate level and significant variation between populations within cropping regions suggested local adaptation to factors like management practices and environmental pressures. A positive correlation with temperature and a negative association with altitude further highlighted that these local environmental pressures are key selection factors for AVR2 and potentially other AVR effectors.

In Papers III and IV, we explored sustainable approaches to control potato late blight, by investigating the potential for the use of microbial biological control agents (MBCAs) to control this disease and the possible mechanisms *P. infestans* utilises to defend itself from attack by such MBCAs. Our literature review revealed that although effective in *in vitro* and *in planta* trials, these methods did not fully translate to field conditions (Paper IV). To unravel potential reasons for the lack of success of specific MBCAs in

agriculture, we investigated the possibility that *P. infestans* could defend itself from mycoparasitic attack (Paper III). Our study identified differentially expressed genes (DEGs) in *P. infestans* that responded specifically to two different *Pythium* species, with a subset of upregulated genes showing conserved responses across both interactions, indicating a possible core role in the defence against oomycete antagonists. We hypothesised that *P. infestans* senses mycoparasitic pressure, triggers signalling pathways, and activates defence responses through the deployment of effector proteins and secondary metabolites. Several GPCRs, RLKs, and an NLR were overexpressed, with GPCR-bigrams suggesting the involvement of the phosphoinositide pathway, which is crucial for cell signalling, membrane trafficking, and cytoskeletal dynamics. Kinase families related to stress, cell wall strengthening, membrane trafficking, and cell death were prominent, indicating key responses from sensing to counter-attack. The over-represented transcription factors identified may potentially maintain structural integrity, adjust metabolism, and activate specific and defence responses during attack. Transporters indicated detoxification as an important process in the response to these antagonists. The overexpression of effector-encoding DEGs, including various RxLRs and CAZymes, emphasised the importance of effectors in the response to mycoparasitic attack. Interestingly, a gene matching *PiAvr2* in PHI-base was overexpressed, suggesting cross-talk between virulence mechanisms during plant infection and mycoparasitic stress, or a dual role for some effectors. Additionally, Kazal-type protease inhibitors, previously identified in another plant pathogen during its interaction with *Py. oligandrum*, may be conserved in oomycetes during mycoparasitic interactions, especially with *Pythium* species.

To conclude, effectors are pivotal in the pathogenicity, population biology, and defence mechanisms of *P. infestans*. Effectors allow the pathogen to manipulate host systems for its advantage, adapt to environmental pressures across diverse geographical regions, and mount defence responses against antagonistic microbes such as mycoparasitic *Pythium*. This study highlights the importance of implementing effective quarantine measures to limit the evolutionary potential of *P. infestans* effectors. Understanding the dual role of effectors in attack and defence has identified potential targets for adaptation of biocontrol treatments, paving the way for more sustainable and effective biocontrol strategies.

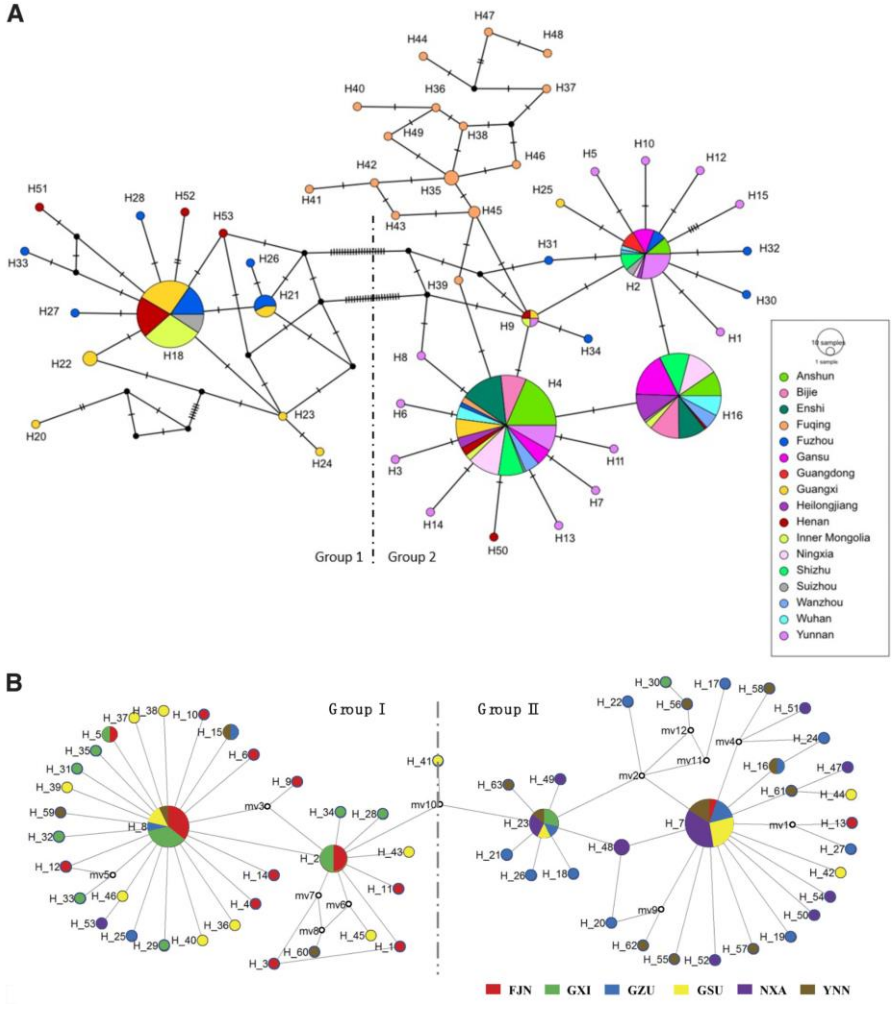


Figure 16. Comparison of the haplotype network of the AVR2 effector from different *P. infestans* populations across China. (A) our study; (B) Yang et al. (2020)

6. Future perspectives

Given the global importance of late blight management, the insights from this thesis can serve as a foundation for developing more sustainable and targeted approaches to disease control. Future research should focus on integrating these findings into holistic disease management frameworks. While this thesis advances our understanding of the molecular mechanisms underlying *Phytophthora infestans* pathogenicity and defence responses, several unanswered questions remain, providing new directions for research.

The proposed model for *P. infestans*-mediated stomatal opening highlights the roles of reduced H_2O_2 and NO levels, lipid and starch catabolism, and increased sugar levels in guard cells, potentially driven by effectors (two in our study). However, additional players are likely involved. Future studies should aim to investigate these mechanisms in greater detail, including the testing of two additional candidate effectors, PITG_03511 and PITG_18396, which were excluded from this study, as well as exploring other yet-to-be-identified candidates. Characterising the specific roles of apoplastic and cytoplasmic effectors in stomatal manipulation remains critical. Proteomics and metabolomics analyses of apoplastic fluid from infected plants could uncover unknown effectors or metabolites involved. Silencing studies could identify redundant effectors with overlapping functions and clarify whether they contribute to other pathogenicity traits. Since stomatal manipulation supports secondary inoculum production, further research on the underlying pathways may inform control strategies, such as chemical or biological inhibitors and enhanced potato varieties could be developed in the future, with more resistant guard cell responses.

Haplotype analysis of the *Avr2* effector revealed populations aligning with Yang et al. (2020), who demonstrated that Group 1 AVR2 proteins were avirulent, had significantly different physiochemical parameters, and a lower

overall percentage of disorder tendency which helps AVR2 to escape R2 recognition in potato. Expanding our current work, holding samples from several other regions and different years, with phenotypic studies could confirm whether these trends hold across China as well as other parts of the world. While this study linked the genetic diversity of *Avr2* to temperature and altitude, future research could examine other factors like UV radiation and soil conditions. As climate change may shift the temperature tolerance and drought survival of *P. infestans*, systematic monitoring of pathogen populations is vital. Additionally, incorporating data from sexual and asexual populations would provide a more robust understanding of AVR2 evolution.

This thesis also explored the defence responses of *P. infestans* to mycoparasitic attack by *Pythium* species at 12 hours post-infection. Testing multiple time points could reveal whether the observed DEGs are truly species-specific or time-dependent instead. Identifying different effector genes expressed at different stages after mycoparasitic infection could provide information on the diverse roles of effectors in microbe-microbe interactions and help shape the way we utilise oomycete antagonists as biocontrol agents. Insights from the *Py. oligandrum – Py. myriotylum* interaction, combined with findings from our study, suggest that Kazal-type protease inhibitors may be conserved among oomycetes during mycoparasitism. Future research should investigate whether specific proteases are correspondingly conserved in the mycoparasites. Additionally, the potential role of *P. infestans* effectors in interactions with other microbes, such as bacteria, or fungi, should be explored. Cloning *P. infestans* effectors and testing their toxicity against various microbes could clarify their role in excluding competitors from the plant environment, securing a competitive edge for infection. The role of *Pythium* effectors in mycoparasitism also warrants investigation, as they may play a role in shaping these interactions. This study also took the first steps in identifying components of the innate immune system in oomycetes. Future work exploring the precise functions of NLRs, RLKs and other components identified here in immunity against a broader range of antagonists or competing microbes will help inform research into oomycete survival in complex ecosystems.

Finally, since this pilot study was conducted *in vitro,* future work should validate these findings under diverse field conditions. Combining molecular and ecological insights will improve the reliability of biocontrol strategies and enhance the sustainability of late blight management.

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Popular science summary

Phytophthora infestans, the pathogenic oomycete responsible for potato late blight, causes devastating crop losses worldwide. Despite advancements in disease management, *P. infestans* remains a significant threat due to its ability to adapt to changing environmental conditions and resist chemical treatments. This thesis explores how *P. infestans* infects host plants, adapts its ability to cause diseases in different populations, and interacts with microbial antagonists in its environment.

One major strategy *P. infestans* uses to thrive is by manipulating the stomata of potato – tiny pores on the leaves that regulate water and gas exchange, playing a crucial role in photosynthesis, the process through which plants harness the energy from the sun to obtain their nutrition. By opening these pores, the pathogen creates an exit point for its spores, facilitating further infection. The research in this thesis reveals that *P. infestans* affects key metabolic pathways in the guard cells surrounding stomata, such as lipid breakdown and the scavenging of reactive molecules like hydrogen peroxide, to open stomata and bypass plant defences, allowing the pathogen to cause disease and providing a means for its reproduction and spread.

Populations of *P. infestans* in China have previously been found to be clonal (very similar to one another genetically), although China experiences large climatic differences across the country. This makes it an ideal location to study the genetic diversity of *P. infestans* using a key pathogenicity protein (the effector *Avr2*) to reveal how the pathogen evolves in response to local environmental factors such as temperature and altitude. By analysing genetic variation in this specific protein, and its association with temperature and altitude, the study highlights the importance of regional differences in pathogen behaviour. These findings emphasize the need for tailored resistance strategies in potato crops, ensuring that disease management is not

only effective but also adaptive to local environmental conditions and a changing global climate. This approach will help develop more targeted and sustainable methods for managing late blight.

Additionally, the study explores the potential of natural antagonistic microbes, like certain species of *Pythium*, as biocontrol agents. By examining how *P. infestans* defends itself against these microbes, the research contributes to developing eco-friendly, non-chemical solutions to combat late blight. This work also provides insights into how antagonistic microorganisms influence the behaviour of the late blight pathogen towards them, offering new directions for strategies where alternative pest management is favoured over chemical treatments.

Together, these findings provide valuable insights into the complex relationship between *P. infestans*, its environment, and its interactions with other organisms. By advancing our understanding of the molecular mechanisms used by *P. infestans* in pathogenicity and defence, this thesis lays the foundation for holistic methods to manage late blight and ensure food security for the future.

Populärvetenskaplig sammanfattning

Phytophthora infestans är en sjukdomsaltrande algsvamp som orsakar potatisbladmögel och ger upphov till stora skördeförluster över hela världen. Trots framsteg med att kontrollera denna sjukdom, så kvarstår *P. infestans* som ett allvarligt hot på grund av sin förmåga att snabbt anpassa sig till miljömässiga förändringar och att motstå kemiska bekämpningsmedel. Denna avhandling utforskar hur *P. infestans* infekterar sin värdväxt, anpassar sin förmåga att orsaka sjukdom i olika populationer samt interagerar med potentiella mikrobiella antagonister i sin omgivning.

En viktig strategi som *P. infestans* använder för att infektera potatis framgångsrikt är att manipulera växtens klyvöppningar – de små porerna på bladen som växten använder för att reglera vatten och gasutbyte med omgivningen, vilket är grundläggande för fotosyntesen där växten utnyttjar solens energi för att få näring. Genom att öppna dessa porer kan patogenen skapa en väg ut för sina sporer, som underlättar ytterligare infektion. Resultaten visar att *P. infestans* påverkar viktiga metabola banor hos klyvöppningarnas läppceller, till exempel nedbrytning av fetter och bortrensning av reaktiva molekyler som väteperoxid, för att öppna klyvöppningarna och kringgå växtens försvarsmekanismer. Detta ökar patogenens möjlighet att orsaka sjukdom och ökar därmed dess reproduktion och spridning.

Populationer av *P. infestans* i Kina har tidigare påvisats vara klonala (väldigt lika varandra genetiskt), trots att Kina uppvisar stora skillnader i klimat. Kinesiska populationer är därför ideala för att studera hur genetisk mångfald hos ett viktigt sjukdomsalstrande protein (effektorn AVR2) hos *P. infestans* anpassar sig genetiskt till lokala miljöfaktorer, som temperatur och altitud. Genom att analysera genetisk mångfald av detta specifika protein och dess samband med miljöfaktorerna temperatur och altitud, belyser studien

vikten av regionala skillnader för patogenens beteende. Dessa resultat betonar behovet av skräddasydda resistensstrategier hos potatis, vilket kan säkerställa att sjukdomshanteringen inte bara är effektiv utan också är anpassad till den lokala miljön och globala klimatförändringar. Ett sådant angreppssätt kan hjälpa oss att utveckla mer målinriktade och hållbara växtskyddsstrategier för att hantera potatisbladmögel

Avhandlingen studerar även potentialen hos naturliga antagonistiska mikrober, som vissa arter av *Pythium*, som biologiska kontrollorganismer. Ökad förståelse för hur *P. infestans* försvarar sig mot angrepp av dessa mikrober kan bidra till utveckling av miljövänliga, icke-kemiska kontrollmetoder av potatisbladmögel. Detta arbete ger också insikter i hur antagonistiska mikroorganismer kan påverka beteendet av en potatisbladmögelpatogen, vilket kan ge upphov till nya växtskyddsstrategier som alternativ till kemisk bekämpning.

Sammantaget bidrar dessa resultat till värdefulla insikter om de komplexa sambanden mellan *P. infestans*, dess miljö och interaktioner med andra organismer. Genom ökad kunskap om de molekylära mekanismer som *P. infestans* använder för att infektera sin värd och för att försvara sig, lägger denna avhandling grunden för mer holistiska metoder för att kontrollera potatisbladmögel och säkerställa vår framtida livsmedelssäkerhet.

பொது அறிவியல் சுருக்கம்

உலகெங்கிலும் உருளைகிழங்கு பயிரின் விளைச்சல் பாதிப்பிற்கு இப்பயிரைத் தாக்கும் லேட் ப்ளைட் எனும் நோயே முக்கியமான காரணமாகும். இந்த நோய் *பைட்டோப்தோரா இன்பெஸ்டன்ஸ்* (*Phytophthora infestans*) என்ற நோய் உண்டு பண்ணுகிற ஊமைசீட்டினால் (Oomycete) ஏற்படுகிறது. நோய் மேலாண்மை வழிமுறைகள் மிகவும் முன்னேற்றம் அடைந்துள்ளபோதிலும் *பைட்டோப்தோரா இன்பெஸ்டன்ஸ்* மிகவும் பயங்கரமானதாகவே விளங்குகிறது. ஏனென்றால் இது சுற்றுச்சூழல் மாறுபாடுகளுக்கு ஏற்ப தாக்குப் பிடிக்க வல்லது மற்றும் இரசாயன மருந்துகளுக்கு எதிர்ப்பு சக்தியினை பெற்றுவிடுகின்றது. *பைட்டோப்தோரா இன்பெஸ்டன்ஸ்* எவ்வாறு தாவரங்களில் நோய் உண்டாக்குகிறது, தான் இருக்கும் வெவ்வேறு இடங்களில் எப்படி அதன் நோய் ஏற்படுத்தும் திறனை மாற்றியமைக்கிறது மற்றும் அதன் சுற்றுச்சூழலில் இருக்கும் எதிரிடையான நுண்ணுயிரிகளுடன் எவ்வாறு வினையாற்றுகின்றது என்பதையும் இந்த ஆய்வறிக்கை ஆராய்கின்றது.

பைட்டோப்தோரா இன்பெஸ்டன்ஸ் செழித்து வளர உருளைக்கிழங்கு பயிரின் இலைகளில் உள்ள நுண் துளைகளை (Stomata) கையாள்வதை ஒரு முக்கியமான உத்தியாக கொண்டுள்ளது. இந்த நுண் துளைகள் நீர் மற்றும் வாயு பரிமாற்றத்தை கட்டுப்படுத்திடவும் ஒளிச்சேர்க்கையின் மூலம் தாவரங்கள் சூரியனிலிருந்து ஆற்றலைப் பயன்படுத்தி தங்கள் ஊட்டச்சத்தைப்

பெறவும் பயன்படுகின்றன. நுண் துளைகளைத் திறந்து தனது வித்துக்களை (Spores) வெளியேற்றி மேன்மேலும் நோய் பரவுவதற்கு வழி வகுக்கின்றது. *பைட்டோப்தோரா இன்பெஸ்டன்ஸ்* இலைகளின் நுண் துளைகளைச் சுற்றியுள்ள பாதுகாப்பு செல்களில் லிபிட் (Lipid) என்ற கொழுப்பின் சிதைவு மற்றும் ஹைட்ரஜன் பெராக்சைடு (Hydrogen peroxide) போன்ற எதிர்வினை மூலக்கூறுகளைக் கைப்பற்றுதல் போன்ற முக்கிய வளர்சிதை மாற்றப் பாதைகளைப் பாதித்து இலைகளின் நுண் துளைகளைத் திறப்பதன் மூலம் உருளைக்கிழங்கு பயிரின் பாதுகாப்பு செயல்முறைகளை தவிர்க்கின்றது என்பதையும் அதன் காரணமாக நோய் உண்டாக்குவதோடு பலுகிப் பெறுகுகிறது என்பதையும் இந்த ஆராய்ச்சி வெளிப்படுத்துகிறது.

சீன தேசத்தின் வெவ்வேறு பிராந்தியங்களில் கால நிலை மாறுபாடுகள் அதிக அளவில் இருப்பினும் *பைட்டோப்தோரா இன்பெஸ்டன்ஸில்* முன்னதாக மரபியல் ஒற்றுமைகள் காணப்பட்டது. இதனால் *பைட்டோப்தோரா இன்பெஸ்டன்ஸின்* மரபியல் பன்முகத்தன்மையினை ஆராய்ச்சி செய்திட இந்த நாடு மிக பொருத்தமானதாகும். *Avr2* என்ற ஒரு முக்கியமான நோய்க்கிருமிதன்மை புரதத்தை பயன்படுத்தி இந்த நோய்கிருமி வெவ்வேறு பிராந்தியங்களில் நிலவும் மாறுபட்ட தட்ப வெப்ப நிலை, கடல் மட்டத்திற்கு மேல் எவ்வளவு உயரத்தில் ஒரு பிராந்தியம் அமைந்துள்ளது முதலிய காரணிகளுக்கு தக்கவாறு எவ்வாறு பரிணாம வளர்ச்சி அடைகிறது என கண்டறியலாம். இந்த புரதத்தில் காணப்படும் மரபியல் மாறுபாடுகளையும் தட்ப வெப்ப நிலை, கடல் மட்டத்திற்கு மேல் எவ்வளவு உயரத்தில் ஒரு பிராந்தியம் அமைந்துள்ளது முதலியவைகளுக்கான தொடர்புகளையும் ஆராய்வதன் மூலம் இந்த ஆராய்ச்சி வாயிலாக வெவ்வேறு பிராந்தியங்களில் இந்த நோய்க்கிருமியின் செயல்பாடுகளில் காணப்படும் வேறுபாடுகள் சுட்டிக்காட்டப்பட்டுள்ளது. இந்த கண்டுபிடிப்புகள் உருளைக்கிழங்கு பயிரில் லேட் ப்ளைட் நோய்க்கு மிகவும்

பொருத்தமான எதிர்ப்பு உத்திகள் தேவை என்று சுட்டிக்காட்டுகின்றன. ஏனெனில் மாறிவரும் சுற்றுச்சூழலுக்கு தகுந்தவாறு நோய் மேலாண்மை பயனளிப்பதாக மட்டுமல்லாது அதுவும் அந்த மாற்றத்திற்கு ஏற்பவும் மேலும் உலகளாவிய பருவநிலை மாற்றங்களுக்கு ஏற்றவாறும் மாற்றி அமைக்கப்படவேண்டும் என்பதின் அவசியத்தை வலியுறுத்துகிறது. இத்தகைய செயல்பாடு லேட் ப்ளைட் நோய் மேலாண்மைக்கான இலக்கு நோக்கிய நிலையான வழிமுறைகளை கண்டறிய உதவும்.

கூடுதலாக, இந்த ஆராய்ச்சி *பித்தியம்* (*Pythium*) போன்ற இயற்கை நுண்எதிரிகளின் பயன்பாடுகளையும் ஆராய்கிறது. ஆராய்ச்சிகளின் முடிவுகளில் *பைட்டோப்தோரா இன்பெஸ்டன்ஸ்* எவ்வாறு *பித்தியம்* போன்ற நுண்எதிரிகளிடமிருந்து தற்காத்துக்கொள்கிறது என்பதைக் கண்டறிந்து அதன்மூலமாக சுற்றுப்புறச் சூழலுக்கு பாதிப்பில்லாத இயற்கையோடு இணக்கமான லேட் ப்ளைட் நோய்க்கான தீர்வை கண்டறிய உதவுகின்றது. இந்த ஆராய்ச்சி லேட் ப்ளைட்டின் நோய்க்கிருமியை எதிரிடையான நுண்ணுயிரிகள் எவ்வாறு பாதிக்கின்றன என்பது குறித்த நுண்ணறிவை வழங்குகிறது. அதன் வழியாக பூச்சி மேலாண்மை உத்திகளில் வேதியியல் சிகிச்சை முறைகளுக்கு மாற்றாக புதிய வழிமுறைகளை காண்பிக்கிறது.

ஒன்றாக பார்க்கும்போது, இந்த கண்டுபிடிப்புகள் *பைட்டோப்தோரா இன்பெஸ்டன்ஸ்* அதன் சுற்றுச்சூழல் மற்றும் பிற உயிரினங்களுடனான அதன் தொடர்புகளுக்கு இடையிலான சிக்கலான உறவு பற்றிய மதிப்புமிக்க நுண்ணறிவுகளை வழங்குகின்றன. இந்த ஊமைசீட்டின் நோய்க்கிருமித்தன்மை மற்றும் பாதுகாப்பு ஆகியவற்றின் மூலக்கூறு செயல்பாடுகள் பற்றிய நமது புரிதலை மேம்படுத்துவதன் மூலம் இந்த ஆய்வறிக்கை லேட் ப்ளைட் நோய் மேலாண்மைக்கும் எதிர்காலத்திற்கான உணவுப்பாதுகாப்பை உறுதி செய்வதற்கும் முழுமையான முறைகளுக்கு அடித்தளம் அமைக்கிறது.

Acknowledgements

My PhD journey has been shaped by the support of incredible people. From intellectual guidance to personal encouragement, each individual made this experience not just a professional achievement but a rewarding life experience.

To my supervisor Laura, thank you for your patience, guidance, and steadfast support. Your encouragement to push boundaries and embrace new challenges has been instrumental in my growth, both academically and personally. It has been a true privilege to work under your supervision.

To my co-supervisor Åsa, your curiosity, enthusiasm, and infectious smile have been a constant source of inspiration. Your guidance extended beyond my PhD and made this journey truly enjoyable.

To my co-supervisor Jiasui and co-authors Li-Na and Yan-Ping, your patience and willingness to share your expertise have helped me navigate unfamiliar territory. I deeply appreciate your support.

To Maja, Ramesh, Sajeevan, and Pruthvi, your friendship and guidance made a huge difference. Thank you for making this journey so much easier and more pleasant.

To Mahboobeh and Poorva, thank you for being a family away from home in Alnarp during COVID-19. Our bond will always be treasured.

To Rickard, Isabel, and Johanna, your kindness and understanding during emotionally challenging times have meant the world to me.

To the SLU PROTECTA gang, our travels, fun moments, and shared pursuit of scientific success have been an unforgettable part of this journey. Thank you Valentina and Noelia for helping me see ice hockey.

To Murilo, thank you for making my transition to Sweden so smooth and comfortable. Your support was invaluable.

To Christian, your friendship and love for science, fish, and bonsai made a lasting impact on me. I am grateful for your presence during both good and tough times.

Thank you Amma and Appa for everything, from helping me find this PhD position to offering unwavering moral support. Your belief in me has been a constant source of strength.

To sweet Nova, your arrival was a gift at the perfect time. Your presence brought calm and joy, teaching me to smile even through the toughest times.

A heartfelt thank you to the Department of Plant Protection Biology for providing a supportive and friendly environment with amazing facilities. It has been a great place to learn and grow.

Lastly, I thank God for bringing all of you amazing people into my life through this journey. எல்லா புகழும் இறைவனுக்கே!

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frontiers in Plant Science

ORIGINAL RESEARCH
published: 12 July 2021
doi: 10.3389/fpls.2021.668797

Pathogen-Mediated Stomatal Opening: A Previously Overlooked Pathogenicity Strategy in the Oomycete Pathogen *Phytophthora infestans*

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Specialty section:
This article was submitted to Plant Pathogen Interactions, a section of the journal Frontiers in Plant Science

Received: *17 February 2021*
Accepted: *18 June 2021*
Published: *12 July 2021*

Citation:
Yang L-N, Liu H, Wang Y-P, Seematti J, Grenville-Briggs LJ, Wang Z and Zhan J (2021) Pathogen-Mediated Stomatal Opening: A Previously Overlooked Pathogenicity Strategy in the Oomycete Pathogen Phytophthora infestans. Front. Plant Sci. 12:668797. doi: 10.3389/fpls.2021.668797

Phytophthora infestans, the most damaging oomycete pathogen of potato, is specialized to grow sporangiophore through opened stomata for secondary inoculum production. However, it is still unclear which metabolic pathways in potato are manipulated by *P. infestans* in the guard cell–pathogen interactions to open the stomata. Here microscopic observations and cell biology were used to investigate antagonistic interactions between guard cells and the oomycete pathogen. We observed that the antagonistic interactions started at the very beginning of infection. Stomatal movement is an important part of the immune response of potato to *P. infestans* infection and this occurs through guard cell death and stomatal closure. We observed that *P. infestans* appeared to manipulate metabolic processes in guard cells, such as triacylglycerol (TAG) breakdown, starch degradation, H_2O_2 scavenging, and NO catabolism, which are involved in stomatal movement, to evade these stomatal defense responses. The signal transduction pathway of *P. infestans*-induced stomatal opening likely starts from H_2O_2 and NO scavenging, along with TAG breakdown while the subsequent starch degradation reinforces the opening process by strengthening guard cell turgor and opening the stomata to their maximum aperture. These results suggest that stomata are a barrier stopping *P. infestans* from completing its life cycle, but this host defense system can be bypassed through the manipulation of diverse metabolic pathways that may be induced by *P. infestans* effector proteins.

Keywords: stomatal immunity, starch degradation, triacylglycerol breakdown, phytophthora infestans, potato defences

INTRODUCTION

Stomata, bordered by a pair of guard cells, play several essential roles in many biological and biochemical processes of terrestrial plants. The size of a stomatal aperture is dynamically regulated by the integration of environmental signals and endogenous hormonal stimuli. Under light stimulation and/or high humidity, stomata open to promote carbon dioxide and oxygen flow for photosynthesis and water evaporation (Berry et al., 2010; Buckley, 2019) and *vice versa*. Biotic

stressors, such as pathogens, also regulate the stomatal aperture of plants. It has long been noticed that many prokaryotic plant pathogens use stomata as a gate to penetrate the inner tissue of plants. Some pathogens, for example, the bacterium *Xanthomonas campestris* pv *armoraciae* (Hugouvieux et al., 1998), fungi from the *Puccinia* Genus (Shafiei et al., 2007), and the oomycete *Plasmopara viticola* (Allègre et al., 2007) are specialized to penetrate and colonize plant tissues only through stomatal pores. For other pathogens, such as the oomycete *P. infestans*, stomata are not essential for invasion and colonization but are required for sporulation (Farrell et al., 1969). To prevent pathogen ingress and reproduction, plants have evolved mechanisms to close stomata upon a perception of pathogens, but adapted pathogens can trigger stomatal reopening to overcome this layer of defense by releasing pathogenicity compounds, such as phytotoxins or effector proteins (Melotto et al., 2017; Ye et al., 2020).

Many compounds, including starch, lipids, and oxidative radicals such as hydrogen peroxide (H_2O_2), are involved in signal transduction to control stomatal movement (Shimazaki et al., 2007; Horrer et al., 2016; McLachlan et al., 2016) and the same compounds may also be manipulated by pathogens to overcome stomata-mediated defenses. Starch, synthesized in plastids in both photosynthetic and non-photosynthetic cells, is the principal carbohydrate storage of higher plants (Zeeman et al., 2010). In guard cells, starch degradation provides organic acids and sugars to increase guard cell turgor pressure and promote the stomatal opening. For example, glucose derived from starch degradation was found to be responsible for rapid stomatal opening in Arabidopsis after exposure to blue light (Flütsch et al., 2020). Furthermore, malate is recognized unequivocally as the predominant donor of the organic anions needed to balance the positive charge of K^+ ions during stomatal opening (Santelia and Lawson, 2016).

Triacylglycerol (TAG), a dominant lipid compound for energy storage present as lipid droplets (LDs) in guard cells, is also involved in the stomatal opening that is stimulated by light illumination (McLachlan et al., 2016). The abundance of TAG in the guard cells is significantly reduced in response to light, and PHOT blue light receptors are involved in this response (McLachlan et al., 2016). Stomatal movement is also an energy-demanding process. The abundant TAGs in guard cells ensure the generation of adequate ATP and activation of the proton pumps required for stomatal opening, such as plasma membrane H^+-ATPases (McLachlan et al., 2016).

The reactive oxygen species hydrogen peroxide (H_2O_2) and the reactive nitrogen species nitric oxide (NO) have a wide range of effects on the developmental processes and stress responses of plants, including seed germination, root development, drought resistance, and defense against pathogens (Liao et al., 2012; He et al., 2013; Smirnoff and Arnaud, 2019). The concurring dynamics of H_2O_2 and NO in most plant organs suggest that they are likely metabolized in parallel and act in tandem (Tanou et al., 2009; Clark et al., 2010), although some results show that NO may function downstream of H_2O_2 (Zhang et al., 2017). In guard cells, H_2O_2 and NO are key regulators that work synergistically or independently in regulating stomatal movement. In the last few years, the roles and mechanisms of ABA-induced stomatal closure modulated by H_2O_2 and NO have been exploited widely (Jannat et al., 2011; Rodrigues et al., 2017). Additionally, H_2O_2 and NO are also involved in darkness-induced stomatal closure (Desikan et al., 2004; Zhang et al., 2017). H_2O_2 and NO concentrations in guard cells increase in darkness but decrease in light.

Phytophthora infestans, the causal agent of potato (*Solanum tuberosum* L.) late blight disease, which was responsible for the Irish potato famine in the 1840s, is to date one of the most devastating plant pathogens known to man (Fry, 2008). *P. infestans* continues to be a major yield-limiting factor in potato production. However, it is also a model species to study the biology, genetics, and evolution of host–pathogen interactions in the oomycetes (Wang et al., 2017; Yang et al., 2019). Despite the existence of sexual reproduction, *P. infestans* still reproduces primarily in an asexual manner by forming sporangia (Zhu et al., 2015). Sporogenesis is an important part of the asexual cycle and massive numbers of sporangia (up to 300,000 per lesion) can be produced rapidly and dispersed across whole fields within days (Fry, 2008). In this process, potato stomata acts as the physical barrier that *P. infestans* must break through, to allow sporangia to be released from the plant, and *P. infestans* sporangiophores are specialized to grow out through stomatal apertures (Farrell et al., 1969).

Recently, a non-specific lipid transfer protein, *StLTP10*, was found to regulate stomatal closure in potato after *P. infestans* infection by physical interaction with the ABA receptor PYL4, indicating that stomatal immunity is important in potato defense against *P. infestans* (Wang et al., 2020). In the current study, cellular interactions between the potato guard cells and *P. infestans* were explored. We found that the antagonistic interactions between the potato guard cells and *P. infestans* started at the very beginning of infection. The non–race-specific stomatal closure caused by guard cell death was found in more than 10 potato cultivars varying in genetic background and quantitative resistance, indicating that guard cell suicide is deployed as a common immune response of potato against *P. infestans* infection. However, we further showed that this immune response is suppressed by the pathogen through the regulation of starch, TAG, H_2O_2, and NO metabolism. We hypothesized that these biochemical processes may be induced by pathogen-effector proteins. The signal transduction pathway of the pathogen-induced stomatal opening may start from H_2O_2 and NO scavenging and TAG breakdown, proceed through starch degradation, and end up with a stomatal aperture maximized for the growth of sporangiophores upward through the stomata, for aerial release of sporangia.

MATERIALS AND METHODS

Growth and Maintenance of Potato and *Phytophthora infestans*

Potato cv Desiree plants were grown at 19°C and 60% humidity in a greenhouse supplemented with 16 light-hours at the intensity

of 120–150 μmol/m^2/s. Leaves used for experiments were taken from 5- to 6-week-old plants. *P. infestans* isolate A21b collected from Fuqing, Fujian Province, in 2016, with high sporangia yield and isolate 88069 (A1 mating type, race 1.3.4.7) retrieved from long-term storage, were grown on Rye B plates at 18°C in dark. After two weeks, the plates were flooded with 5 ml of sterilized water and scraped with a plastic rod to make a sporangial suspension. The suspension was calibrated to ∼80,000 sporangia/ml using a hemocytometer and was sprayed evenly on the abaxial side of potato leaves to ensure sufficient and uniform infection. After the inoculation, the potato leaves were kept in sealed boxes to maintain moisture and they were placed at 18°C in dark. The inoculation was replicated at least three times for each treatment.

Apoplastic Fluid Collection and Infiltration

Apoplastic fluids (AFs) were collected from potato leaves infected with *P. infestans* A21b when disease symptoms were visible but the detached leaves were still green, which usually occurred around 20 h post inoculation (hpi). After removal of sporangia and mycelia, the infected leaves were immersed in distilled water in a beaker and then infiltrated by placing them into a vacuum desiccator for 5 min. Excess water droplets on leaf surfaces were removed with tissue paper. The infiltrated potato leaves were rolled up and inserted into 30 ml tubes with small holes at the bottom. Each tube was then slipped into a larger, 50 ml tube and centrifuged at 1,000 × *g* for 10 min at 10°C. The harvested AF from the larger tubes was filtered through 0.22 μm Millex sterile filters and used to infiltrate potato leaves. Three leaves per potato plant were infiltrated with the AF and three plants were included for each treatment, bringing nine leaves in total for each treatment. AF collected from uninfected healthy potato leaves was used as a control. Diphenyl methylphosphonate (DMP), when used, was co-infiltrated with AFs at a concentration of 25 μM (from a 25 mM stock in DMSO).

Stomatal Aperture Measurements

Unless stated otherwise, all experiments were conducted in dark and the potato leaves used in the study were kept in the dark at 18°C for 2 h before use to ensure stomata closure. During the infection time course, the detached leaves with the abaxial side up were attached to glass slides using double-sided adhesive tape and photographed at 20X magnification using a microscope (NIKON Ni-U). Epidermal strips were manually peeled off from the infection sites at 12 and 24 hpi, and incubated in KCl/MES buffer (10 mM MES, 5 mM KCl, and 50 μM $CaCl_2$) with ABA, $CaCl_2$, H_2O_2, Sodium Nitroprusside (SNP), and Na_3VO_4 at 18°C in dark. After 2 h of incubation, the epidermis was photographed using a microscope (NIKON Ni-U) at 20X magnification. Stomatal apertures were measured using the software ImageJ 1.50. All the experiments were repeated in at least three independent treatments, and three leaves from different plants were used per treatment.

Starch Quantification in Guard Cells

Starch in guard cells was quantified by the pseudo-Schiff propidium iodide (PS–PI) staining protocol as described previously (Horrer et al., 2016) with some minor modifications. Briefly, the epidermis was manually peeled off the detached potato leaves that had either been infected with *P. infestans*, infiltrated with AF, or treated with light incubation and fixed in 50% (v/v) methanol, 10% (v/v) acetic acid at 4°C overnight. The epidermal peels were rinsed briefly with sterilized water and incubated in 1% periodic acid at room temperature for 40 min. They were rinsed again with sterilized water and stained with Schiff reagent (100 mM sodium metabisulfite and 0.15 N HCl) and propidium iodide [0.1 mg/ml (w/v) final concentration] for 1–2 h. The stained epidermal peels were affixed to microscope slides and submerged in chloral hydrate solution overnight. Excess chloral hydrate on the epidermal peels was removed from the microscope slides and the epidermal peels were fixed with Hoyer's solution. The images of fluorescent activity in the guard cells were taken using a NiKON (Ni–U) fluorescence microscope with an excitation wavelength of 540 nm and an emission wavelength of 605 nm. The starch granule area was quantified by measuring fluorescent areas in the guard cells with ImageJ 1.50. This experiment was repeated using three independent treatments, and three leaves from different plants were used in each treatment.

Lipid Droplet Quantification in Guard Cells

After *P. infestans* infection, AF infiltration or light treatment, the leaf epidermis was manually peeled from the detached leaves, at the specified time points described above, and incubated in 30 μM Nile Red (NR; McLachlan et al., 2016) for 40 min and washed in KCl/MES buffer (10 mM MES, 5 mM KCl, and 50 μM $CaCl_2$) for 5 min. Images of NR fluorescence activity in the guard cells were taken using a NiKON (Ni–U) fluorescence microscope with excitation wavelengths of 465–495 nm and emission wavelengths of 512–558 nm and the LD volume was quantified using ImageJ 1.50. This experiment was repeated using three independent treatments, and with three leaves from different plants in each treatment.

H_2O_2 and NO Accumulation Measurement

After *P. infestans* inoculation, the epidermis was manually peeled off from the detached leaves at each specified time point and incubated for 20 min in KCl/MES buffer with 50 μM H_2DCF DA for H_2O_2 measurements or in KCL/MES buffer with 10 μM DAF-FM DA for NO measurements and then washed with KCl/MES buffer twice. The images of H_2O_2 and NO fluorescent activity in the guard cells were taken using a Nikon fluorescence microscope (Ni–U) with excitation wavelengths of 465–495 nm and emission wavelengths of 512–558. H_2O_2 and NO concentrations were quantified by measuring their fluorescence density in the guard cells using ImageJ 1.50. Again, this experiment was repeated using three independent treatments, and with three leaves in each treatment.

The accumulation of H_2O_2 in mesophyll cells was measured by histochemical analysis *via* 3,3′-diaminobenzidine (DAB) staining. Potato leaves which were inoculated with or without (control, CK) *P. infestans* sporangia of isolate A21b were incubated in DAB solution (1 mg/ml, pH 3.8) for 16 h at 25°C in the dark, then soaked in 95% ethanol overnight to remove chlorophyll (Thordal-Christensen et al., 1997). Photos were taken using a digital camera.

RNA Extraction

RNA was extracted as described previously (Resjö et al., 2017). Briefly, the total RNA was extracted from frozen samples ground in liquid nitrogen using a Qiagen RNeasy Plant Mini kit following the protocol set by the manufacturer. Samples were derived from a time-course of potato leaves (cultivar Desiree) inoculated with *P. infestans* strain 88069, or from pre-infection structures collected *in vitro* as described (Grenville-Briggs et al., 2008). Before cDNA synthesis all samples were DNase treated using the Ambion Turbo DNA-free kit, according to the protocol set by the manufacturer. RNA samples were assessed for purity and integrity by agarose gel electrophoresis and Nanodrop Spectrophotometry. First strand cDNA was synthesized from 20 g total RNA by oligo(dT) priming using the Superscript IV Reverse transcriptase cDNA synthesis kit (Thermo Scientific).

Quantitative RT-PCR Assays

The primer pairs that annealed specifically to each of the candidate effectors PITG_11755 (Meijer et al., 2014) and PITG_15152 (de Vries et al., 2017) were used to quantify gene expression *in vitro* and *in planta* as described previously (Resjö et al., 2017). A template cDNA for *in planta* analysis over an infection time course was derived from mycelium grown for 72 h in liquid pea broth as well as from potato leaves inoculated with *P. infestans*. Samples were taken at 6, 12, 24, and 48 hpi. Pre-infection samples of non-sporulating mycelium, sporangia, zoospores, germinating cysts, and germinating cysts with appressoria were collected as described by Grenville-Briggs et al. (2008). The actA gene from *P. infestans* was used as constitutively expressed endogenous control and the abundance of each transcript in mycelium was determined relative to the actA transcript as described previously (Grenville-Briggs et al., 2008). All qRT-PCR assays were performed using three biological replicates. The results from each assay were analyzed using the modified $\Delta\Delta$cT method, and relative expression was determined relative to a calibrator sample (mycelium) as described previously (Resjö et al., 2017).

Plasmid Construction and Transient *in planta* Expression

The full-length sequences of PITG_11755 and PITG_15152 without their signal peptides were cloned from the gDNA of *P. infestans*, ligated into pEarlyGate 101 (C-terminal GFP tag) and pEarlyGate 104 (N-terminal GFP tag), respectively, using Vazyme ClonExpressII One Step Cloning Kit, and then transformed into *Agrobacterium tumefaciens* strain AGL1. Overnight, *A. tumefaciens* cultures were harvested by centrifugation and resuspended in infiltration buffer (10 mM MES, 10 mM $MgCl_2$, and 200 mM acetosyringone). The resuspended *A. tumefaciens* cells with an optical density (OD_{600}) of 0.5 were infiltrated into leaves of 5- to 6-week-old potato plants. Stomatal apertures were measured 3–5 days post infiltration.

Statistical Analyses

Analysis of variance (ANOVA) for stomatal aperture and concentrations of starch, lipids, H_2O_2, and NO in the guard cells were performed using the general linear model embedded in SAS 9.4, and significant differences between treatments in these parameters were evaluated using a Duncan test. Standard deviation was estimated separately for each parameter using the data generated from different replicates and is shown as error bars in the displayed charts.

RESULTS

Starch Degradation and Triacylglycerol Breakdown Are Associated With Light-Induced Stomatal Opening in *Solanum tuberosum*

It has been documented that LDs (Sakaki et al., 1995) and starch (Pallas, 1964) are present widely in both higher and lower plants, and their catabolism is associated with light-induced stomatal opening (Horrer et al., 2016; McLachlan et al., 2016). To verify these reports in potato (*Solanum tuberosum*), we measured the LD and starch contents in cv Desiree leaves under both dark and light conditions. We found that large amounts of starch and LDs are present in potato guard cells (**Figure 1A**), and that the contents of the two compounds were significantly decreased under light conditions (**Figure 1B**) when stomatal apertures increased (**Figure 1D**). The H_2O_2 and NO contents of the guard cells were also reduced under the same conditions (**Figure 1C**). Artificial supplements of H_2O_2, NO, ABA, $CaCl_2$, and the H^+-ATPase inhibitor Na_3VO_4, all significantly impaired the light-induced stomatal opening (**Figure 1E**). These results indicate that starch, TAG, H_2O_2, and NO are involved in stomatal opening and closing in potato.

Stomatal Defense Is Inhibited by *Phytophthora infestans*

Many pathogens, particularly prokaryotic microbes, such as bacteria, rely on plant stomata for penetration and infection initiation (Melotto et al., 2006). To prevent the attack, guard cells can perceive bacteria and trigger stomatal closure (Melotto et al., 2017), and even specifically commit suicide, for example, in response to rust fungal invasion (Ye et al., 2020). The appressorium and invading hypha of *P. infestans* can be formed 8–12 hpi (**Supplementary Figure 1**). The life cycle of the pathogen can be completed within 5 days on potato foliage (**Supplementary Figure 2**) and the whole field can be transformed from slightly diseased to nearly completely

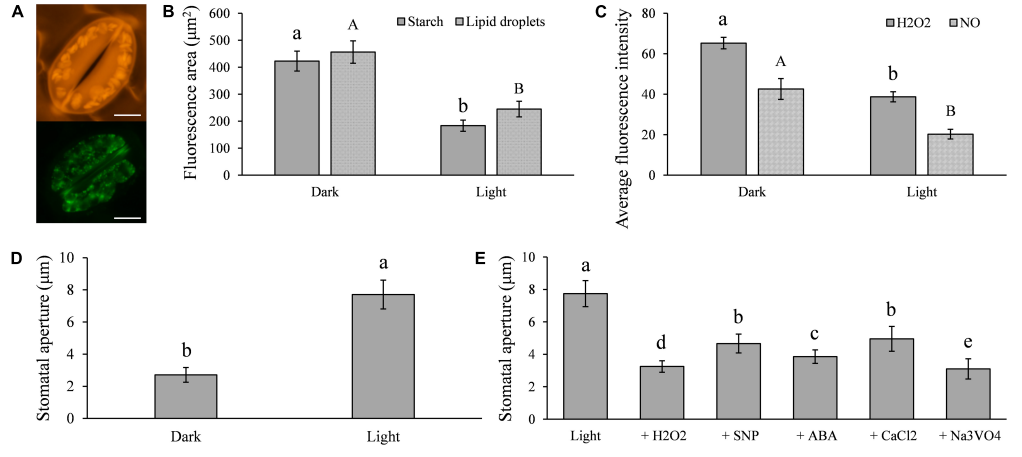

FIGURE 1 | Light-induced stomatal opening in potato is associated with the metabolisms of triacylglycerols (TAGs), starches, H_2O_2, NO, ABA, $CaCl_2$, and plasma membrane H^+-ATPase in guard cells: **(A)** images showing potato guard cells containing a large amount of starch (up) and TAGs (below); **(B)** compared with the results seen in dark conditions, the quantities of starch and TAGs in guard cells were significantly less in light (100 μmol/m^2/s^1 for 6 h); **(C)** H_2O_2 and NO contents in guard cells were significantly decreased in light compared with those in dark conditions; **(D)** the size of stomatal aperture significantly increased in light compared with those in dark conditions; and **(E)** light-induced stomatal opening was impaired by the artificial supplement of 2 μM H_2O_2, 1 μM NO, 10 μM ABA, 5 mM $CaCl_2$, and 1 mM Na_3VO_4. The marker = 20 μm. Photos within a panel are from a representative of a single replicate while photos among panels are pooled from multiple replicates.

destroyed within ∼2 weeks under ideal conditions (Fry, 2008). Recently, it was demonstrated that stomatal defense may also play a role in potato immunity to *P. infestans* (Wang et al., 2020). Here we monitored the stomata–*P. infestans* interaction over the infection time course under dark conditions to determine whether *P. infestans* can perturb this process. Interestingly, we found a hypersensitive-like response of ∼50% guard cells at the infection sites, where they turned dark brown, atrophied, and eventually died. This process started usually between 4 and 8 h post sporangial inoculation (hpi) in cv. Desirée, leading to the permanent closure of these stomata (**Figure 2**). No sporangiophores were observed to emerge through the dead stomata. Besides Desiree, the same pattern was found in 18 other potato cultivars with varying resistance levels against *P. infestans* (**Supplementary Figure 3**). Thus, this phenomenon appears to be a general potato response to *P. infestans* infection and not race-cultivar specific.

On the other hand, we found the majority of leaf stomata at the infection sites started to open at 8 hpi of *P. infestans*, reached their maximum aperture at 48 hpi and afterward remained fully open (**Figures 3A,B**). Although initially some guard cell death (∼50%) was observed, no further dead guard cells were found after the pathogen-induced stomatal opening started. Sporangiophores started to emerge from opened stomata at the infection sites around 72 hpi and large amounts of sporangia were observed after 4–5 days post inoculation in cv. Desirée leaves (**Figure 3C** and **Supplementary Figure 4**). The infection-induced stomatal opening was also found in 18 other potato cultivars (**Supplementary Figure 3**). These results suggest that potato plants can sense and defend against *P. infestans* infection by stomatal closure, while *P. infestans* can suppress these defenses in potato as found in other plant–pathogen interactions.

Phytophthora infestans Infection Induces Potato Stomatal Opening by Degrading Starch in Guard Cells

As starch degradation is strongly connected to stomatal opening (Horrer et al., 2016) and potato guard cells contain a large amount of starch (**Figure 1A**), we hypothesized that *P. infestans* may manipulate potato stomatal movement by inducing starch degradation in the guard cells. To test this hypothesis, we examined starch dynamics in the guard cells during *P. infestans* infection. Indeed, we found that starch metabolic processes in the guard cells were altered by *P. infestans* infection. Starch in guard cells at the infection sites started to degrade at 12 hpi (**Figure 4**). At 24 and 48 hpi, only remnant starch grains were observed in the guard cells of stomata, indicating that they are almost completely degraded (**Figure 4**).

The metabolism of both glucose and malate has previously been reported during stomatal opening (De Angeli et al., 2013; Santelia and Lawson, 2016). This led us to hypothesize that the significant increase of stomatal aperture 12–24 hpi may result from glucose or malate accumulation. To test this, we treated epidermal strips collected at 12 hpi, that is, the time starch started to degrade, with 2.5 mM glucose and 5 mM malate, respectively, and found that glucose and malate supplements indeed facilitated stomatal opening significantly

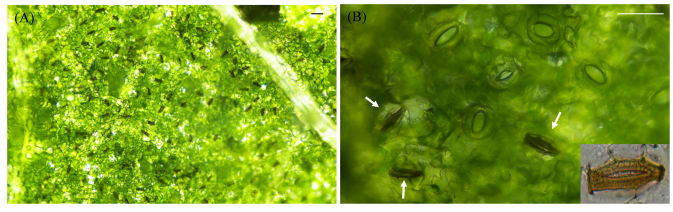

FIGURE 2 | Images showing that potato defenses against *P*hytophthora *infestans* infection at the interface of stomata: **(A)** hypersensitive-like cell death occurred specifically in guard cells 8 h post infection; and **(B)** permanent closure of stomata during the whole infection course after the death of guard cells. The marker = 50 μm. Photos within a panel are from a representative of a single replicate while photos among panels are pooled from multiple replicates.

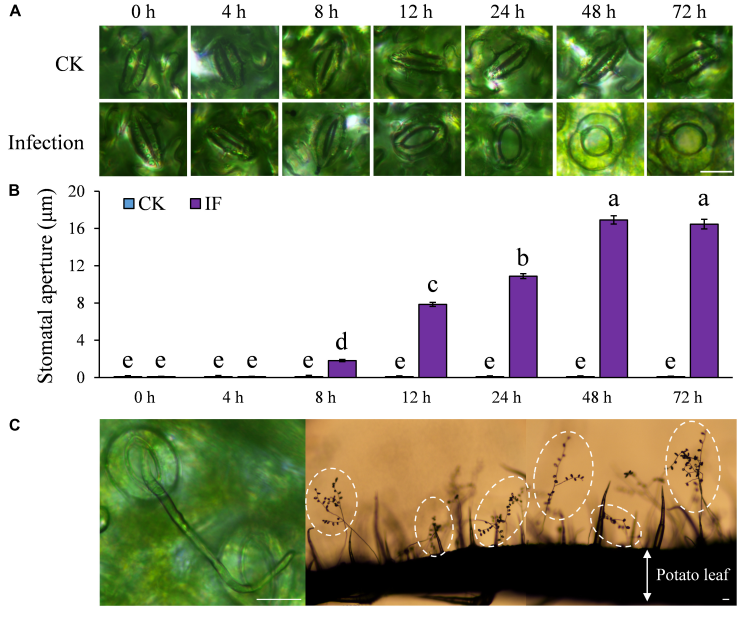

FIGURE 3 | Stomatal aperture was significantly increased after *P. infestans* inoculation under dark condition: **(A)** Images showing stomatal aperture of potato leaves increased after the successful colonization of *P. infestans*; **(B)** stomatal aperture started to increase from 8 h post infection (hpi), and reached the maximum size at 48 hpi; and **(C)** images showing that sporangiophores emerged through opened potato stomata 72 hpi and a large amount of sporangia (the lemon-shaped spots in dashed circles) were formed 4–5 days post inoculation. The marker = 20 μm. Photos within a panel are from a representative of a single replicate while photos among panels are pooled from multiple replicates.

(**Figure 5**), although the stomatal aperture did not reach the same size as that seen at 48 hpi (at the time starch was almost completely degraded). Interestingly, after starch degraded completely and the apertures reached maximum, which occurred at ∼48 hpi, stomata lost responsiveness to the stimulation of ABA, NO precursor SNP, $CaCl_2$, and Na_3VO_4. The epidermal strips supplemented with malate at 12 hpi showed the same phenotype (**Figure 5**). Glucose supplement also weakened the stimulation of ABA, NO precursor SNP, $CaCl_2$, and Na_3VO_4 (**Figure 5**). These results indicate that starch degradation was affected by *P. infestans*, which we hypothesized may induce stomatal opening, possibly aided by soluble sugars, such as glucose and malate.

Phytophthora infestans Infection Induces Potato Stomatal Opening by TAG Breakdown in Guard Cells

One interesting finding is that stomata at the infection sites started to open at 8 hpi but starch degradation in guard cells was

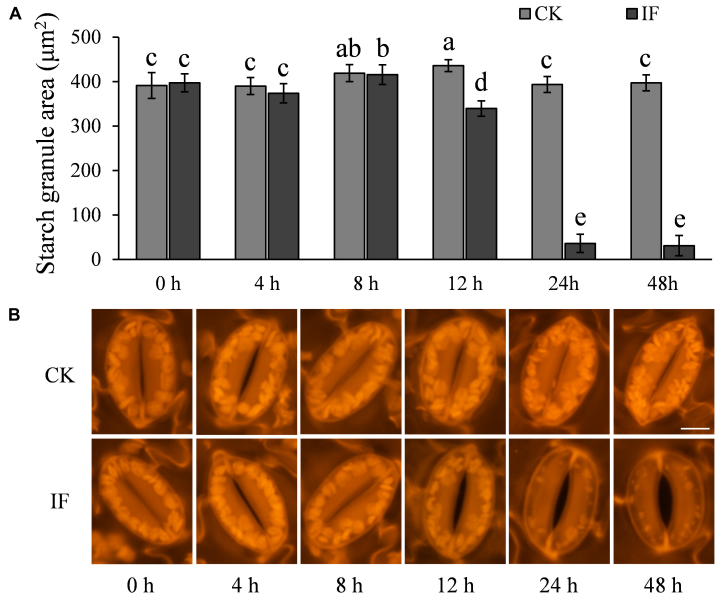

FIGURE 4 | *P. infestans*-induced stomatal opening correlates with starch degradation in guard cells: **(A)** starches in guard cells of *P. infestans*-infected potato leaves started to degrade at 12 hpi in dark, and at 24–48 hpi nearly no starch was visible in the guard cells; **(B)** images show starch in the guard cells of control (CK, no *P. infestans* inoculation) and infected (IF) potato leaves. Starches in the guard cells around the infection sites started to degrade 12 hpi. The marker = 10 μm. Photos within a panel are from a representative of a single replicate while photos among panels are pooled from multiple replicates.

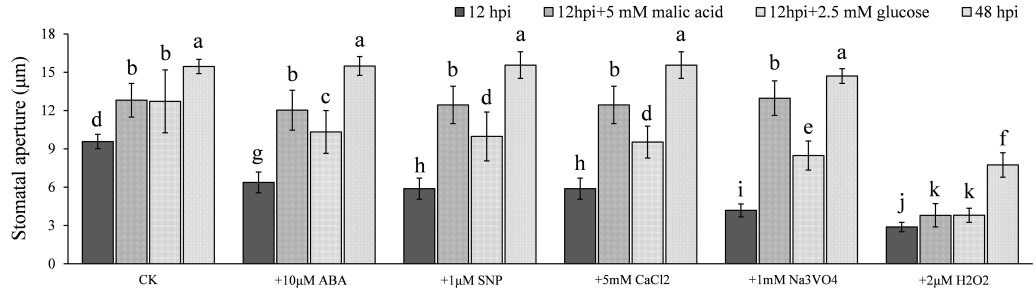

FIGURE 5 | Glucose and malate accumulations were responsible for the full stomatal opening during *P. infestans* infection. Stomatal apertures reached the maximum 48 hpi and failed to the stimulation by ABA, NO, $CaCl_2$, and Na_3VO_4. Glucose and malate supplement to epidermal strips collected 12 hpi showed a similar trend. Photos within a panel are from a representative of a single replicate while photos among panels are pooled from multiple replicates.

not observed until 12 hpi. Therefore, we hypothesized that there must be other metabolic pathways that are also involved in the initiation of the stomatal opening process. It has been shown that TAG in guard cells is the energy source used to activate the proton pump H^+-ATPase involved in light-induced stomatal opening (McLachlan et al., 2016). We thus monitored TAG dynamics in the guard cells of *P. infestans*-infected and control (CK, not challenged by the pathogen) potato leaves over a 48-h period under dark conditions. We observed that TAG breakdown in the infected leaves started to occur at 8 hpi or earlier (**Figure 6**), which was almost coincident with the starting time of stomatal opening (**Figure 3B**). After 24 hpi, TAG was largely absent in the guard cells of the *P. infestans*-infected leaves (**Figure 6**). These results indicate that TAG breakdown in potato guard cells occurs earlier than starch degradation during the response to *P. infestans* infection.

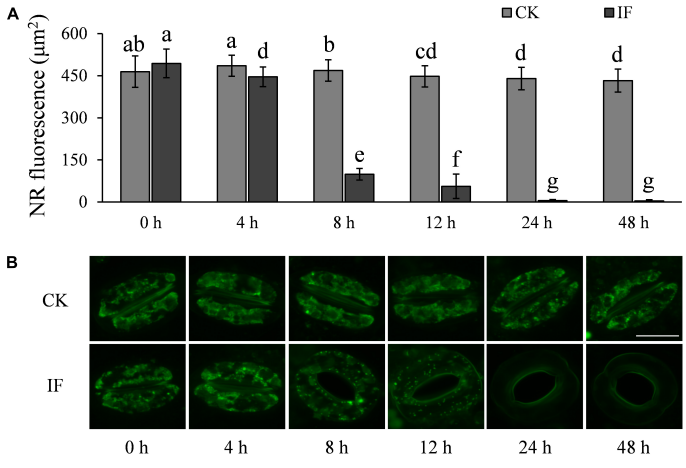

FIGURE 6 | *P. infestans*-induced stomata opening correlates with TAG breakdown in guard cells: **(A)** TAGs in the guard cells of *P. infestans*-infected (IF) potato leaves started to decrease significantly 8 hpi; and **(B)** images showing the volume of TAGs in guard cells of the *P. infestans*-infected (IF) and not-infected (CK) potato leaves. The marker = 20 μm. Photos within a panel are from a representative of a single replicate while photos among panels are pooled from multiple replicates.

H_2O_2 and NO Accumulation in Potato Guard Cells Is Disturbed by *Phytophthora infestans* Infection

H_2O_2 and NO are important signaling molecules involved in stomatal movement, especially in ABA- and dark-induced stomatal activity (Desikan et al., 2004; Jannat et al., 2011; Rodrigues et al., 2017; Zhang et al., 2017). H_2O_2 and NO are accumulated in guard cells in response to ABA enrichment and dark stimulation, but are reduced when stomata open in light (Desikan et al., 2004; She et al., 2004). To investigate whether *P. infestans*-induced stomatal opening is associated with H_2O_2 and NO metabolism, we measured the two compounds in potato guard cells using the fluorescent dyes dichlorodihydrofluorescein diacetate (H_2-DCFDA) and 3-amino, 4-aminomethyl-2′, 7′-difluorescein, diacetate (DAF-FM DA), respectively. In contrast to mesophyll cells (**Supplementary Figure 5**), H_2O_2 and NO were found to be significantly reduced in guard cells at the time stomata started to open, that is, 8 hpi (**Figures 7**, **8**). After 8 hpi, NO levels in the infected guard cells continued to fall but H_2O_2 levels basically flattened out (**Figures 7**, **8**). These results suggest that the metabolism of H_2O_2 in the guard cells is independent from that of the mesophyll cells, and a reduction of both H_2O_2 and NO in the guard cells may participate in the *P. infestans*-induced stomatal opening process in potato plants.

Apoplastic Fluids and Effector Overexpression Induce Stomatal Opening

During infection, *P. infestans* secretes a large number of apoplastic and cytoplasmic effector proteins which are targeted to the cytoplasm or apoplast of host cells (Haas et al., 2009). To check whether infection-derived molecules, such as effector proteins participate in the antagonistic interactions between potato guard cells and *P. infestans*, AFs that contain a mixture of *P. infestans* secreted effectors were collected from diseased plants and infiltrated into healthy potato leaves. We found that the stomatal aperture was significantly increased, and TAG in the potato guard cells was significantly decreased at 15 h after the infiltration (**Figures 9A,C,D**). Because AF extraction is known to often damage some of the plant cells, and plant materials leaking from the cytoplasm may complicate the results, we controlled for this by comparatively infiltrating AFs collected from control (uninoculated) plants. The results showed that stomatal apertures and TAG levels were not disrupted by the control AF infiltrations. When we co-infiltrated the AFs from diseased plants with DMP, the LD mobilization inhibitor that acts early in the β-oxidation pathway, the stomatal opening was significantly inhibited (**Figures 9B,C**). We also found that the content of both H_2O_2 and NO decreased in most of the guard cells infiltrated with AF from disease plants (**Supplementary Figure 6**). However, starch content in the guard cells did not change at 15 h after these AF infiltrations (**Figure 9E**), and only decreased in some of the cells at 24 h after infiltration when leaves withered (**Supplementary Figure 7**). These results indicate that infection AFs contain metabolites or proteins, such as effectors of the pathogen, that either directly or indirectly (e.g., general suppression of immune responses) trigger stomatal opening.

The apoplastic candidate effector PITG_11755 and cytoplasmic candidate effector PITG_15152 were both highly expressed in the pre-infection stages and during infection (**Figures 10A,B**). During a time course of infection, both of these effector genes followed a similar expression pattern, which is

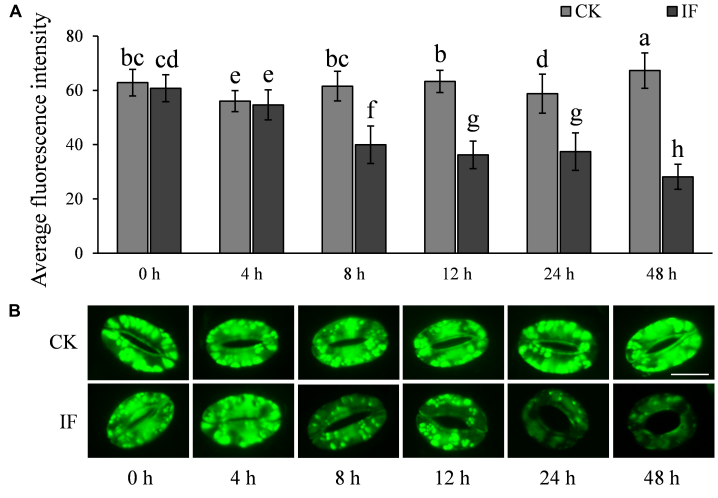

FIGURE 7 | H_2O_2 accumulation changes in guard cells during *P. infestans* infection: **(A)** H_2O_2 dynamics in the *P. infestans*-infected (IF) and non-infected (CK) potato guard cells; and **(B)** images showing the fluorescence intensity of H2-DCFDA in IF and CK potato guard cells. The marker = 20 μm. Photos within a panel are from a representative of a single replicate while photos among panels are pooled from multiple replicates.

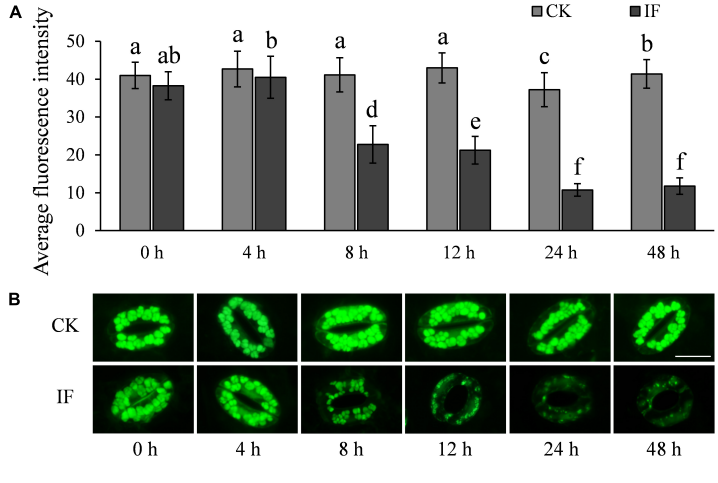

FIGURE 8 | NO accumulation changes in potato guard cells during *P. infestans* infection: **(A)** NO dynamics in the *P. infestans*-infected (IF) and non-infected (CK) potato guard cells; and **(B)** images showing the fluorescence intensity of DAF-FM DA in IF and CK potato guard cells. The marker = 20 μm. Photos within a panel are from a representative of a single replicate while photos among panels are pooled from multiple replicates.

highly elevated early on in infection at 6 hpi, rising to a peak at 12 hpi and reduced at 24 hpi before rising again at 48 hpi (**Figure 10B**). These results suggest that these effectors may have roles both early (at a similar time point to our observations of the onset of stomatal opening) and later on in infection (when sporangiophores are produced and begin to grow out of stomatal openings). To test our hypothesis that effector proteins may be involved in the infection-induced stomatal opening, PITG_11755 and PITG_15152 were non-endogenously overexpressed in potato leaves. We found that both the effector proteins significantly increased stomatal opening at 3 days after infiltration (**Figures 10C,D**), supporting our hypothesis that

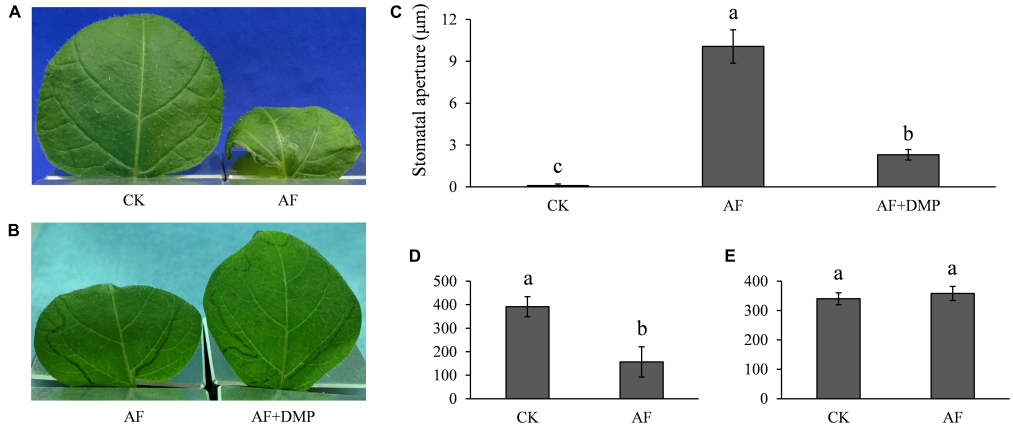

FIGURE 9 | Apoplastic fluids (AFs) induced stomatal opening and TAG breakdown: **(A)** accelerated water evaporation in AF infiltrated potato leaves; **(B)** accelerated water evaporation by AF infiltration was reversed by DMP; **(C)** stomata was opened by AF treatment and the aperture size was compromised by DMP; **(D)** reduced TAGs in potato guard cells by AF treatment; and **(E)** AF had no effect on starch content at 15 h post inoculation in potato guard cells. Photos within a panel are from a representative of a single replicate while photos among panels are pooled from multiple replicates.

effectors of both apoplastic and cytoplasmic origin may have a role, directly or indirectly, in regulating the stomatal opening of potato after *P. infestans* infection.

DISCUSSION

Stomata are the battle field of molecular and physical interactions between plants and pathogens. On the one hand, many plant pathogens, in particular bacterial pathogens, rely on plant stomata as the natural gate of invasion and colonization (Melotto et al., 2006). On the other hand, since stomata are an inseparable part of the integral innate immune system (Melotto et al., 2017), plants can sense the chemical and physical presence of pathogens and force the closure of stomata to prevent pathogen entrance. This stomatal closure can be achieved in a very short time (< 1 h) through the expression of pattern recognition receptors in guard cells (Robatzek et al., 2006; Liu et al., 2009), such as FLS2, EFR, and CERK1 which can recognize flg22, elf18, elf26, lipopolysaccharide, and chitin of bacterial pathogens (Murata et al., 2015). Recent studies have documented that highly adapted pathogens can produce phytotoxins, such as COR (Bender et al., 1999), or secrete effectors (Jiang et al., 2013; Hurley et al., 2014; Lozano-Durán et al., 2014; Zhou et al., 2015; Wang et al., 2016) to circumvent the host defense system through stomatal closures.

The available knowledge on pathogen-mediated stomatal movements and defenses are exclusively derived from systems involving prokaryotic pathogens and fungi. It was found that plants can defend against bacteria and rust fungi through stomatal closure or guard cell death (Melotto et al., 2006, 2017; Ye et al., 2020). *P. infestans* can penetrate potato epidermal cells directly through the formation of appressoria, therefore stomata are not essential for the penetration and colonization of the pathogen (Farrell et al., 1969,Grenville-Briggs et al., 2008). However, the stomatal opening is required for the discharge of sporangia, the main secondary inoculum source leading to late blight epidemics. In this study, we demonstrate that stomatal movement and defense also exist in the plant–oomycete interactions. The specific cell death in potato guard cells and locked stomatal closure during the *P. infestans* infection process indicate that stomata are indeed involved in the potato defense response to *P. infestans* (**Figure 2**). The findings that stomata close shortly after *P. infestans* inoculation and that this closure significantly affects the ability of the pathogen to colonize and grow in potato also support the phenomenon of stomata-regulated immunity response in *P. infestans*–potato interaction (Wang et al., 2020). However, our finding that stomata open in response to the presence of *P. infestans* and/or AF from disease plants confirms that the pathogen may in some way be able to overcome or suppress the stomata-mediated defense systems.

Lipids and starch are among the main compounds involved in plant stomatal movement. Their metabolisms in plant guard cells are regulated by environmental conditions, such as light, as seen in our study as well as the reported literatures (Horrer et al., 2016; McLachlan et al., 2016). Under light conditions, TAG is catalyzed to provide ATP for the stomatal opening process, such as the activation of a plasma membrane H^+-ATPase (McLachlan et al., 2016), while starch degradation changes cell turgor to trigger stomatal opening (Santelia and Lawson, 2016). Similar to those in other plants, potato guard cells contain a large amount of starch and TAG, and apparently, *P. infestans* deploys the same mechanisms to regulate potato stomatal movement by

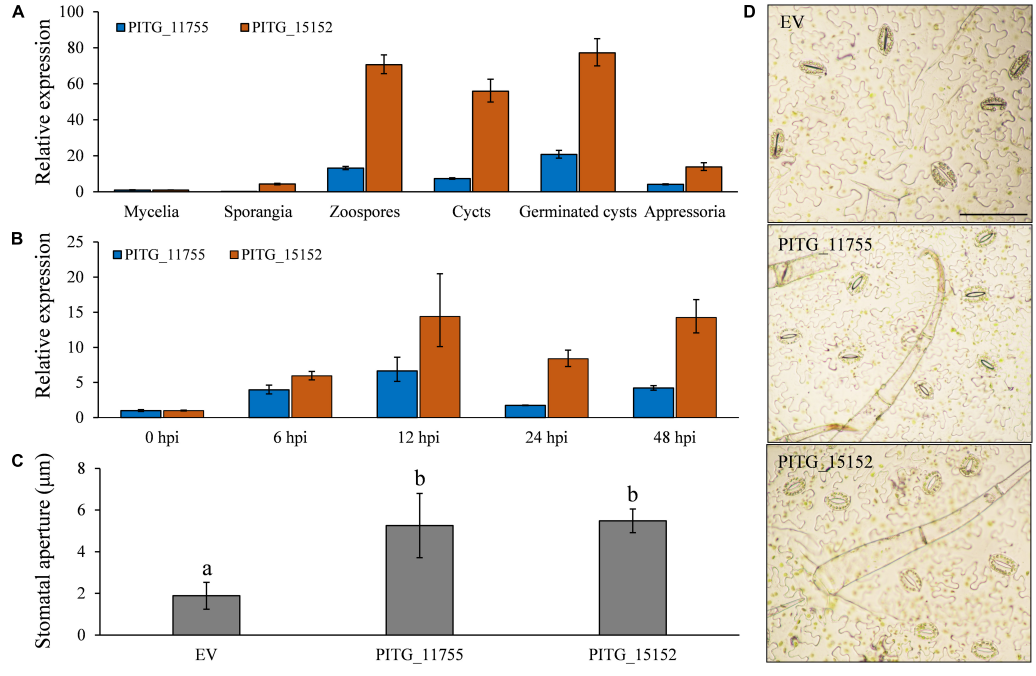

FIGURE 10 | Both extracellular and intracellular effector proteins participate in *P. infestans* infection-induced stomata opening. **(A)** The putative effectors PITG_11755 and PITG_15152 were highly expressed during pre-infection development and **(B)** at both early and late time points *in planta*; **(C)** the overexpression of PITG_11755 and PITG_15152 significantly increased the stomatal aperture; and **(D)** images show increased stomatal apertures after effector overexpression. Photos within a panel are from a representative of a single replicate while photos among panels are pooled from multiple replicates.

altering metabolic activities of lipids and starch as supported by the reverse relations between the size of stomatal apertures and the abundance of TAG and starch that we observed in the *P. infestans*-infected guard cells. This theory is further supported by the observed associations of stomatal opening with TAG breakdown after the infiltration of healthy potato plants with AF from disease plants, the impaired stomatal aperture size by co-infiltration of the AF with DMP, and the increase of stomatal aperture size by malate supplement. Although this appears to be the likely pathway that is affected by the presence of either *P. infestans* or molecules secreted by this pathogen, the exact mechanism by which *P. infestans* regulates this process is not yet known.

It appears that lipid breakdown and starch degradation are involved in the *P. infestans*-induced stomatal opening cascade at different time points. TAG breakdown was found starting from 8 hpi, which was parallel to the starting time of stomatal opening in potato leaves. While degradation of starch in potato guard cells was first observed at 12 hpi and exhausted by 48 hpi, stomatal apertures reached their maximum diameter and failed to respond to stimulation by ABA, SNP, $CaCl_2$, Na_3VO_4, and H_2O_2. It is likely that the energy generated by TAG catabolism (McLachlan et al., 2016) activates a proton-pump H^+-ATPase and initiates stomatal opening processes, while the subsequent starch degradation reinforces the opening process by strengthening guard cell turgor to maximize stomatal opening (De Angeli et al., 2013).

H_2O_2 and NO are important signaling molecules in regulating plant defense responses. Their production in many parts of plants can be triggered upon the recognition of pathogens (Mehdy, 1994; Bolwell, 1999). However, we observed that H_2O_2 and NO concentrations in guard cells were sustainably reduced after *P. infestans* infection and AF infiltration, indicating that the metabolism of these molecules in guard cells is independent from their metabolism in other parts of potato plants, such as mesophyll cells. We noticed that H_2O_2 and NO reduction in the *P. infestans*-infected guard cells occurred slightly earlier than TAG breakdown, suggesting that the lipid catabolism might be induced by H_2O_2 and NO scavenging. Although we did not have statistical support for the relationship in this study, lipid metabolism induced by oxidative radicals has been documented recently in several species (Xie and Roy, 2012; Jin et al., 2018; Becerril et al., 2019). Further study is needed to confirm this hypothesis.

During plant–pathogen interactions, successful pathogens secrete a range of effectors that act inside (cytoplasmic effectors) or outside (apoplastic effectors) plant cells to suppress or manipulate host defense systems (Haas et al., 2009; Giraldo and Valent, 2013) and promote infection. Forced stomatal opening after the infiltration of AF from disease plants into healthy potato leaves suggests that the pathogen-induced stomatal movement is likely mediated chemically either by molecules released from the pathogen, or by molecules produced in the plant in response to pathogen-derived signals. The AF we applied was extracted from *P. infestans*-infected potato leaves. In addition to ions, metabolites, and proteins of potato, this AF may also contain an array of apoplastic effectors secreted by *P. infestans* and we hypothesized that these apoplastic effectors may turn on the stomatal opening pathway through H_2O_2 and NO scavenging and TAG breakdown in potato. Cytoplasmic effectors, or other as yet unknown molecules may also directly or indirectly participate in the *P. infestans*-induced stomatal opening pathway since our experiments revealed that the AF alone did not induce starch degradation or stomatal opening to the same degree as that seen during infection (**Figure 9**).

Since stomatal opening appears to be important for the production of sporangia (secondary inoculum) and not race specific (**Supplementary Figure 3**), we hypothesized that conserved effectors that are essential for oomycete virulence may have a role in directing stomatal opening. PITG_11755 (protein ID D0NIG7) has been demonstrated to be secreted from *P. infestans* haustoria and has a hypothesized function in the apoplast (Meijer et al., 2014; Wang et al., 2018). *Phytophthora* suppressor of silencing 2, PSR2, encoded by PITG_15152 in *P. infestans* is one of only four effectors so far identified as conserved across members of the *Phytophthora* Genus (Win et al., 2007), suggesting that this cytoplasmic RXLR effector may have an essential role in oomycete pathogenicity. PSR2 and the related *P. infestans* gene PITG_14054, have been shown to function as suppressors of host gene silencing in *P. infestans*–host interactions (de Vries et al., 2017,Vetukuri et al., 2017). The increase of stomatal aperture after PITG_11755 and PITG_15152 overexpression suggests that both of them might participate in stomatal opening during the complex antagonistic interactions between potato guard cells and *P. infestans*. However, further experiments are required to confirm this hypothesis.

Based on these observations, we conclude that potato mounts a defense response against *P. infestans* infection by closing stomata and that *P. infestans* has evolved mechanisms to overcome this defense response. We propose that a series of chemical cascades are involved in the stomatal opening pathway induced by *P. infestans* (**Supplementary Figure 8**). It starts from the released effector proteins, or other molecules from *P. infestans* that inhibit H_2O_2 and NO biosynthesis or promote their catabolism. The lipid breakdown that follows then generates ATP for stomatal opening processes, including through the activation of a plasma membrane H^+-ATPase. Subsequent starch degradation and glucose and malate accumulation reinforce the stomatal opening to maximum, which later allow the pathogenic sporangiophores to exit the plant and disperse sporangia for the subsequent spread of the disease.

From the perspective of disease epidemiology, quick discharge of enough sporangiophores is essential for rapid spread of potato late blight in a field. In this study, we found that the antagonistic interactions between potato guard cells and *P. infestans* started at a very early time point of the infection course. The closure of potato stomata caused by guard cell suicide in several potato varieties with different resistance levels suggests that potato guard cells can actively respond to the *P. infestans* infection and prevent the releasing of sporangiophores from stomata. However, *P. infestans* can bypass this impediment by manipulating diverse cell processes (directly or indirectly) to open other stomata to maximize apertures for sporangiophore release. The underlying mechanisms of this zig-zag of interactions between potato guard cells and *P. infestans* are likely to be complex and may involve several effectors. Although its detailed mechanisms are not clear yet, we have shown that the stomatal opening is an important pathogenicity strategy for *P. infestans*. Manipulation of stomatal immunity may be an important strategy for future control of potato late blight without agrochemical inputs. More research should be focused on this pathogenicity process to uncover the specific underlying mechanisms that *P. infestans* uses and how they might be disrupted to sustainably control late blight disease.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**. Further inquiries can be directed to the corresponding author/s.

AUTHOR CONTRIBUTIONS

JZ, L-NY, and ZW: conceived and designed the experiments. L-NY, HL, Y-PW, and JS: performed the experiments. L-NY, JZ, and LG-B: analyzed data and wrote and critically revised the manuscript. All authors reviewed the manuscript.

FUNDING

This work was supported by the National Natural Science Foundation of China (grant no. 31901861). This project has also received funding from the European Union's Horizon 2020 Research and Innovation Programme under grant agreement no 766048 to LG-B, which supports JS, the Carl Tryggers Foundation for Swedish Research (grant CTS:17:169 to LG-B), and the Swedish Research Council Formas through grants 2015-00430 and 2019-00881 to LG-B.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2021.668797/full#supplementary-material

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

IV

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Fungal Biology Reviews

Review

The hunt for sustainable biocontrol of oomycete plant pathogens, a case study of *Phytophthora infestans*

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ARTICLE INFO

Article history:
Received 30 March 2021
Received in revised form
5 November 2021
Accepted 8 November 2021

Keywords:
Biological control
Evolutionary IPM
Integrated disease management
IPM
Late blight disease
Potato
Tomato

Abbreviations:
BCA
Biological control agent

ABSTRACT

Late blight caused by the oomycete *Phytophthora infestans* is considered to be one of the most severe diseases of potato and tomato worldwide. Whilst current synthetic fungicides are efficient at controlling this disease, they are an environmental and economic burden. In line with EU directives to reduce the use of synthetic pesticides and increase the use of sustainable alternative disease control strategies that can form part of integrated pest management systems, practical biological control solutions are urgently needed. Despite the fact that there has been a large body of scientific research into microorganisms with potential for the biological control of late blight disease, relatively few commercial biocontrol agents, licensed to control late blight, exist. Furthermore, the practical uptake of those in Europe is lower than might be expected, suggesting that such solutions are not yet feasible, or effective. Here we review the scientific literature, focusing on the most recent developments in the hunt for efficient and sustainable biological control of late blight disease. We discuss the progress in our mechanistic understanding of mycoparasite–prey interactions, in the context of late blight and the challenges and limitations to the use of such knowledge in practical disease control within a European context.

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https://doi.org/10.1016/j.fbr.2021.11.003

IPM
Integrated pest management
IDM
Integrated disease management
In-planta
Studies conducted in the host plant, under controlled conditions, for instance in greenhouse
In-agro
Field studies
DSS
Decision support system
VOC
Volatile organic compound

1. Introduction

1.1. *A rational for the hunt: the need for biocontrol*

In the last decades, chemical pesticides have been widely used to diminish yield losses caused by plant pathogens and pests (Hillocks, 2012). However, their continuous and abusive application has been associated with harmful side effects which have led to environmental and human health concerns. As a result, the number of registered synthetic chemical pesticides has decreased within the EU through restrictions imposed by directives 2009/128 and 2019/782 to reduce pesticide application risks on human health and environment (Baker *et al.*, 2020; Junaid *et al.*, 2013; Popp *et al.*, 2013). These directives promote the use of Integrated Pest Management (IPM) and alternative approaches to synthetic pesticides, such as biological control and low-risk compounds with the aim of achieving a sustainable and ecologically sound use of plant protection measures. Combined with a heightened public awareness of sustainability issues, research into alternative control strategies, particularly biological control, is a rapidly growing area (Barratt *et al.*, 2018).

Biological control of plant diseases can be defined as the application of beneficial micro, (or macro) living organisms to control aerial or soilborne plant pathogens. We consider biological control in the strictest sense of the definition, i.e., always involving a living organism that targets the pest directly or indirectly (Heimpel and Mills, 2017; Stenberg *et al.*, 2021). The term biocontrol agent (BCA) refers to the organism (typically a bacterium, fungus, oomycete, nematode, insect, virus or occasionally plant) that is used to control the disease-causing agent (Stenberg *et al.*, 2021). Mechanisms of biocontrol are broadly divided into four categories (Köhl *et al.*, 2019). 1) Competition for resources, including nutrients, space, and/or water and often for rare nutrients such as iron. Biocontrol agents that use this mode of action are termed competitive saprophytes. Here, disease suppression by mixed communities of microbes or other organisms in the soil in combination with physiochemical soil properties that discourage the growth of disease-causing microbes may also occur (e.g., through the action of suppressive soils) 2) Mycoparasitism, whereby the biocontrol agent is termed a facultative hyperparasite and where direct infection of the plant pathogen (prey species) occurs. 3) Antibiosis, also a feature of facultative hyperparasites, where antimicrobial compounds or toxins and lytic enzymes are produced to destroy the prey (plant pathogen). 4) Induced host resistance, whereby plant hormone mimics or precursors are produced and/or innate immunity is triggered by a facultative plant symbiont to confer resistance to incoming pathogens. Biocontrol ability, and mode of action, is generally strain-specific depending on the host, plant, pathogen, and environmental factors and most BCAs employ more than one mode of action to control pathogens (Köhl *et al.*, 2019). The methods through which biocontrol is used can be classified into four main categories depending on whether native BCA species are utilized, with or without targeted human intervention (conservation biological control and natural biological control, respectively) or if BCAs are added into the agroecosystem for permanent or temporary establishment (classical biological control and augmentative biological control, respectively) (Stenberg *et al.*, 2021).

1.2. *Know your prey: oomycete threats to our crops and the environment*

Oomycetes, or water moulds, are fungus-like eukaryotic microorganisms that genetically belong to the Stramenopila (which includes brown algae) but resemble fungi in both their filamentous growth and absorptive nutrition (Baldauf *et al.*, 2000). The oomycete lineage contains both pathogenic and non-pathogenic species. The pathogenic species have a wide range of hosts and affect plants, insects, crustaceans, fish, vertebrate animals, and various microorganisms (Judelson and Ah-Fong, 2019). Oomycetes are able to swiftly develop resistance to synthetic fungicides, overcome the resistance genes that have been bred into crop plants (Vleeshouwers *et al.*, 2011) and are known to deploy a vast array of effectors to facilitate destruction of their hosts (Bozkurt *et al.*, 2012; Hamed and Gisi, 2013; Schornack *et al.*, 2009).

Phytopathogenic oomycetes, such as *Phytophthora infestans*, are the causal agents of some of the most devastating plant diseases known to man (Birch *et al.*, 2012). *P. infestans*, the causal agent of potato and tomato late blight now has an almost global reach (Birch *et al.*, 2012) and is arguably the most destructive of the oomycete plant diseases both in terms of economic damage

(Haverkort *et al.*, 2016) and of fundamental food loss (Fisher *et al.*, 2012). *P. infestans* is able to trigger stomatal opening, through an as yet unknown mechanism (Yang *et al.*, 2021). This allows asexual propagation to occur via the emergence of aerial sporangiophores containing sporangia. A single potato late bight lesion can contain more than 300 000 *P. infestans* sporangia that can be rapidly dispersed via the wind, or after cleavage into motile zoospores, through air or soil -borne water droplets (Fry, 2008). The biflagellated wall-less zoopores are able to swim towards host plant cues, and differentiate to form walled cysts that germinate upon contact with the host, producing a thick-walled penetration structure, the appressorium (Grenville-Briggs *et al.*, 2008). The pre-infection stages of the asexual lifecycle occur outside of the host plant, and thus *P. infestans* is particularly vulnerable to fungicides or to attack by other microbes, such as potential biocontrol agents during these stages of development. The infection potential of an oomycete spore on plant tissue is probably much lower than 100% (Kong and Hong, 2016), meaning that not all of the 300 000 sporangia produced in a lesion will continue to propagate the disease. Thus, the progress of an epidemic, and to some extent the effectiveness of control treatments, will be influenced by factors that raise or lower this infection potential (Judelson and Ah-Fong, 2019; Willocquet *et al.*, 2017). This can include aspects of the cultivation system (Brylińska *et al.*, 2016), temperature (Lurwanu *et al.*, 2021; Wu *et al.*, 2020), UV index (Wu *et al.*, 2019), carbon dioxide levels (Plessl *et al.*, 2007) and population structure, including sexual reproductive capacity (Klarfeld *et al.*, 2009). There are indications that over the past 20 years, the aggressiveness of *P. infestans* has increased (Cooke *et al.*, 2011; Lehsten *et al.*, 2017), whilst at the same time global populations of the pathogen are in flux, with sexual recombination and diverse rapidly evolving genotypes contributing to unexpectedly severe epidemic outbreaks worldwide (Fry *et al.*, 2015). Thus *P. infetans* can be considered to be a reemerging pathogen (Fry *et al.*, 2015) and under a warming climate, we will require new and/or more adapted control measures in the different growing regions of the world. However, the common feature of those control measures must be in line with the UN sustainability goals and the EU directives, to reduce the burden of synthetic inputs in agricultural systems, whilst maintaining biodiversity and effective disease control. Biological control has the potential to be an important component of such an integrated disease management program. Thus, here we discuss progress and challenges to the development and deployment of BCAs against late blight disease of potato and tomato.

2. Preparing and executing the hunt: towards biocontrol of *Phytophthora infestans*

In the hunt for new or improved biocontrol agents, most studies firstly employ *in vitro* assays of growth inhibition or death of the prey species, typically in confrontation assays (e.g., Fig. 1 lower panel). This approach allows mechanistic understanding of direct interactions between BCAs and their prey to be elucidated under laboratory settings. In the case of *P. infestans*, this allows researchers to test potential BCAs for direct control of the vulnerable pre-infection stages of the lifecycle. The "*in vitro* first" approach is attractive for several reasons, including the low cost, high throughput nature of such assays, and the ease of experimental set up and data analysis for individual experimental factors under controlled conditions. However, such approaches run the risk of years of work failing to identify BCAs that perform effectively in an agricultural setting. In fact, it may be argued that research starting from field (*in agro*) or greenhouse (*in planta*) (e.g., Fig. 1 middle and top panel) studies has a higher chance of implementation and may be more informative for practitioners than *in vitro* analysis; since, differences in efficacy of disease control in controlled environments versus in the field, coupled with the high costs of field trials mean that some BCAs never move beyond *in vitro* or *in-planta* studies to field applications. Furthermore, promising agents that may work in the field but not *in vitro* can be mistakenly eliminated from screens. *In vitro*, and in particular associated molecular and 'omics studies may stimulate new research avenues including important questions regarding microbe–microbe interactions and generate important fundamental knowledge including mechanistic understanding of BCAs and their interactions with their prey. However, if we want to bring about meaningful change in pest management practices, in terms of a reduction in the reliance on synthetic pesticides and an increase in the use of BCAs, we need more applied studies. The knowledge obtained from these applied studies is essential to the development of durable formulations of BCAs for sustainable disease control.

In the last 20 years, there have been more than 95 peer-reviewed scientific publications in which the potential of a microorganism for the biocontrol of *P. infestans* has been investigated (Table S1). Here, we review that literature with particular emphasis on research from the last 7 years, in the hunt for sustainable biocontrol of late blight disease of potato and tomato. Our hunt takes us from mechanistic studies or "target practice" as revealed by *in vitro* studies, through to *in planta* and *in agro* (full agricultural field) studies to bring down our prey *P. infestans*, as summarised in Fig. 1. Finally, we discuss "the struggle to make the kill" – the challenges we face in the use of biocontrol against oomycete diseases and prospects for the future, focusing on the integration of biocontrol into IPM strategies (summarised in Fig. 2) and the current situation in Europe.

2.1. *Target practice: biocontrol mechanisms revealed by in vitro studies*

Despite the fact that *in vitro* biological control studies often fail to translate to field applications, valuable mechanistic knowledge can be gained from such experiments. One of the most studied and most well-known fungal genera harbouring BCAs is *Trichoderma* (De Silva *et al.*, 2019; Rai *et al.*, 2019). The biocontrol mechanisms used by *Trichoderma* spp. against *P. infestans* encompass classical mycoparasitism and antibiosis behaviour, by coiling around prey hyphae, and secreting lysing enzymes, secondary metabolites and/or other toxins that directly inhibit *P. infestans* growth and sporulation *in vitro* (Kariuki *et al.*, 2020; Yao *et al.*, 2016).

Within the oomycetes, *Pythium oligandrum* is known for its antagonistic properties and its mycoparasite behaviour of a

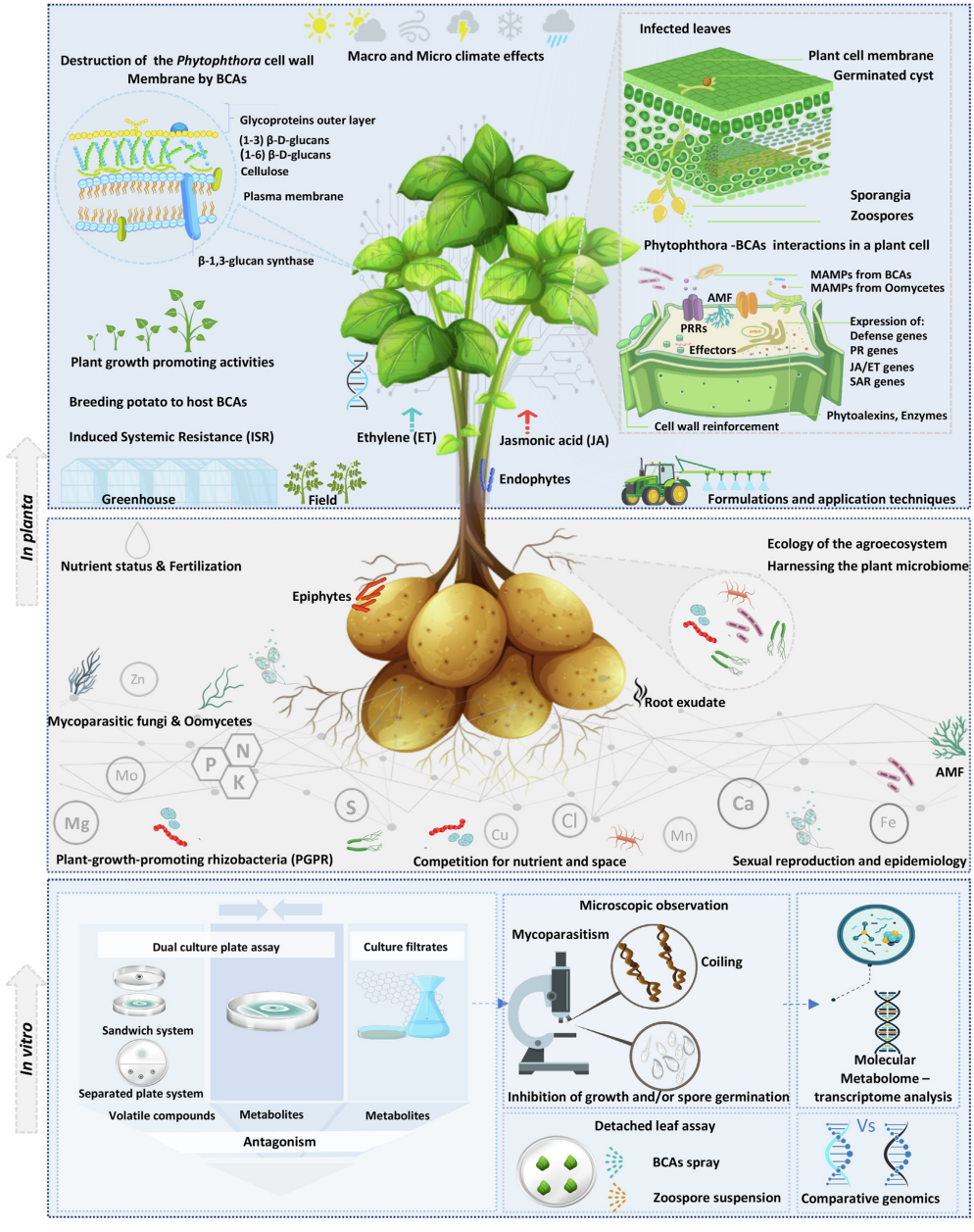

Fig. 1 – Towards both a mechanistic understanding and practical use of biocontrol agents against potato late blight disease. *In vitro* assays may lead to the identification of metabolites, volatiles or direct mycoparasitic effects of BCAs, which, combined with molecular tools such as metabolomics, transcriptomics or comparative genomics provide a mechanistic understanding of mycoparasite-prey interactions between BCAs and *P. infestans* (bottom panel). *In planta* and *in agro* experiments allow us to understand these interactions within the agroecosystem, and in the context of plant responses to both the pathogen and the potential BCAs (top panels). Within the soil BCAs have to compete for space and nutrients with the indigenous plant microbiome and with each other, and may be affected by the nutritional status of the plant (middle panel).

diverse set of fungal and oomycete prey species (Gerbore *et al.*, 2014). It displays mycoparasitic behaviour and secretes cell wall degrading enzymes and putative effectors during colonisation of *P. infestans in vitro* (Horner *et al.*, 2012; Liang *et al.*, 2020). Interestingly, a recent comparative genomics study revealed *P. oligandrum* may have evolved its mycoparasitic capabilities by tandem gene duplication and horizontal gene transfer of specific carbohydrate-active enzymes (CAZy) from fungal and bacterial species, giving it the potential to utilize fungal and oomycete species for nutrition (Liang *et al.*, 2020).

Many genera of bacteria display biocontrol characteristics and the major genera that have been investigated in relation to control of *P. infestans* are *Bacillus, Pseudomonas* and *Streptomyces* (Table S1). Several Bacillus species show direct antagonism toward *P. infestans* providing effective growth reductions *in vitro* (Cray *et al.*, 2016; Caulier *et al.*, 2018; Wang *et al.*, 2020a, 2020b). Bacterial volatile organic compounds (VOCs) play important ecological roles in both soil microbial and host plant interactions (De Vrieze *et al.*, 2015) and have also been shown to be important for inhibition of *P. infestans* growth (Anand *et al.*, 2020; Bailly and Weisskopf, 2017; Guyer *et al.*, 2015; Joller *et al.*, 2020; Lazazzara *et al.*, 2017). Among these, hydrogen cyanide, long-chain aldehydes, alkenes and short-chain ketones as well as sulphur-containing compounds and some longer-chain ketones all have an inhibitory effect on *P. infestans* growth, spore development and germination *in vitro* (Bailly and Weisskopf, 2017). The majority of these VOCs have been identified from *Pseudomonas* species that are native inhabitants of the potato rhizosphere, and do not negatively affect potato growth (Bailly and Weisskopf, 2017).

Numerous other bio-active metabolites are produced by bacterial BCAs, and those from species of *Bacillus* and *Pseudomonas* have been particularly well studied. For example, cyclic lipopeptides produced by several *Pseudomonas fluorescens* strains have specific activity against the vulnerable wall-less zoospores of *P. infestans* (De Vrieze *et al.*, 2020; Zachow *et al.*, 2015) and siderophore production has also been associated with anti-oomycete activity, in the *Pseudomonas* genus (De Vrieze *et al.*, 2020). Iron acquisition genes including those linked to the production of Pyoverdines have been linked to both the ability of *Pseudomonas* strains to survive in the soil and colonise plant roots, as well as to direct antagonism of *P. infestans* (De Vrieze *et al.*, 2020) suggesting iron competition could be one of the mechanisms used by some *Pseudomonas* species to inhibit *P. infestans* development. Although members of both the *Pseudomonas* and *Bacillus* genera are known to be prolific producers of numerous bioactive compounds including those with anti-oomycete activities, the *in vitro* activities of such compounds are not easily transferable to *in planta* or *in agro* assays (Caulier *et al.*, 2018), thus new methods for selection of BCAs need to be developed. One possibility for the screening of potential bacterial BCAs could be to screen for the production of biosurfactants and siderophores, since in their large-scale analysis of over 2800 bacterial isolates (Caulier *et al.*, 2018), identified that the strains that were effective *in planta* at controlling late blight were prolific producers of both, and indeed the production of such compounds might be the significant factor contributing to the success of these strains as BCAs. As microbial competition for nutrients and ecological niches, on, or within plants, contributes to the antagonistic activity of competent bacterial strains (Vorholt, 2012), isolates naturally associated with potato plants, such as some of these *Bacillus* and *Pseudomonas* species, have the highest chance to be artificially reintroduced to a crop for control purposes, through classical biocontrol strategies.

Molecular and genomic studies, in combination with *in vitro* assays can also reveal important information on the biology, modes of action, and genetics of BCAs. For example, comparative genomics studies have revealed that specific loci in the genomes of some *Pseudomonas* species control aggressiveness of these species towards *P. infestans*. Furthermore, this aggressiveness can increase through increasing exposure to the prey *in vitro* (De Vrieze *et al.*, 2020), meaning that in the future it may be possible to genetically engineer hyper-aggressive strains for use in the field. Additionally, such studies can determine the life history of BCAs, for example, mycoparasitic *Pythium* species likely acquired their facultative hyperparasitic abilities by horizontal gene transfer and tandem gene duplication, meaning the ancestral state of these species was likely to be as phytopathogens (Liang *et al.*, 2020). In contrast, *Trichoderma* species are likely to have an ancestral state as facultative hyperparasites, with some members of the genus later developing abilities to parasitise plants (Kubicek *et al.*, 2011).

Understanding the biology of both the BCA and the prey is important for the development of effective biological control strategies. For example, the *P. infestans* cell wall consists predominantly of β-D-glucans and cellulose, and the correct formation of the cell wall during encystment of wall-less zoospores and subsequent differentiation into appressoria, is required for establishment of disease (Grenville-Briggs *et al.*, 2008). The cell wall is already the target of several anti-oomycete fungicides, such as the CAA fungicides including Mandipropamid (Blum *et al.*, 2010). Breaching the cell wall is also necessary for BCAs that act as facultative hyperparasites, and thus many bacterial (Caulier *et al.*, 2018), fungal (Karlsson *et al.*, 2015; Kubicek *et al.*, 2019), and oomycete (Grenville-Briggs *et al.*, 2013; Liang *et al.*, 2020) BCAs secrete extensive cocktails of cell wall degrading enzymes, (CWDEs) which are likely to contribute to their success as mycoparasites. Indeed it has recently been proposed that such CWDEs from such hyperparasites are major pathogenicity determinants (effectors) in these species, since they display the genetic hallmarks of rapidly evolving effectors (Linag *et al.*, 2020; Karlsson *et al.*,

Within the leave, *P. infestans* produces effectors to manipulate the host and evade immunity, whilst the plant responds with defence related genes that are often hormonally regulated. Within this battle ground, BCAs have to succeed to break open the cell wall of the prey and to obtain the nutrients they require (top panel). Finally, this knowledge must be harnessed and BCAs manipulated to withstand both macro and micro climate effects, to produce correctly formulated products for practical plant protection within greenhouse or open field systems (top panel) in order to successfully control late blight disease.

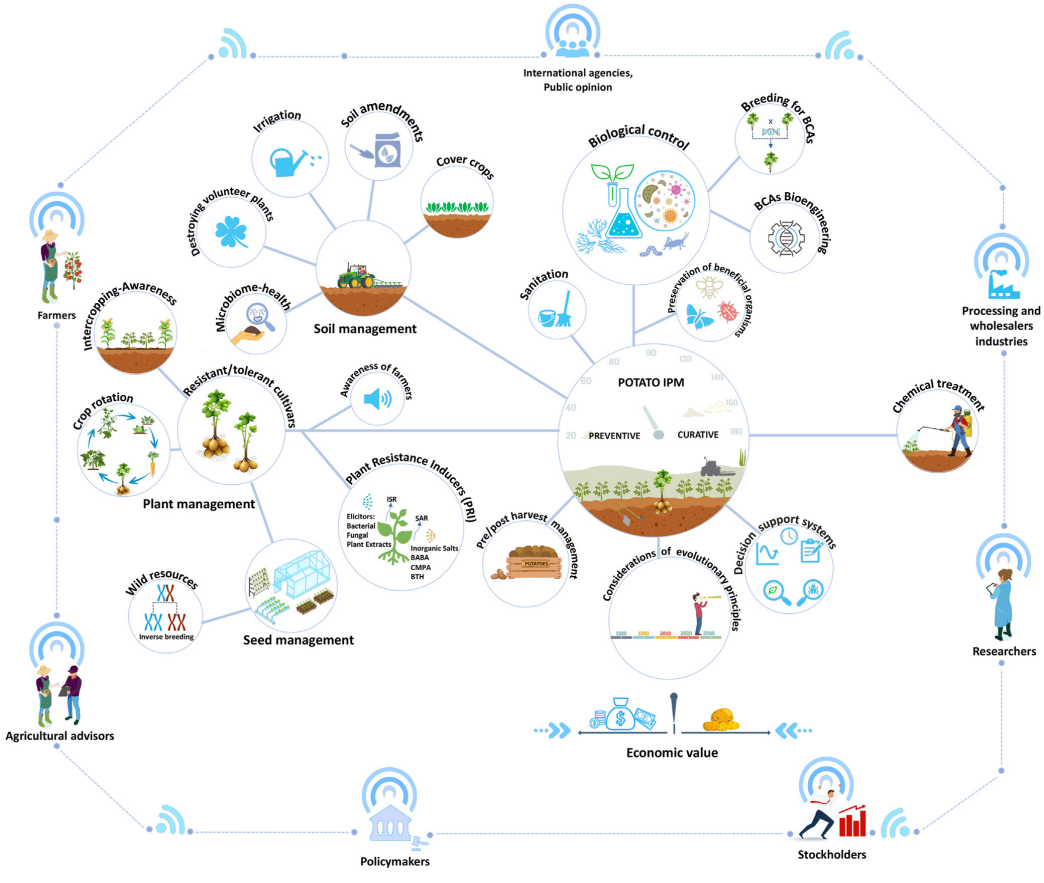

Fig. 2 – Integrated Pest Management (IPM) in potato. A schematic of the potential components of an IPM system for the protection of potato from pests such as late blight disease. For successful implementation of IPM in potato, greater emphasis should be placed on preventing pest problems (traveling left from the central bubble) than curative measures (traveling right from the central bubble). Bubble size reflects the importance of the action in the system. Biological control should be a central component of IPM that can be applied both preventatively and curatively, and should include methods to preserve beneficial microbes and breeding of potato to host beneficial microbes including BCAs. Soil management, should include microbiome health, as well as traditionally included measures such as soil amendments, irrigation and destruction of volunteer plants. Plant management should include crop rotations, resistant cultivars and the use of low-risk compounds that act as plant resistance inducers. IPM needs to take into account evolutionary principles and the use of curative synthetic pesticides should be a last resort, and they should be applied only when needed, with the help of decision support systems. IPM involves many actors, including farmers, advisors, policymakers researchers, and the processing and wholesale industries.

2015). The secretion of the most potent mixtures of CWDEs could therefore be used to select Eukaryotic microbes that will be efficient BCAs in the future.

2.2. *The thrill of the chase:* in planta *studies of late blight disease control by microbial BCAs*

A substantial number of studies have tested BCAs against late blight *in planta*, either in controlled environment detached leaf assays, or in whole plant greenhouse bioassays (Table S1), although the efficacy of control varies. Since commercial tomato cultivation in Northern Europe is almost exclusively carried out in greenhouses, it is relatively easy to mimic this production system to accurately analyse BCA efficacy in a research setting. Valuable knowledge that might also translate into potato cultivation systems can also be gained in this manner. In addition to direct disease control measures, *in planta* assays have the advantage of evaluating biostimulation, for example Kariuki *et al.* (2020) observed that both *T. harzianum* and *T. asperellum* stimulated growth of

Table 1 – Table of approved products against Phytophthora *infestans* in potatoes or tomatoes. Product name, licence approval, regislation, active components and Application is displayed.

Product Name	License approved	Licence expire	Approval under Reg. (EC) No 1107/2009	Component(s)	Application method
TAEGRO®	2017-06-01	2032-06-01	Yes	*Bacillus subtilis* var. *Bacillus amyloliquefaciens* strain FZB24	Sprayed product.
Polygandron WP®	2009-05-01	2022-04-30	Yes	*Pythium oligandrum*	Sprayed product.
Sonata®	2014-09-01	2024-08-31	Yes	*Bacillus pumilus* QST 2808	Sprayed product.

above ground tomato plants and increased the biomass by over 30% and 19%, respectively compared to control plants as well as providing protection against late blight, reducing disease symptoms to 40% of that seen in control plants. Stephan *et al.* (2005) used the commercial product Trichodex® based on *Trichoderma harzianum*, in greenhouse and detached leaf assays to assess the potential for this product to control potato late blight, but despite a previously reported *in vitro* growth reduction of *P. infestans* by 40% when co-cultured with this species (Fatima *et al.*, 2015), there was no significant effect of the commercial preparation on late blight disease development.

Endophytes, or other antagonistic indigenous soil microbes from healthy plants in a habitat where disease is a problem are more likely to be competitive BCAs, than introduced species, since such endophytes are already adapted to the appropriate environment (Collinge *et al.*, 2019). The large-scale screening study conducted by (Caulier *et al.*, 2018) took this approach. They first isolated 2800 strains of *Bacillus*-like and *Pseudomonas*-like bacteria from potato agroecosystems and then tested a subset of them for *in planta* activity against *P. infestans*. Of the 11 strains tested *in planta*, four strains (*Bacillus amyloliquefaciens* 17A-B3, *Bacillus subtilis* 30B-B6, *Pseudomonas brenneri* 43R-P1 and *Pseudomonas protegens* 44R-P8), decreased disease symptoms in the greenhouse study, and one of these, *B. subtilis* 30B-B6, significantly decreased late blight disease severity *in agro*, in a small-scale field trial (Caulier *et al.*, 2018), validating the endophyte-based approach for the identification of new BCAs.

2.3. *Bringing down the prey: translation of these data to an* in agro *setting*

Since potato is the third most important food crop globally (Fisher *et al.*, 2012), potato late blight arguably has a greater potential impact on food and economic security than tomato late blight, and thus to actually transform late blight disease control in a sustainable manner, biocontrol strategies that are durable in open field agriculture (*in agro*) are desperately needed.

Many *Trichoderma* species have been tested for potato late blight disease control *in agro* (Al-Mughrabi, 2008; de Souza *et al.*, 2015; El-Naggar *et al.*, 2016; Wharton *et al.*, 2012; Yao *et al.*, 2016). For example, Al-Mughrabi (2008) showed a significant effect on late blight incidence when tubers were pretreated with Trichoderma in field trials. The commercial product Planter-Box® containing *Trichoderma harzianum*, is effective against late blight seed pierce incidence on potato tubers, and microscopic investigations confirm *T. harzanium* coiling around *P. infestans* hyphae and thus exhibiting classical mycoparasitic behaviour *in agro* (Wharton *et al.*, 2012). Yao *et al.* (2016) showed that Trichoderma can significantly reduce late blight disease severity in potato field trials and, most recently, Lal *et al.* (2021) tested Neem, a bioextract, in combination with *Trichoderma viride* and found a significant reduction of potato late blight *in agro*. However these studies did not determine if secondary metabolites also played a role in the efficiency of these treatments.

In agro studies with other fungal BCA have also been carried out, for example, Shanthiyaa *et al.* (2013) demonstrated that application of a spore suspension of *C. globosum Cg-6* as a tuber, soil and foliar treatment inhibited late blight infections by 72% *in agro*. The application of *C. globosum* even resulted in an increased tuber yield. They further identified a metabolite "Chaetomin" belonging to epidithiodioxopiperazine potentially responsible for the antagonistic activity; a great example of antibiosis working *in agro*. This study also highlights the importance of secondary metabolites produced by BCAs in effective disease control.

Within the oomycetes, the BCA *Pythium oligandrum* has been formulated into the commercial products Polyversum® and Polygandron®. These products have been tested *in agro* against potato late blight through applications as both tuber dressings and as foliar sprays. A multi-year field trial in Poland demonstrated a significant yield increase and foliar protection against late blight from these applications (Kurzawińska and Stanisław, 2007), and a Swedish study demonstrated effective control of potato late blight for the first 20–30 days of the growing season after applications of Polygandron® early in the season (Wiik *et al.*, 2020).

The effects of bacterial BCAs under field conditions have been reported in several publications (El-Naggar *et al.*, 2016; Huang *et al.*, 2007; Wang *et al.*, 2020a; Yan *et al.*, 2021). Moreover, their application as a seed treatment for late blight control under storage conditions has been investigated (Cray *et al.*, 2016; Wharton *et al.*, 2012). However, the results are variable. For instance, Yan *et al.* (2021) showed that *in agro* application of a low concentration of the fungicide Fluopimomide and a high concentration of the antagonistic bacteria *Bacillus velezensis* SDTB038 can be effective in controlling potato late blight. *Bacillus subtilis* applied both to the soil and as a foliar treatment has significantly reduced late blight occurrence rate *in agro* compared to control plants (Kumbar *et al.*, 2019). Open field foliar applications of crude bacterial suspensions of *Pseudomonas protegens* strain 44R-P8 and *B. subtilis* strain

30B-B6 were shown to decrease late blight severity by approximately 20% (Caulier *et al.*, 2018). However, most BCAs do not perform to the same level as synthetic chemical fungicide treatments (Caulier *et al.*, 2018; Kumbar *et al.*, 2019). It may therefore be more promising currently, to combine BCAs with low-risk fungicide treatments, such as resistance inducers or biopesticides in an integrated disease management approach. For example, it may be effective to apply BCAs early on, i.e., before the onset of disease, whilst the infection pressure within the field is low, and before the crop canopy has closed. After canopy closure, when living BCAs might find it difficult to access all areas of the plant and when the infection pressure is higher, (bio)fungicide treatments may well be more effective. This combinatorial approach proved successful when the bacterium *Rhodopseudomonas palustris*, GJ-22 was combined with the two synthetic fungicides cymoxanil and mancozeb (Zhang *et al.*, 2020). The challenge for the future is to provide effective disease control not only when combining BCAs with synthetic fungicides, but rather with biopesticides, such as those refined from secondary metabolites produced by BCAs, resistance inducers or other low-risk compounds that are more sustainable.

3. The struggle to make the kill: challenges and limitations for the development of biocontrol solutions to late blight

P. infestans displays the nature of a super pathogen, adapting and evolving, to constantly stay on top of the host-pathogen evolutionary arms race, in terms of overcoming both host resistance and synthetic fungicides through adaptive evolution of effectors and fungicide target genes (Dong and Ma, 2021). Nevertheless, sustainable control measures are still needed and for the future, this should include biological control as a major component of disease management.

3.1. *Licence to kill: registration of BCAs*

Recently the International Organisation for Biological Control (IOBC) reviewed the barriers to uptake of biological control by practitioners and advisors, and concluded that the reasons why biological control is not widely used are the difficult and risky regulatory processes involved, as well as bureaucratic barriers to access to biocontrol agents (Barratt *et al.*, 2018). The same barriers serve as limiting factors for developing and registering BCAs for late blight disease control.

The EU Commission Regulation No 546/2011 of 10 June 2011, implementing directive (EC) No 1107/2009, specifically regulates the use of plant protection products, including biological control agents, in agriculture. Requirements for registration to ensure safe usage are important and needed. However, these regulations also create an unwanted effect, namely that it is a long and potentially expensive process to get a product approved in the EU (Hauschild, 2017). The process is more extensive in the EU than in other parts of the world, (taking on average 65,7 months in the EU compared to 15,7 months in the USA) (Balog *et al.*, 2017; Frederiks and Wesseler, 2019; Kiewnick, 2007). In general the US approval process is much faster since microbial BCAs are registered under the regulatory framework of Biopesticides, and the US is accustomed to such. To formally have a microbial BCA approved in the EU, it needs to pass two steps within the legislation. The first step is the evaluation of the active substances. This step consists of three phases, the rapporteur member state phase, the risk assessment phase and the risk management phase (Frederiks and Wesseler, 2019). Generally, during the first step applicants provide a dossier of documents regarding the active substance to a member state. After evaluation of the dossier, the European Food Safety Authority starts assessing the risks in all aspects of food safety and the risk management is then carried out by the European Commission. The second step is to approve and register the plant protection product at a member state level. BCAs used in field crops are usually registered and approved to be used within specific zones of Europe, however country-specific or cross-zonal approval occurs too. EU Approved active substances and BCAs can be found here: https://ec.europa.eu/food/plant/pesticides/eu-pesticides-db_en.

Dominating factors that slow the EU registration process are investigations into the environmental fate, toxicity and resilience of the BCA, questions that reappear throughout literature and are seldom studied (Ehlers, 2011; Hajek and Eilenberg, 2018; Köhl *et al.*, 2019). Based on the assumption that an increase in population levels of microbial species can have adverse effects on other organisms and ecosystems, data on the environmental fate and persistence of microbial BCAs are required (Deising *et al.*, 2017). However, long-term experiments show that applications of non-pathogenic microorganisms into the environment have not generated situations where the released organisms became overwhelming and the dominating species within the habitat (Alabouvette and Cordier, 2011; Köhl *et al.*, 2019; Sundh and Goettel, 2013).

3.2. *The armoury: formulation of microbial BCAs*

A major challenge in using living microorganisms as BCAs is that they can be difficult to formulate in a way so they can both be sprayed efficiently and still be storable over longer time, whilst remaining viable. Formulation is often beyond the scope of most research projects, even those with applied aspects, and thus to move from research to practice, practitioners may be reliant on larger agrochemical companies seeing the value and need in a BCA and taking on both the formulation and registration process for these organisms. Very few of the BCA presented in this review have been commercially formulated for late blight control (Table 1), considering the amount of research conducted (see S.1). To produce a successful formulation it is necessary to take into consideration how the microbial BCA can be affected by temperature, humidity, soil type, pH and UV light. Some notable species that have been commercialised such as *Clonostachys rosea*, and members of the *Bacillus*, *Pseudomas* and *Trichoderma* genera have been well studied in this regard (Costa *et al.*, 2016; Maruyama *et al.*, 2020; Panpatte *et al.*, 2016; Wang *et al.*, 2018; Zin and Badaluddin, 2020). Such data is essential to allow formulation of microbial BCAs that need to establish themselves and proliferate within agroecosystems, where they

are expected to be effective within a single growing season, and then may be disturbed by tillage and crop rotation in a standard intensive European farming system. This has led practitioners to use BCAs in a curative manner, utilising existing machinery used for application of synthetic pesticides. However, given the lack of success in translating *in vitro* studies to *in agro* applications, it might be time to rethink this approach. Future efforts should be focused on better understanding the modes of action and environmental interactions of current BCAs, and reformulating them for use at or before planting, to help stabilise host plants before pathogens enter the agroecosystem. Furthermore, for those species where active secondary metabolites have been identified from BCAs, isolating and stabilising those active metabolites in a formulation suitable for spray applications would allow practitioners to use only the active substance from the BCA in curative applications where needed.

3.3. *The weapons factory: utilising microbial secondary metabolites for biological control*

A range of secondary metabolites with activity against plant pathogens such as *P. infestans* have been identified from many microbial BCAs. Therefore, we suggest that microbial BCAs could be utilized as cell factories to produce compounds with anti-pathogen properties such as secondary metabolites, and indeed screening for the ability to produce biosurfactants, siderophores or other secondary metabolites may be a more successful approach to identifying competent BCAs rather than *in vitro* confrontation assays. Many secondary metabolites from BCAs have been demonstrated to have good efficacy in the suppression of late blight disease. For example, the cyclic dipeptide 2,5-diketopiperazine cyclo (L-Pro-L-Tyr) from *Lysobacter capsici* AZ78 can directly inhibit development of *P. infestans* sporangia (Puopolo *et al.*, 2014). Bikaverin and fusaric acid from *Fusarium oxysporum* EF119 are effective at controlling tomato late blight in greenhouse settings (Son *et al.*, 2008) and *Trichoderma* species are well known to produce a wide variety of metabolites and other compounds such as peptaibols (i.e., trichokonins, alamethicin), small non-ribosomal peptides (NRP) (i.e., gliotoxin, siderophores), polyketides (PK) (i.e., aspinolides, trichodermaketones), terpenes (i.e., trichothecenes), and pyrones (i.e., 6-pentyl-2H-pyran-2-one (6-PP) (Hermosa *et al.*, 2014; Vinale *et al.*, 2014).

Commercial formulations such as Serenade®, a broad spectrum biofungicide based on *Bacillus subtilis*, are thought to derive much of their success from the secreted metabolites and lipopeptides produced by the bacterium and included in the commercial formulation (Stephan *et al.*, 2005). This sets a precedent for the development of low risk biopesticides that are based solely on secreted metabolites and that thus will not be as sensitive to environmental changes as formulations containing living BCAs.

Trichoderma species have been harnessed for novel plant biotechnology approaches. One notable example is the use of *T. atroviride* as a "cell factory" to produce selenium nanoparticles (SeNPs) as an eco-friendly plant protection product. These fungal-derived SeNPs have an inhibitory effect on *P. infestans* growth and spore production *in vitro* (Joshi *et al.*, 2019) and *in planta* when used as a seed coating in tomato, where they prime the plant defence responses and stimulate induced systemic resistance (ISR) (Joshi *et al.*, 2021). The SeNP treated tomato plants showed a significant deposition of callose and lignin as well as elevated H_2O_2 consistent with an ISR response, and an upregulation of general defence enzymes such as lipoxygenases, and cell wall degrading enzymes, with 72% of the primed plants showing complete disease protection against late blight (Joshi *et al.*, 2021). Such bioengineering approaches are highly promising for the future of plant protection.

3.4. *Hunting in the wilderness: agroecological effects on BCA efficacy*

Even if natural secondary metabolites are not necessarily suitable for direct use as commercial fungicides, the identification of such mechanisms are key for the development of control strategies, in both Integrated Pest Management systems and organic agricultural production systems. However deeper knowledge about the adaptation to environmental conditions, and an understanding of microbiome interactions is crucial to find a better approach for BCA selection.

The variability in efficacy of BCAs *in planta* or *in agro* is generally attributed to climatic variations (temperature, humidity, radiation) encountered in field conditions, a lack of ecological competence (survival, colonization ability) of the biocontrol agent, intrinsic traits of the antagonistic microbe (variable production of required metabolites or enzymes) and/or an unstable quality of the formulated product (Bardin *et al.*, 2015; Mark *et al.*, 2006; Ruocco *et al.*, 2011). Another factor contributing to varying efficacy of BCAs is interactions with the native microbial community in the soil, or leaf, microbiome associated with the host plant. Studies have shown that for instance the bacterial community in potatoes are recruited from the soil (Buchholz *et al.*, 2019; De Vrieze *et al.*, 2015). Abiotic factors such as environmental conditions (Rasche *et al.*, 2006) or soil types (Inceoğlu *et al.*, 2012) are known to influence the structural and functional diversity of the bacterial microbiota of potato plants. Similar trends have been seen for fungi. Hou *et al.* (2020) reported that the change of the microbiome in potato plants was most significant at seedling stage, and that potato root exudates contributed to the growth of the rhizobiome. Zimudzi *et al.* (2018) reported that the rhizospheric fungal microbiome of potatoes were different between the two seasons and in the different plant growth stages within a given season, indicating the significance of the rhizosphere in shaping microbial communities.

Individual BCAs have different survival capacities in the rhizosphere or in host plants and this is an area where we still lack a lot of knowledge. *Pseudomonas protegens* has been reported to survive down to 2 m below the soil surface (Troxler *et al.*, 2012) and, whilst some studies have shown that *Trichoderma* species can survive for up to two years after inoculations in soils (Longa *et al.*, 2009), other studies show that these BCAs are not able to persist long-term (Feng *et al.*, 2011). Hence, it matters greatly into which environment and

existing interactive microbial community the BCA will be amended, and thereby to what extent it will persist in the environment and provide effective disease control.

3.5. *Timing the shot: when and how to apply BCAs*

Targeting *P. infestans* at the right moment, of both the epidemic within the field and also of the lifecycle, is challenging. Directly combatting late blight when already established in the host by augmented release of a BCA is possible, but, as discussed above, can have varying results. To increase the efficacy of many of the microbial BCAs discussed in this review, we may have to move away from using BCAs curatively as we do synthetic fungicides and start using them as fertilisers or soil improvers, ensuring they can establish themselves in the field at or before planting. For example, Stephan *et al.* (2005) reported that a combination of preventive and curative application of the BCA had a better effect than just a curative application alone. It is likely that the BCA needs to establish itself in the agroecosystem first, and then a population threshold may need to be met before it will be effective at disease suppression or controlling a pathogen. The population dynamics and survival of BCAs in agroecosystems is a huge gap in the biocontrol research field. Future research therefore needs to investigate this aspect in agroecosystems. There are several disease predictions models of late blight in potatoes, many of which are widely used by agricultural advisers and farmers, to predict the best timing for synthetic fungicide applications based on local weather conditions and potato phenology (Cooke *et al.*, 2011), although they may also need to be adapted to local agroecosystems and climates e.g., (Lehsten *et al.*, 2017). The challenge for the future is to incorporate the biology and ecology of potential BCAs into such models so that they can be used more effectively in the field and crucially at the right time.

3.6. *Multifunctional weapons: combining multiple modes of action and creating synergistic effects*

Most of the BCAs discussed in this review display a combination of different modes of action, although many of the mechanistic studies focus on a detailed understanding of one of these, probably due to the notorious difficulties of elucidating modes of action in microbe–microbe interactions. Indeed, a combination of several modes of action is likely to ensure better phytopathogenic disease control (Köhl *et al.*, 2019). Resistance to BCAs or their metabolites has been reported in pathogens such as *Botrytis cinerea* (Ajouz *et al.*, 2011), which makes BCAs with several modes of action advantageous in terms of limiting the risk of resistance emergence among phytopathogens such as *P. infestans*, which is well known to adapt resistance to synthetic fungicides and to overcome resistance bred into commercial potato and tomato cultivars (Bardin *et al.*, 2015). Furthermore, targeting more than one stage of the lifecycle may also enhance the efficacy of a BCA.

Given the adaptability of *P. infestans* towards fungicides with only one mode of action, it is worth considering combining more than one BCA to combat late blight, or at least using a BCA with several different modes of action. Synergistic effects of combining two or more BCAs against late blight in potatoes and tomatoes have been reported (De Vrieze *et al.*, 2018; El-Naggar *et al.*, 2016; Kumar *et al.*, 2015; Lourenço Júnior *et al.*, 2006; Maksimov and Khairullin, 2016; Wharton *et al.*, 2012). Combinations of metabolites produced by BCAs can also be effective at disease control, particularly if these metabolites function through synergistic modes of action. For example, Fengycin B from *Bacillus pumilus* is directly toxic to *P. infestans*, whereas the surfactin metabolites produced by the same organism induce defence responses in potato, and the combination of these two metabolites is more effective at treating late blight in potato than either metabolite alone (Wang *et al.*, 2020a). Tripartite combinations of resistance inducers such as chitinosan, with BCAs and low doses of fungicides, should be considered in an integrated pest management (IPM) program, and have recently shown promising results in protecting potato from *P. infestans* (Shukla *et al.*, 2021). Combinations of fungicides, such as metalaxyl, and BCAs, such as *Trichoderma asperellum*, have shown effective disease control and allowed the intervals between fungicide applications to be prolonged in potato (Jackson *et al.*, 2020). However, whilst this might be a workable approach currently, moving away from combinations that include synthetic fungicides should be seen as a long term goal, that will allow IPM to become more environmentally sustainable.

In some studies, the combination of two BCAs was not effective in controlling late blight disease, even though each individual BCA was effective separately (Zegeye *et al.*, 2011). It is important to note that even though strategies based on combinations of two BCAs may be currently unrealistic in practice, given the high registration costs for BCAs, we need to better understand any potential added value of combining different BCAs or their metabolites to control diseases such as late blight. Such research may lead to more effective biological control strategies that may become more affordable in the future.

3.7. *The magic bullet? IPM solutions in practice*

Integrated pest management (IPM) is now recognised as the most sustainable pest management practice, in most cases, and is therefore now mandatory in all EU member states, being regulated through directives 2009/128 and 2019/782. It is a complex, knowledge-intensive management practice, that needs to be optimised for every crop and location. Potato lags behind many other crops in terms of reductions in the use of synthetic pesticides, (which are currently very effective against late blight) (Eriksson *et al.*, 2016) and in the uptake of IPM. For example, whilst potato typically occupies around 0.9% of the cultivated land in Sweden, at least 20% of the synthetic fungicide usage in Swedish agriculture is directed to protecting potatoes (Eriksson *et al.*, 2016). Thus, we urgently need more sustainable IPM practices for the management of late blight disease in potato.

Decades of potato and tomato resistance breeding have led to the commercial use of dozens of Resistance to *Phytophthora infestans* (Rpi) genes (Vleeshouwers *et al.*, 2011). However, little to no attention has been given to potential plant genetic components of biological control. Since most BCAs need to form close associations with plants in order to be effective at controlling phytopathogens, this is a hugely overlooked area

with great potential to enhance the effectiveness of biological control. Plant growth promotion by *Trichoderma* species, is highly dependent on plant genetics (Schmidt *et al.*, 2020) and thus future breeding efforts in crops such as potato would benefit from approaches that include genetic compatibility with biological control agents. This should be considered a key component in the IPM of potatoes, as shown in Fig. 2.

In Fig. 2 we present an overview of an IPM strategy for potato, in which biological control is a central component. Progress is being made towards more integrated disease control approaches, for example, as discussed above, combinations of BCAs, resistance inducers, and if absolutely necessary, reduced doses of fungicides show promising results for the future (Shukla *et al.*, 2021). Such treatments are likely to be most effective in cultivars that display some level of resistance, as is the case with the use of the resistance inducer phosphite (Liljeroth *et al.*, 2016). However, as with resistance breeding and the use of synthetic fungicides, BCAs are likely to have a pest load beyond which the pathogen develops resistance and thus, IPM strategies should be designed with evolutionary principles in mind, to ensure sustainability (Karlsson Green *et al.*, 2020). Using genetic information from wild resources for inverse breeding – keeping in mind various evolutionary factors (Egan *et al.*, 2018; Thormann *et al.*, 2014), maintaining intermediate levels of both tolerance and defence in plants (Fornoni *et al.*, 2004), increasing spatial (Yang *et al.*, 2019) and temporal (Mariotte *et al.*, 2018) genetic diversity, manipulating the off-season survival of the pathogen in the agroecosystem (Vetukuri *et al.*, 2020) and optimising plant health and the resilience of BCAs within the plant microbiome are some tactics that, when combined, can help reduce the risk of oomycetes developing resistance to IPM strategies such as biological control. For instance, very little is known about the longevity and survival of BCAs in the field, and more importantly about the time for a BCA to establish in the field. Nemec (1997) evaluated the longevity, survival and compatibility of *Bacillus subtilis*, *Trichoderma harzianum*, *Streptomyces griseoviridis*, and experimental single isolates of *Serratia plymuthica*, a *Pseudomonas flouorescens* parent, and its lacZY mutant, with the mycorrhizal fungus *Glomus intraradices* in a commercial planting mix. The study showed that the number of *Trichoderma* isolates increased slightly within 2 weeks after application and were stabilized through to the end of the test, around 8 weeks. In this study, *Bacillus* and *Trichoderma* species were the microorganisms with higher survival rates in a mix for potential use as BCAs in tomato. There are not many IPM strategies that include an evolutionary approach. It is, therefore, crucial to develop novel IPM strategies, that also fit the *P. infestans* pathosystem.

3.8. Conclusions

Large-scale *in vitro* screening approaches have had some notable successes in the identification of BCAs that reduce late blight disease severity *in agro* (Caulier *et al.*, 2018), however these successes are often down to the fact that large numbers of isolates have been screened to find a single successful BCA, or that the microbes tested were identified as potato or tomato endophytes initially. Whilst *in vitro* approaches are very useful for the identification of the modes of action of BCAs, new studies should focus on testing BCAs *in agro* both in terms of disease control as well as in terms of environmental survival in the agroecosytem. A better understanding of the interactions between BCAs and the soil microbiome will provide valuable ecological risk data and allow better formulations of existing BCAs. Furthermore microbiome studies, which are just in their infancy in potato, have the potential to allow us to identify potentially new BCAs that are adapted to the potato rhizosphere or live endophytically in close association with potato, and are crucially already adapted to the correct agroecological environment. Screening thousands of new microbes in the same manner as Caulier *et al.* (2018) whilst admirable is not always practical. Rather, we propose that such microbes should be screened for their production of secondary metabolites and CWDEs as well as their evolutionary potential in the agroecosystem. This is likely to aid in the identification of new BCAs with traits already adapted to the same environment as the pathogen. Further utilising these or existing well characterised BCAs as cell factories to bioengineer effective formulations of secondary metabolites as biopesticides is also a promising new direction in the hunt for sustainable control of late blight disease. Finally, practitioners should be encouraged not to simply replace their synthetic pesticide sprays with BCAs, but to utilise BCAs preventatively as soil amendments before or at planting, to better allow the establishment of healthy rhizosphere soil, in a similar manner to the use of pro and pre-biotics to support human health.

Funding

This work was supported by the European Union's Horizon 2020 research and innovation programme [grant agreements No 766048, (MSCA-ITN PROTECTA) and 774340 (OrganicPLUS)] and The Swedish research council Formas [grant number 2019-00881].

Declaration of competing interest

This manuscript has not been published previously, and is not under consideration for publication elsewhere and we have no conflicts of interest to disclose.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fbr.2021.11.003.

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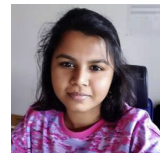
Maryam Hashemi obtained her BSc. In Agricultural Engineering-Agronomy and plant breeding from Isfahan University of Technology-Isfahan, Iran and pursued her MSc studies in the same university focusing on Agricultural Engineering- Plant Breeding. Maryam is currently a PhD student at the LRSV in Toulouse under the supervision of Bernard Dumas. Her work concerns the commercialization of oomycetes for biocontrol and bio-protection with an emphasis on using *Pythium oligandrum* as a biocontrol agent to fight against oomycetes diseases and is carried out in collaboration with the Biofungicide company, De Sangosse.

Dania Tabet is originally from Lebanon where she completed her bachelor's degree in Agricultural engineering specialization in plant protection at The Lebanese University, faculty of agriculture. She completed her her master's degree in Integrated Pest Management of Mediterranean fruit tree crops (IPM) from the Mediterranean Agronomic institute of Bari (CHIAM-Bari-Italy). Her master thesis focused on soil borne citrus and olive pathogens in nurseries. Currently she is pursuing her doctoral studies at Tuscia University in Italy under the supervision of Anna-Maria Vettraino. Dania hopes to identify new Eco-friendly control strategies against *Phytophthora* species and is investigating various species as biocontrol agents.

Murilo Sandroni has a background in Agricultural Engineering which he studied in his home country, at the University of São Paulo, Brazil. Murilo undertook a professional science master degree in Agricultural production at the University of Illinois Champaign–Urbana, USA followed by a double masters through the Erasmus Mundus program from the Polytechnic University of Valencia, Spain and the Georg-August-University of Göttingen, Germany, specialising in Plant Health in Sustainable Cropping Systems. Murilo is a PhD student at the Department of Plant Protection Biology, SLU, under the supervision of Erik Alexandersson and Laura Grenville-Briggs. Murilo is studying alternative methods of resistance to *Phytophthora infestans* in potato, by investigating the effects of Plant Resistance Inducers (PRIs) as low-risk alternatives to synthetic pesticides.

Clara Benavent-Celma has a background in Forestry Engineering, which she studied at the Polytechnic University of Valencia, Spain. She is studying for her PhD at the University of Aberdeen under the supervision of Steve Woodward and Pieter van West. Clara is interested in the study of alien invasive pest and pathogens, their detection, the damage they cause, how they are spread globally and the mitigation methods that can be used to reduce their impacts on natural ecosystems and is thus studying the biocontrol properties of *Pythium oligandrum* for the control of Phytophthora species in nurseries and the plants for planting pathway.



Jenifer Seematti completed a Bachelor of Technology in Genetic Engineering at SRM University, India. She completed her BSc thesis on kidney toxicology in mouse models at Harvard Medical School, USA. Following this, she completed an MSc in Plant Biotechnology at Wageningen University and Research, Netherlands. She is currently a PhD student at in Laura Grenville-Briggs' lab at the Department of Plant Protection Biology, at SLU, Alnarp. Her PhD project focus on functional characterization and population genetics of *Phytophthora infestans* effectors. She hopes to identify effectors to use in resistance breeding and biocontrol against potato late blight.

Christian Benjamin Andersen graduated from the University of Copenhagen with a master's degree in Agronomy before joining SLU as a PhD fellow. Christian is pursuing his PhD studies in Laura Grenville-Briggs' lab at the Department of Plant Protection Biology, SLU. He researches oomycetes for biological control of various plant pathogens and the effector biology of the mycoparasitic oomycete *Pythium oligandrum*. He also aims to improve the use of *P. oligandrum* as a biocontrol agent. Through his investigations into the ecological risk of using conventional pesticides and biological control agents in commercial agriculture he hopes to benefit both producers and society alike.

Laura Grenville-Briggs was educated at the Universities of Bath and Birmingham (PhD, 2003). After a post-doctoral period at the University of Aberdeen, UK, and a Marie-Curie Intra-European Fellowship at the Royal Institute of Technology, Stockholm, Laura joined the Swedish University of Agricultural Sciences (SLU) as an independent researcher in 2014 and was appointed Professor of Integrated Plant Protection in 2018. Her research group focuses on the molecular and genetic determinants of disease in oomycete and fungal phytopathogens and their interactions with microbial biocontrol agents. The goal is to develop more durable, environmentally sustainable integrated pest management strategies for plant diseases.

Acta Universitatis agriculturae Sueciae
Doctoral Thesis No. 2025:7

Potato late blight, caused by the oomycete *Phytophthora infestans*, remains a major agricultural threat. This thesis explores new roles for effectors from this pathogen. Firstly, in pathogenicity, by manipulating stomatal opening; secondly in the local adaptation of *P. infestans* populations; and thirdly in response to attack by mycoparasites. These findings emphasise the need to integrate molecular insights from the battle between biocontrol agents and their prey, along with region-specific strategies to achieve sustainable disease control.

Jenifer Seematti Sundar received her doctoral education at the Department of Plant Protection Biology, Swedish University of Agricultural Sciences and obtained her MSc in Molecular Plant Breeding and Pathology at Wageningen University and Research, Netherlands.

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SLU generates knowledge for the sustainable use of biological natural resources. Research, education, extension, as well as environmental monitoring and assessment are used to achieve this goal.

ISSN 1652-6880
ISBN (print version) 978-91-8046-442-0
ISBN (electronic version) 978-91-8046-492-5