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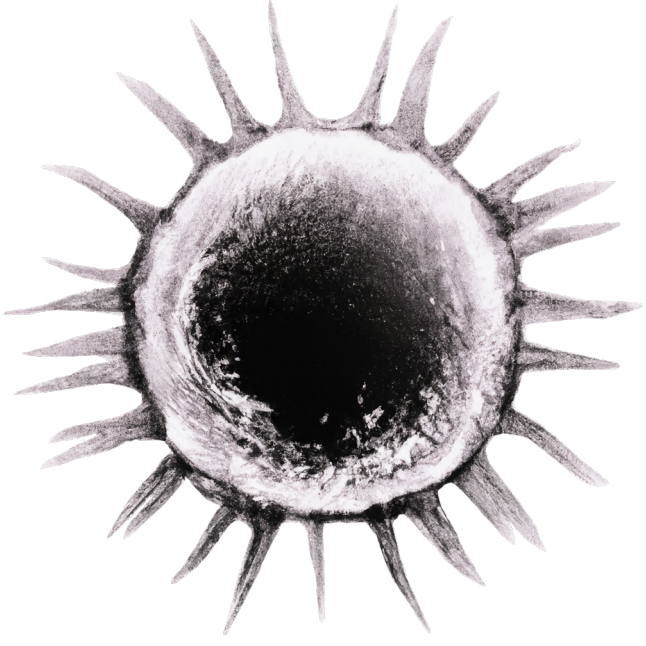
Doctoral Thesis No. 2023:20
Faculty of Landscape Architecture, Horticulture and Crop Production Science

Friend or Foe? Biocontrol interactions of *Pythium oligandrum* within the potato cropping system

Christian Benjamin Andersen



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Cover: Schematic representation of an oospore of *Pythium oligandrum*
(photo: Christian Benjamin Andersen & Mark Aron Thomsen, with assistance by Dall E 2)

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Abstract

The potato crop is a valuable but vulnerable food source on a global level. It is under constant pressure from destructive plant diseases such as early and late blight. Traditionally to control these diseases farmers have relied heavily on synthetic fungicide use. It is evident that the use of agrochemicals such as synthetic fungicides can cause unwanted effects on the environment, and human health. Thus, there is a need for a more sustainable approach to control diseases in the potato cropping system. One approach could be to use biological control agents, (BCAs) such as mycoparasitic *Pythium* species to control the disease. However, to utilize these BCAs in the best way we need to increase our fundamental knowledge about their biology and interactions within potato cropping systems. Therefore, the aim of this thesis is to broaden our fundamental knowledge of the mycoparasitic *Pythium* species, *Pythium oligandrum* and *Pythium periplocum*, and how they differentiate from their phytopathogenic counterparts. Further, the thesis also pinpoints characteristics that determine mycoparasitism in these two *Pythiums*, and how this relates to their biocontrol abilities, at the molecular level. This was elucidated using comparative genomics, transcriptomics. The result showed that ABC transporters, AVH-like RXLR effectors, and an expanded CAZyme among other things, are conserved and important for mycoparasitism, by *P. oligandrum* and *P. periplocum* both when preying on phytopathogens but also in comparison to closely related plant pathogens. This thesis also explores if *P. oligandrum* can be used as an antagonist against early blight disease on potato plants. The results showed that *P. oligandrum* did induce disease suppression in potato in controlled greenhouse environments, however in the field trials when the disease pressure was high the effects disappeared. The thesis also explores if and how *P. oligandrum* interacts with the potato plants. The results showed that *P. oligandrum* induced biostimulation in potato both in controlled environments and field studies, although the biostimulation was in a plant-genotype dependent manner. Finally, the thesis investigates the impact on the resident rhizosphere microbiome of field grown potato plants when *P. oligandrum* was applied. The results showed that *P. oligandrum* has a very limited impact on the diversity and community structure in the rhizosphere of potato. Overall, the work in this thesis has generated a deeper understanding of the fundamental biology of the mycoparasitic *Pythium* species and how they differ from plant pathogens. An increased understanding of the environmental consequences and how *P. oligandrum* can be utilized as a BCA in the potato cropping system has been elucidated. This work also contributes with pivotal knowledge about how *P. oligandrum* can be utilized in an IPM strategy in the potato agricultural cropping system.

Keywords: biocontrol agents, biostimulation, comparative genomics, early blight, late blight, microbiome, mycoparasitism, oomycetes, rhizosphere, transcriptomics

"Vän eller fiende? Biokontrollinteraktioner med *Pythium oligandrum* inom potatisodling"

Abstrakt

Potatis är en värdefull men sårbar livsmedelskälla på global nivå. Den är ständigt utsatt för skadliga växtsjukdomar såsom torrfläckssjuka och bladmögel. Traditionellt har lantbrukare förlitat sig på användning av syntetiska fungicider för att kontrollera dessa sjukdomar. Agrokemikalier som syntetiska fungicider kan dock orsaka oönskade effekter på miljön och människors hälsa. Därför finns det ett behov av en mer hållbar metod för att kontrollera sjukdomar hos potatis. En metod kan vara att använda biologiska bekämpningsmedel (BCA), såsom mykoparasitiska *Pythium*-arter, för att kontrollera sjukdomar. För att kunna använda dessa BCA på bästa sätt behöver vi öka vår grundläggande kunskap om deras biologi och biotiska interaktioner inom potatisodlingen. Syftet med denna avhandling är att bredda vår grundläggande kunskap om de mykoparasitiska *Pythium*-arterna, *P. oligandrum* och *P. periplocum*, och hur de skiljer sig från sina fytopatogena motsvarigheter. Avhandlingen pekar också ut egenskaper som är länkade med mykoparasitism hos dessa två arter och deras koppling med biokontrollförmåga på molekylär nivå. Detta studerades med hjälp av jämförande genomik och transcriptomanalys. Resultatet visade bland annat att ABC-transportörer, AVH-liknande RXLR-effektorer och ett utökat CAZyme är konserverade och viktiga för mykoparasitism hos både *P. oligandrum* och *P. periplocum* när de prederar på fytopatogener i jämförelse med nära besläktade växtpatogener. Avhandlingen utforskar också om *P. oligandrum* kan användas som antagonist mot torrfläckssjuka på potatisplantor. Resultaten visade att *P. oligandrum* reducerade angreppen hos potatis i kontrollerade växthusmiljöer, men i fältförsöken när sjukdomstrycket var högt försvann de positiva effekterna. Avhandlingen utforskar också om och hur *P. oligandrum* interagerar med potatisplantorna. Resultaten visade att *P. oligandrum* inducerade biostimulering av tillväxten hos potatis både i kontrollerade miljöer och fältstudier, även om biostimuleringen var beroende av potatisgenotyp. Slutligen undersöker avhandlingen effekten av att applicera *P. oligandrum* på mikrobiomet i rhizosfären hos fältodlade potatisplantor. Resultaten visade att *P. oligandrum* har en mycket begränsad påverkan på diversiteten och samhällsstrukturen hos mikroorganismerna i rhizosfären hos potatis. Sammanfattningsvis har denna avhandling genererat kunskap om grundläggande biologi hos mykoparasitiska *Pythium*-arter och hur de skiljer sig från växtpatogener. Avhandlingen har också belyst hur *P. oligandrum* kan användas som BCA inom potatisodling samt ökat förståelsen av miljömässiga konsekvenser av användningen. Denna kunskap är central för hur *P. oligandrum* kan användas i en IPM-strategi inom potatisodlingen.

Nyckelord: biokontrollmedel, biostimulering, jämförande genomik, mikrobiom, mykoparasitism, oomyceter, rhizosfär, sen bladmögel, tidig bladmögel, transkriptomik.

Dedication

I dedicate this work to my family and my friends. Thank you all for being there when the boat was rocking.

“Not all those who wander are lost”
J.R.R. Tolkien

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. Dong Liang[*], **Christian B. Andersen[*],** Ramesh R. Vetukuri, Laura J. Grenville-Briggs. New insights into mycoparasitism in *Pythium* species revealed by comparative genomics and transcriptomics (Manuscript). [*]Co-first author.

II. Dong Liang, **Christian B. Andersen**, Ramesh R. Vetukuri, Daolong Dou, & Grenville-Briggs, L.J. (2020). Horizontal Gene Transfer and Tandem Duplication Shape the Unique CAZyme Complement of the Mycoparasitic Oomycetes *Pythium oligandrum* and *Pythium periplocum*. *Frontiers in Microbiology*, *11*, 2609. https://doi.org/10.3389/fmicb.2020.581698. (Published)

III. **Christian B. Andersen[*]**, Kristin Aleklett, Garima Digdarshika , Åsa Lankinen[*] and Laura J. Grenville-Briggs. *Pythium oligandrum* induces growth promotion in starch potato without significantly altering the rhizosphere microbiome. (Manuscript). [*]Corresponding authors

IV. **Christian B. Andersen[1*]**, Simon B. Lassen, Maja Brus-Szkalej, Linnea J. Stridh, Åsa Lankinen, Laura J. Grenville-Briggs. Resilience of the potato rhizosphere microbiome during both early blight disease and application of the biocontrol agent *Pythium oligandrum* (Manuscript). [*]Corresponding author.

V. Linnea J. Stridh, Hadis Mostafanezhad, **Christian B. Andersen,** Firuz Odilbekov, Laura J. Grenville-Briggs, ÅsaLankinen, & Erland Liljeroth. (2022). Reduced efficacy of biocontrol agents and plant resistance inducers against potato early blight from greenhouse to field. *Journal of Plant Diseases and Protection*, *129*(4), 923–938. https://doi.org/10.1007/s41348-022-00633-4 (Published)

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The contribution of Christian Benjamin Andersen to the papers included in this thesis was as follows:

I. Wet lab work including setting up interaction assays and RT-qPCR, interpretation of the results and co-writing of the manuscript.

II. Wet lab work including setting up interaction assays, growth assays and RT-qPCR, partial data analysis and co-writing of the manuscript.

III. Co-design of experiments, planning and execution of field, greenhouse and microbiome assays, DNA extractions, data analysis and writing of the manuscript.

IV. Co-design of experiments, planning and execution of field, greenhouse and microbiome assays, DNA extractions, data analysis and writing of the manuscript.

V. Wet-lab work, including production and application of *P. oligandrum* inoculum, partial writing, and editing.

Abbreviations

A. solani	*Alternaria solani*
ABC	ATP-binding cassette (transporter)
AUDPC	Area Under the Disease Progression Curve
AVH	avirulence homolog
B. cinerea	*Botrytis cinerea*
BCA	Biological Control Agent
bp	base pair
CAZymes	carbohydrate-active enzymes
CWDE	Cell Wall Degrading Enzymes
cv.	cultivated variety
DEGs	Differentially Expressed Genes
hpi	hours post interaction
IAA	Indole-3-acetic acid
LCA	Last Common Ancestor
MBCA	Microbial Biological Control Agent
MRCA	Most Recent Common Ancestor
NGS	Next Generation Sequencing
NMDS	Non-Metric Multidimensional Scaling
P. oligandrum	*Pythium oligandrum*
P. Periplocum	*Pythium periplocum*
PCoA	Principal Coordinate Analysis
PFAM	Protein families database
Ph. Infestans	*Phytophthora infestans*
rAUDPC	relative Area Under the Disease Progression Curve
RNA-seq	RNA sequencing
ROS	Reactive Oxygen Species

RT-qPCR	Real Time quantitative PCR
VOC	Volatile Organic Compound
WDG	Water Dispensable Granules

1. Introduction

With the worldwide rapid growth of the human population, there is an increased demand for stable and sustainable food sources. In fact, projections have estimated that global demand will increase between 50-60 % by 2050 (Falcon et al., 2022). On the contrary, the amount of arable land to produce food crops has declined per capita since 1961 (Richard et al., 2022). Therefore, there is a need for high yielding crops to meet the demand for more food. One staple food crop that is highly important in this context is potato. Potato (*Solanum tuberosum* L.) is the fourth most cultivated crop in the world (Lovat et al., 2016), and has a high nutritional value (Burgos et al., 2020). Potatoes have strongly benefited from agricultural intensification; the availability of agrochemicals, fertilizers and heavy machinery for crop management have increased the yield potential by almost 190 % since the 1960s (Meno et al., 2021). However, potatoes are vulnerable to destructive plant diseases such as early blight caused by fungi of the genus *Alternaria* and late blight caused by the oomycete *Phytophthora infestans*. These diseases are known to inflict heavy yield losses in potatoes (Odilbekov et al., 2020; Savary et al., 2019) and it has been estimated that the cost to control late blight alone exceeds 7 billion euros annually (Haverkort et al., 2016).Additionally, the intensive application of chemical fungicides has been correlated with risks to both human health and the environment (Ivanov et al., 2021). Thus, in 2014 the EU mandated that the use of agrochemicals should be reduced and the use of Integrated Pest Management (IPM) strategies should be promoted (Barzman et al., 2014; Deguine et al., 2021).
IPM is thought of as a sustainable approach to manage pests and diseases, which involves using a combination of techniques to minimize damage to the crops while also minimizing the use of synthetic agrochemicals.

One of the key tools in the IPM management portfolio is biological control (biocontrol) (Hashemi et al., 2022). Examples of biocontrol agents (BCAs) that can potentially be used in potato are the mycoparasitic *Pythium* e.g., *Pythium oligandrum* and *Pythium periplocum,* as they have shown antagonistic effects against a broad range of plant diseases (Bělonožníková et al., 2022). However, to successfully harness the potential of these mycoparasites it is important to understand the fundamental biology and the modes of action that these mycoparasites exhibit. Expanding knowledge of these organisms on a genome level can unravel mycoparasitic determinants and this knowledge can potentially be exploited to increase the efficacy of the biocontrol agents. To do so, effective assays must be established, where modern day methods can be utilized. Plant microbe interactions have traditionally been investigated as binary systems, but viewing and investigating the plant as a holobiont, could broaden our understanding of the pathosystem of potato diseases and the general health of the plants. This, in turn, can be translated to an increased efficacy of the BCAs that can benefit plant productivity. BCAs are viewed upon as being an eco-friendly approach. However, although they have been well studied in controlled growing systems, we lack information on how they interact with the host and impact the environment and the non-target species within cropping systems in open field agriculture. Analysis of, for example, the rhizosphere microbial communities (the microbiome) of potato plants, has the potential to enhance our understanding and importance of these interactions. This thesis offers an insight into these topics and investigates the potential benefits and risks related to the use of the BCAs *P. oligandrum* and *P. periplocum* within the potato cropping system.

2. Background

2.1 What is biological control?

In the process of agricultural development, agrochemicals, such as synthetic pesticides, have been widely used to combat plant disease and other pests in agricultural production systems (Sharma et al., 2019). However, their continuous and abusive application has been associated with harmful side-effects which have led to concerns about environmental and human health (Sharma et al., 2019). Therefore, the EU has fostered the idea that the use of agrochemicals in agricultural production systems should be reduced. The Directives 2009/128 and 2019/782, adopted by the EU, not only restrict the use of synthetic chemicals, but also promote the use of sustainable and environmentally friendly plant protection methods. These directives encourage the use of Integrated Pest Management (IPM), which includes alternative approaches to synthetic pesticides, such as biocontrol. As a consequence, there has been a significant growth in the field of research on biocontrol (Barratt et al., 2018).

Biocontrol is not a newly discovered approach. In fact, the application of natural enemies to control pest populations has been known and used for centuries (Smith, 1919). More recently, biocontrol agents have been found to suppress Dermatophytes related to humans (Gabrielová et al., 2018), rodent pests in agriculture (Schlötelburg et al., 2020) , insect pests (Meyling & Eilenberg, 2007), and plant diseases (Raymaekers et al., 2020). In relation to agriculture, biocontrol has been defined in a broad sense as any method, product or organism used in the context of integrated crop protection and derived from another living organism (Stenberg et al., 2021). Biocontrol of

plant diseases can be defined as the application of beneficial micro (or macro) living organisms to control aerial or soilborne plant pathogens (Hashemi et al., 2022; Köhl et al., 2019a) The mode of action (MOA) of microbial biocontrol agents (BCAs) can be classified into five distinct groups, which are briefly summarized below:

1) Induced resistance:

BCAs can induce resistance in plants by activating innate immunity or can produce plant hormone precursors or analogues that protect the host plant from incoming pathogens.

2) Competition:

BCAs can outcompete potential plant pathogens in the search for scarce resources, for instance limited nutrients such as iron, water or simply space.

3) Hyperparasitism/Mycoparasitism:

BCAs can directly infect and/or feed on the plant pathogen.

4) Antibiosis:

Antibiosis is often associated with hyperparasitism. However, antibiosis does not require direct physical contact between the BCA and the pathogen. The BCA instead secretes antimicrobial compounds, for instance toxins or lysing enzymes (cell wall degrading enzymes), restricting the growth of the pathogen.

5) Mixed MOAs:

Most biocontrol agents contain several of the above-described MOAs, *e.g.* the BCA *Trichoderma harzianum* (combination of induced resistance, competition, hyperparaitism and antibiosis) (Elad, 2000; López-Mondéjar et al., 2011).

Apart from the MOAs the biocontrol efficacy of a BCA depends on several additional factors, *e.g.* the prey it antagonises, the host plant and the environment that it is released into (Köhl et al., 2019).

2.2 The oomycetes: not just destructive pathogens

The oomycetes are a group of organisms that are found in multiple habitats. They are unicellular eukaryotes and belong to the Kingdom Chromista (Cavalier-Smith, 1981). The class of oomycetes contains many important and destructive pathogens, including plant pathogens e.g. *Phytophthora infestans*, fish pathogens such as *Saprolegnia parasitica*, and human pathogens e.g. *Pythium insidiosum* (**Fig. 1**). The socioeconomic impact of the diseases caused by oomycete pathogens is huge and accounts for several billions of dollars across industries (Derevnina et al., 2016; Haverkort et al., 2016; Pavić et al., 2022). The most notorious and well-studied oomycete is *Ph. infestans*, the causal agent of late blight on potato and tomato, and the biological agent behind the Great Irish (and European) Potato Famine of the 1840s (Goss et al., 2014). It has, therefore, been described as the oomycete with the greatest impact on humanity and has been developed as a model species for the study of oomycetes at the molecular level (Kamoun et al., 2015). However, not all oomycetes are disease causing agents. Within the oomycete class, there are species with biocontrol properties, such as *Pythium oligandrum* and *Pythium periplocum* (**Fig. 1**, group 2). These are closely related species that are mycoparasitic on a wide range of fungal and oomycete prey.

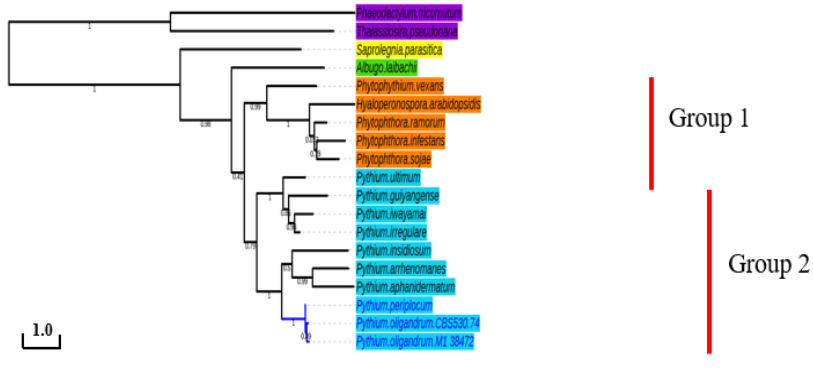

Figure 1. Phylogenetic relationship among mycoparasitic oomycetes and plant pathogenic oomycetes, *Phaeodactylum tricornutum* and *Thalassiosira pseudonana* were used as outgroups (displayed in purple). Group one shows the phytopathogenic plant pathogens (orange). Group 2 shows phytopathogenic *Pythium* species in black and the mycoparasitic *Pythium* species in dark blue.

Historically, oomycetes were classified as fungi. However, several distinct characteristics separate them from the Kingdom Fungi. Phylogenetic analyses revealed that fungi share a common ancestor with animals, whereas the closest relatives of the oomycetes are the heterokont golden-brown algae (Baldauf et al., 2000). There are important morphological and physiological differences between fungi and oomycetes as well. Oomycetes are diploid in contrast to fungi which for most of their lifecycle are haploid or dikaryotic. Oomycete hyphae are coenocytic (non-septate), whereas fungal hyphae contain septa. Most of the oomycetes display sterol autotrophy, while fungi do not (Latijnhouwers et al., 2003). Several other characteristic differences exist including the composition of the cell wall; the most important structural component of the oomycete cell wall is cellulose, along with 1,3-β-glucans, some 1,6-β-glucans and 1,4-β-glucans (Sietsma et al., 1969), whereas the major component of the fungal cell wall is chitin (Latijnhouwers et al., 2003). The reproduction of oomycetes occurs when the female organ the "oogonia" fuses with the male reproductive organ the "antheridia". Within the oogonia another cell wall is made, surrounding the new nucleus. This results in the formation of the oospore. The oospore is a tough and environmentally resilient spore that can remain dormant for years, until the conditions become favourable for germination. This means that soil-borne pathogens may survive many years as dormant oospores, which are generally resistant to pesticides. The resilient characteristics of oospores are, however, an advantage for the mycoparasitic *P. oligandrum and P. periplocum*, since they appear to be a stable inoculum source which, if formulated correctly, can be utilised in the production of commercial biocontrol products.

2.3 Insights into the genomes of oomycetes and mycoparasitic fungi

The genome of an organism holds a wealth of information about its evolution, physiology and ecology. Thus, genomic data is especially valuable for the fundamental understanding of how and why plant pathogens and biocontrol agents work.

With the development of next generation sequencing (NGS) platforms, it is now possible to rapidly and economically sequence and compare a large number of genomes (Adhikari et al., 2013). One tool that broadens our

understanding of the genomic features of an organism is comparative genomics. Comparative genomics involves comparisons of genomic features between different or related organisms (Massart et al., 2015). The specific approach has been used to compare the genomes of two mycoparasitic *Trichoderma* species (*T. atroviride* and *T. virens*) to the saprotrophic (non-mycoparasitic) *Trichoderma reesei* (Kubicek et al., 2011). Comparison of the genomes of these species revealed that certain gene families, such as proteases, the CAZyome complement, chitinolytic enzymes, etc. were expanded only in the mycoparastitic species. In addition, the saprotrophic *Trichoderma* species had lost more than half of the genes coding for the production of secondary metabolites in comparison to the mycoparasitic species (Kubicek et al., 2011). Interestingly, Karlsson *et al.* (2015) used a comparative genomic approach to compare the same *Trichoderma* mycoparasites with the mycoparasite *Clonostachys rosea.* Although the authors expected that the species would share similar mycoparasitic mechanisms due to their similar lifestyles, they found that the majority of the mycoparasitic genes in *C. rosea* did not evolve in the same manner as those in *Trichoderma* species. Specifically, members of the ABC transporter families, probably associated with exposure to mycotoxins produced by fungal prey and/or chemical fungicides (Karlsson et al., 2015; Kosawang et al., 2014), were expanded in *C. rosea.* Additionally, in contrast to the results from Kubicek *et al.* (2011), the CAZyome complement of *C. rosea* was found to be less important as a mycoparasitic feature for this species (Karlsson et al., 2015). These studies demonstrate how valuable comparative genomics is in elucidating specific mechanisms important for the lifestyle and behaviour of an organism.

So far, at least 65 genomes of both plant and animal pathogenic oomycetes are available in public repositories (McGowan & Fitzpatrick, 2020). By combining comparative genomic studies of various species along with transcriptomic studies, gene families responsible for the virulence of phytopathogenic oomycetes have been identified (Dong et al., 2015). A breakthrough in the understanding of the virulence of phytopathogenic oomycetes was the identification of effector proteins, and specifically, the Crinkler (CRN) and the RXLR effectors. These effectors are thought to enter host cells and change the cellular environment, thereby facilitating disease and helping the pathogen evading the host inherent immunity (Haas et al., 2009; Kiselev et al., 2022; Lévesque et al., 2010; Whisson et al., 2007) . The

Crinkler (Crn) genes in *Phytophthora* spp. encode a significant family of effector proteins that are secreted and contain a consistent motif in their amino-terminal domain (Haas et al., 2009). Similar amino-terminal domains have also been discovered in the proteins of other oomycete pathogens (Gaulin et al., 2008; Win et al., 2007). It has been proposed that this N-terminal sequence motif in these proteins is responsible for their transfer into the plant cells, ultimately being fatal for the plant cell (Schornack et al., 2010).

Another group of effectors that have received a lot of attention is RXLR-effectors. RXLR effectors were first predicted in 2005 from comparative analyses of genes encoding oomycete proteins recognised by plant resistance genes (Rehmany et al., 2005) . In 2007, Whisson *et al.* (2007) used the Hidden Markov model and regular gene expression and identified RXLR effectors in *Phytophthora* species. Since then, many RXLR effectors have been identified in oomycete phytopathogens. However, until recently, they were not identified in the mycoparasitic *Pythium* species. Ai *et al.* (2020) identified, cloned and tested a total of 73 putative RXLRs from the mycoparasitic *Pythiums* in *Nicotiana benthaminana* to discover if these effectors triggered cell death. Only three out of 73 putative RXLRs were found to trigger cell death and plant defense responses. This indicates that the remaining 70 effectors may have a different role, for instance in mycoparasitism or interactions with other organisms. Thus, RXLR effectors might be important mycoparasitic determinants. As of today, comparative genomics between the mycoparasitic *Pythium* species and their oomycete phytopathogenic relatives remains underexplored. This could provide valuable information about the mycoparasitic lifestyle and provide key knowledge on exactly what mechanism determines the mycoparasitism for these BCAs.

2.4 The biology of mycoparasitic *Pythium* species

The oomycete class contains approximately 130 different *Pythium* species, inhabiting both terrestrial and aquatic environments (LéVesque & De Cock, 2004). Many species within the *Pythium* genus are phytopathogens, however, a few species have received attention in phytopathology due to their mycoparasitic behaviour. The mycoparasitic *Pythium* species are a

group of oomycetes that can infect and kill phytopathogenic oomycetes and fungi. They have been found to decrease plant disease in a wide variety of crops and account for the disease suppression of more than 40 different plant pathogens, from bacterial, fungal and oomycete origin (Bělonožníková et al., 2022). They were first morphologically described by Drechsel (1943), as species with spiny oogonia parasitizing the plant pathogenic *Pythium* species. The mycoparasites described included *Pythium oligandrum, Pythium periplocum* and *Pythium acanthicum.* Since then four other mycoparasitic *Pythium* species have been discovered including *Pythium nunn, Pythium mycoparasiticum, Pythium acantophoron* and *Pythium lycopersicum* (Benhamou et al., 2012). Among these mycoparasites, *P. oligandrum* and to some extent *P. periplocum* are the most researched, and efficient, in terms of mycoparasitism and agronomic relevance. Thus, in this thesis the term mycoparasitic *Pythium* species refers specifically to those two species.

P. oligandrum is recognized as a mycoparasite of a wide range of plant pathogens, as recently reviewed in Bělonožníková *et al.*(2022). It is fast-growing (up to 35 mm/day), which may indicate an ability to outcompete other species for space and nutrients in the plant rhizosphere (Takenaka et al., 2008). *P. oligandrum* is a homothallic species, which means it can self-fertilize. It has an asexual and sexual life cycle. In the asexual cycle, it exists as a diploid zoospore which is a flagellated cell that can move through water. In the sexual cycle, it forms thick-walled oospores which are the result of fertilization of oogonia by antheridia. The majority of oogonia develop parthenogenetically, but some form sexually. Oospores can survive for long periods before germination, they are resilient to environmental changes and thus serve as a good source for formulation of the BCA to be used in agriculture. In **Figure 2 A-E**, the development process of an oospore of *P. oligandrum* can be observed. The immature oospore stage, as seen in **Figure 2 A**, is followed by the stage where the formation of spikes is observed (**Fig. 2 B**). The mature resting oospores (**Fig. 2 C**) are the common source for formulation of *P. oligandrum* used in agricultural cropping systems. When conditions are favourable the oospores can germinate forming several hypha as seen in **Figure 2 D-E**

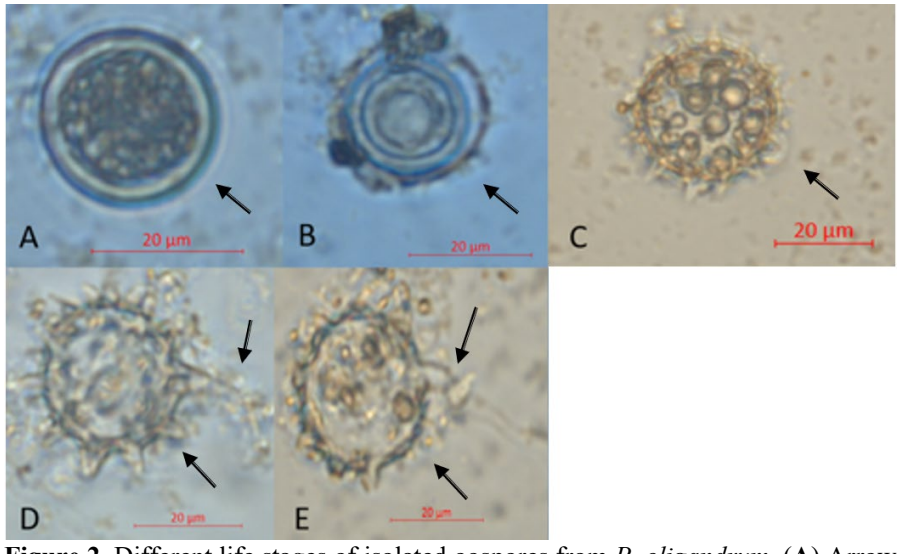

Figure 2. Different life stages of isolated oospores from *P. oligandrum*. (**A**) Arrow points towards an unripe oospore. (**B**) Intermediate ripening, arrow points towards initial spike formation. (**C**) Ripe oospore ungerminated but metabolic active, the spherical circles are possibly lipid formation. (**D - E**) Ripe oospores that are germinating with one or several hyphae. Picture acquisition performed with a Zeiss Axio observer inverted microscope at 40X magnification.

In the tip of the hyphae inflated sporangia can form and these sporangia can differentiate to release motile wall-less zoospores. These zoospores can move in soil in damp environments, after which they can encyst by secreting material to form a new cell wall. Cysts can germinate, forming new hyphae, or can directly infect plant tissue.

The mycoparasite *P. periplocum* was originally isolated in the USA, and found to cause blossom end-rot in watermelon plants (Drechsler, 1939). However, in 1992 Hockenhull *et al.* demonstrated the mycoparasitic abilities of this species, against damping-off in cucumbers. Since then it has been recognized as an aggressive mycoparasite against *Botrytis cinerea* (Paul, 1999) and many other fungal pathogens (Ribeiro & Butler, 1995). *P. periplocum* has a similar lifecycle to that of *P. oligandrum.* However, there are a few distinct morphological characteristics that can be used to separate the two species. In comparison to *P. oligandrum,* the oospores of *P. periolocum* are significantly smaller (Ribeiro & Butler, 1995). It has also been proposed to be the only member of the *Pythium* genus that possesses

ornamented oogonia and a filamentous inflated type of sporangia (Paul, 1999)

2.5 Determinants of mycoparasitism in Pythium species

Antagonism of prey

Direct antagonism has been identified to be a key determinant of the antagonistic ability of the mycoparasitic *Pythium* species (Benhamou et al., 2012; Daly et al., 2021; He et al., 1992; Picard et al., 2000). The first step is chemotrophic growth towards the prey. In **Figure 3 A**, an example of the early stages of mycoparasitism can be seen, where growth of *P. oligandrum* towards *Phytophthora infestans* (prey) is followed by initial adhesion and direct penetration around 6 hours post interaction (hpi). This is initiated by the sensing of the prey. In 2012 Horner *at al.* identified protease encoding transcripts when *P. oligandrum* was feeding on dead *Ph. infestans* tissue, leading to the hypothesis that these proteases are involved in the sensing of the prey. Similar mechanisms have been speculated for *Trichoderma* species (Seidl et al., 2009). The recognition events between an antagonist and a prey typically involves the use of cell surface molecules from both entities. In the case of *P. oligandrum* and the fungus *Fusarium oxysporum*, it appears that a matrix containing chitin from the prey is involved in the recognition process (Benhamou et al., 1999). This suggests that *P. oligandrum* has cell surface peptide receptors that are capable of binding to N-acetylglucosamine (Benhamou et al., 2012). Transcripts encoding tyrosine- and glycine-rich proteins, found in the cell wall of *P. oligandrum* are accumulated, during interactions with *Ph. infestans* hyphae, leading to the assumption that these proteins are involved in the recognition of the prey and attachment to it (Horner, et al., 2012). We recently proposed that the cellulose-binding elicitor lectin family (CBEL) is also important for sensing and adhering to the oomycete prey, *Ph. infestans*, based on the fact that the respective genes were upregulated in *P. oligandrum* during the interaction with the prey (Liang et al., 2020). However, more research is needed to pinpoint the exact mechanism the mycoparasites use to sense and adhere to their prey. After adhesion, the mycoparasites coil around prey hyphae, a behaviour observed

in interactions with fungal pathogen *F. oxysporum* (Benhamou et al., 2012), the oomycete *Ph. infestans* (Horner et al., 2012), as well as with phytopathogenic *Pythium* species (Berry, Jones & Deacon, 1993; Daly et al., 2021). In **Figure 3B** the start of coiling of *P. oligandrum* hyphae is observed around *Ph. infestans* hyphae. Once coiled around the prey, *P. oligandrum* penetrates the cell wall. It has been observed that this phase is accompanied by formation of several papilla-like structures at sites of potential penetration (Benhamou et al., 2012). After 48 hours, the prey hyphae have typically been degraded due to the extensive feeding of the mycoparasite (**Fig. 3 D**).

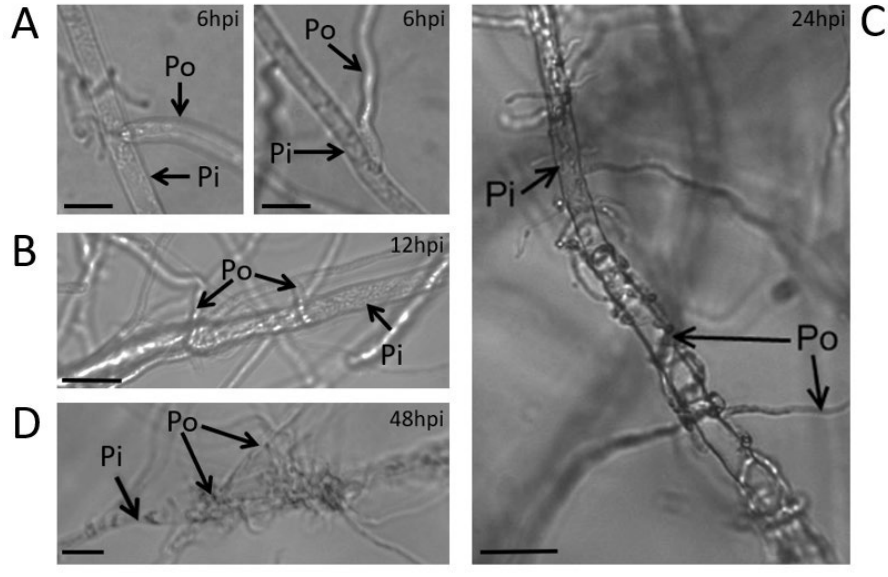

Figure 3. Hyperparasitism by *P. oligandrum* when interacting with *Ph. infestans.* (**A**) At 6hpi directional growth towards prey occurs, contact initiates coiling and/or direct penetration of prey hypha. (**B**) At 12hpi hyphae from the mycoparasite converge on the prey hypha and begin coiling. (**C**) At 24hpi coiling of mycoparastic hyphae along the prey hypha can be clearly seen. (**D**) 48hpi the prey hypha begins to be degraded by a mass of mycoparasitic hyphae feeding on the prey tissue. Scale bar = 20um. Here, *P. oligandrum* (Po) and *Ph. infestans* (Pi). With permission from Laura Grenville-Briggs.

Degradation of the prey cell wall

Oomycetes including the mycoparasitic *Pythium* species, can be characterised by their osmotrophic lifestyle, thus secreting enzymes for direct or indirect acquisition of nutrients for degradation of organic debris or host cells (McGowan & Fitzpatrick, 2017). Indeed, degradation of host cell wall is likely pivotal for the mycoparasitic lifestyle. Firstly, for successful penetration of prey cell walls and secondly for acquisition and absorption of nutrients released during lysis of the cells. Production of degrading enzymes by *Pythium* species was first reported by Krátká & Veselý (1979). Since then, the repertoire of known hydrolytic enzymes from mycoparasitic *Pythium* species has been widely expanded (Faure et al., 2020; Kushwaha et al., 2017a, 2017b; McGowan & Fitzpatrick, 2017). Among the most important enzymes responsible for cell lysing, is a repertoire of cell wall degrading enzymes (CWDE) belonging to the carbohydrate active enzyme complement, the CAZyome. The CAZyome is the collection of predicted genes coding for enzymes which are involved in carbohydrate metabolism in an organism (CAZymes). These enzymes are involved in the modification of structural components of the cell wall, synthesis of cell wall components, and degradation or turn-over of cell wall components (Ospina-Giraldo et al., 2010; Zerillo et al., 2013). The CAZyome is important for phytopathogens, when infecting host plants, aiding in the degradation of the host cell wall and thus easing the penetration of the epidermis (Ospina-Giraldo et al., 2010; Zerillo et al., 2013). However, the CAZyome has also been identified to be important for mycoparasites, when feeding on prey, for instance in *Trichoderma* ssp. (Kubicek et al. 2011) and in *Clonostachys rosea* (Tzelepis et al., 2015). In the mycoparasitic *Pythium* species the CAZyome may also be important for feeding on prey species (Horner et al., 2012) and it was actually found to be enriched in the genome of *P. oligandrum* (Faure et al., 2020). Thus, the CAZyome of the *Pythium* species should be investigated thoroughly as an important mycoparasitic determinant, e.g. using comparative genomic and transcriptomic approaches. This will increase our understanding of the mechanisms behind the hydrolysis of prey cells by the mycoparasitic *Pythium* species, thus broadening our understanding of their mode of action and our potential to utilize this to increase their biocontrol abilities in the future.

Defense against prey toxins

Mycoparasitic BCAs need a mechanism to detoxify secondary metabolites and/or toxins produced by their preys, released upon hyphal lysis or secreted as defence molecules. The ABC transporter gene family is common and abundant in eukaryotes and they are vital in several biological processes, such as transport of molecules across cell membranes against concentration gradients using ATP hydrolysis. ABC transporters have been identified to be more abundant or the expression of their encoding genes was higher in mycoparasitic fungi during feeding on different prey species (Kim & Vujanovic, 2022; Marra et al., 2006; Zhao et al., 2020). BCAs belonging to the *Trichoderma* genus have a variety of gene families belonging to the ABC-supergroup that have been associated with the ability to withstand toxic compounds produced by pathogenic fungi (Ruocco et al., 2009). Horner *et al*. (2012) identified several ABC-transporters expressed in *P. oligandrum* when feeding on *Ph. infestans* tissue. Although not much is known about the role of ABC-transporters role in mycoparasitic *Pythium* species, they might possess a similar function to that reported in fungal BCAs and could also be important mycoparasitic determinants.

Antibiosis by mycoparasitic *Pythium* species

Antibiosis has been reported for *P. oligandrum* although it is hypothesised to be of minor importance for this species. Bradshaw-Smith *et al.* (1991) found that a volatile organic compound (VOC) was responsible for reduced growth rate of *Phoma medicagini* and *Mycosphaerella pinodes.* Degradation of *Phytophthora megasperma* has been observed without physical contact by *P. oligandrum,* indicating antibiosis (Benhamou et al., 1999). However, to date, the compounds responsible for antibiosis have not yet been identified and characterised (Bělonožníková et al., 2022). An arsenal of VOCs was identified in *P. oligandrum,* four of which were found to have a strong inhibition on growth of *Pythium myriotylum.* The strongest inhibition was identified for methyl heptenone (96%), d-limonene (85%), 2-undecanone (70%), and 1-octanal (66%) (Sheikh et al., 2023).

2.6 Symbiont-plant interactions of mycoparasitic *Pythiums*

Both mycoparasitic *Pythium* species described in this thesis have been isolated from the rhizosphere of many plants species, as reviewed in Gerbore *et al.* (2014). Cytological studies have given an insight into how they colonize the roots of tomato plants. Le Floch *et al.* (2005) showed that the colonization of tomato roots starts with massive penetration and proliferation across the root epidermis, and colonization of the root cortex. Electron microscopic examination and cytochemical labelling showed that after inoculation with *P. oligandrum*, the oomycete rapidly and deeply colonized the cortex of tomato roots within 12 hours. Afterwards, the hyphae began to deteriorate and only a few host responses were observed. By the end of the experiment, after 48 hours, most hyphae looked distorted, sometimes collapsed, with empty shells (Gerbore et al., 2014). According to the authors, the most interesting observation was the formation of oogonia, that seemed to be alive, in cortical, parenchyma and xylem vessels, without causing any symptoms within the root system (Le Floch et al., 2005). This indicates that the mycoparasitic *Pythium* species can form symbiotic relationships with plants from the family of *Solanaceae* and are not *per se* recognized as pathogens

Induction of plant defences

Plant defence-related genes were upregulated when the cell wall fraction from *P. oligandrum,* specifically containing the elicitin like proteins POD-1 and POD-2, was applied on tomato seedlings (Takenaka et al., 2011). The molecule oligandrin, as well as other elicitin-like proteins, isolated from *P. oligandrum* can induce plant defence or resistance responses which help plants to better withstand infections from both fungal and oomycetal phytopathogens (Bělonožníková et al., 2022; Louet al.,, 2011; Ouyang et al., 2015)fter treatment with oligandrin physical strengthening of the plant cuticle and cell wall was observed in tomato plants. The authors also reported that the plants treated with oligandrin exhibited significantly decreased infection by *Phytophthora parasitica* (Picard et al., 2000). Altered cell wall, appositions were also observed in oligandrin-treated tomato plants after infection with *Fusarium oxysporum* (Benhamou et al., 2001). Collectively,

this highlights that elicitors from *P. oligandrum* can induce resistance or prime the plants to better tolerate pathogen attacks.

Biostimulation

In the present thesis biostimulation will be defined as the stimulation of plant growth mediated by the addition of a BCA. Biostimulation by the mycoparasitic *Pythium* species has only been reported for *P. oligandrum*, which has been shown to promote growth in a variety of vegetable and other crop species. This growth promotion has been demonstrated in *Solanaceae*, specifically in tomatoes (Le Floch et al., 2003), in greenhouse production of pepper plants (Al-Rawahi & Hancock, 1998), in field cultivated sugar beets (Veselý, 1989), cucumbers (Kratka et al., 1994), and even in monocots, such as rice plants (Cother and Gilbert, 1993). The benefits of *P. oligandrum* in terms of plant growth have been linked to many positive outcomes, including increased yield of tomatoes (Le Floch et al., 2003) and improved plant health (Bělonožníková et al., 2022). The mechanisms behind the observed biostimulation have been investigated in detail in tomatoes. Le Floch *et al.* (2003) showed that biostimulation was a result of increased tryptamine availability. Tryptamine is a precursor of the well-known plant-growth stimulating phytohormone auxin. Le Floch *et al.* (2003) found that *P. oligandrum* can produce tryptamine (TNH2) in a culture medium amended with auxin precursors, including tryptophan and indole-3-acetaldehyde. *P. oligandrum* metabolizes tryptophan and indole-3-acetaldehyde into tryptamine through the tryptamine pathway (Le Floch et al., 2003). The TNH2 produced in this way is highly bioavailable, and can be absorbed by the root system, leading to both an increased root weight and the formation of secondary roots. This tryptamine pathway also exists in tomato plants, and a moderate influx of TNH2 from an external origin has been shown to trigger the synthesis of indole-3-acetic acid (IAA) by the plant, leading to increased plant growth (Benhamou et al., 2012). Seed coating of rapeseed with *P. oligandrum* was found to increase the level of auxin in the rapeseed plants, which was hypothesized to be correlated with biostimulation (Bělonožníková et al., 2020). Generally, the availability of tryptophan in the rhizosphere is low, but research has shown that *P. oligandrum*, like other soilborne microorganisms, obtains nutrients from root exudates. If these nutrient sources contain appropriate precursors, like tryptophan the

oomycete is able to produce TNH2, and thus induce growth in the associated plants (Benhamou et al., 2012). In conclusion, the mycoparasitic *P. oligandrum* induces biostimulation in several plant species, but whether similar effects can be observed for potato plants remains unclear.

2.7 Biocontrol in relation to the microbiome of plants

Historically, plants have been seen as standalone entities, but there is a paradigm shift in research, where a more holistic perception is needed (Vandenkoornhuyse et al., 2015). Plants can be seen as complex systems, which live in close association with diverse communities of animals, insects, microbes and viruses that continuously interact with each other and their surrounding environment. Thus, the concept of a plant as a holobiont, an organism hosting a multitude of both beneficial and pathogenic species that are affected by biotic and abiotic stresses, is currently being recognized (Mannaa & Seo, 2021; Vandenkoornhuyse et al., 2015). As seen in **Figure 4**, the plant microbiome, the totality of microbes living in association to the host plant, includes both the symbiome, the beneficial plant-associated microbes, and the pathobiome, the sum of pathogens (Mannaa & Seo, 2021). Within the symbiome, microbes have key functions vital for plant growth, nutrient acquisition and resistance to biotic and abiotic stresses (Vandenkoornhuyse et al., 2015).

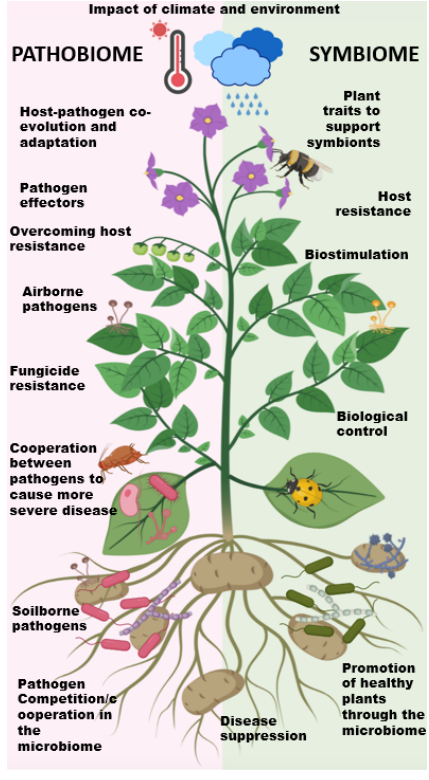

Figure 4. Schematic representation of the potato plant as a holobiont. A plant (here potato) is affected by a multitude of biotic and biotic factors. These can broadly be divided into 2 catagories. The symbiome, which a plant beneficial factors and the pathobiome which are factors contributing to plants stresses. Adapted from (Mannaa & Seo, 2021).

The pathobiome of plants can be defined as a collective set of pathogens that can interact with the host and negatively affect its health (Bass et al., 2019). In that sense BCAs or synthetic fungicides help to suppress the pathobiome effects on the host plant. It has been predicted that understanding how the symbiome and the pathobiome form and interact will become crucial for future disease control and crop production (Bass et al., 2019). The majority of microbes that form associations with the plant below ground come from the surrounding soil, thus the soil microbiome is tightly connected to plant microbiome.

The microbiome of the soil has been recognized as pivotal for plant health and vigour (Souza et al., 2015), which may be of even more importance for tuber crops such as potato. In relation to the plant as a holobiont, the

microbiome residing in the soil is of pivotal importance, since it is the source of microbes that eventually will form associations with the host plant. The rhizosphere can be defined as the metabolically active soil zone, which is in close proximity to the plant roots (Berendsen et al., 2012). Soil type affects the composition of the rhizosphere microbiome in plants (Berg & Smalla, 2009). In addition, spatial, temporal and climatic conditions also impact the rhizosphere microbiome (Fierer, 2017). Plants release organic compounds through the root system and these compounds attract heterotrophic microbes, including plant symbionts (Philippot et al., 2013). Different plant species have been found to both influence the composition of the microbiome in the rhizosphere, but also the function of the microbes residing within (Berg & Smalla, 2009; Ofek-Lalzar et al., 2014)

Such effects have also been demonstrated for potato plants. Soil type has been found to influence the potato rhizosphere microbiome in several studies (Buchholz et al., 2021; Elsayed et al., 2021). In addition, spatial changes can also impact the bacterial community structure in the potato rhizosphere (Lukow et al., 2000), while temporal changes have been observed in bacterial and fungal potato rhizosphere microbiomes across the cultivation period (Hou et al., 2020; Loit et al., 2020). Interestingly, Lukow *et al*. (2000) found a significant effect of plant age on the bacterial microbiome in the potato rhizosphere. The bacterial and fungal microbiomes differ also depending on the potato cultivar (İnceoğlu et al., 2012; Loit et al., 2020). Cropping systems, such as continuous monocultures of a single potato genotype have been found to impact the soil bacterial microbiome, where diversity both measured as Shannon-indices and species richness measured as Chao1 have declined as years progressed. However, continuous potato monocropping appeared not to significantly affect the fungal community diversity and richness (Liu et al., 2014). A comparison of the fungal rhizosphere microbiome between organic and conventional potato cropping systems revealed a higher fungal diversity in the organic farms (Sugiyama et al., 2010). However, there is limited knowledge of the impact of conventional versus organic farming on the rhizosphere community of potatoes.

As potatoes are under constant attack by pathogens, treatments including biological control agents and conventional pesticides are used for disease control. Recently, the impacts of these treatments on the rhizosphere microbial community have been studied. Early blight is typically managed in conventional potato production using synthetic chemical fungicides.

These fungicides have been found to significantly alter the rhizosphere of potato (Santísima-Trinidad et al., 2018; Zhang et al., 2021; Zhang et al., 2014). The impact of biocontrol agents, as alternative treatments against potato diseases, on the potato rhizosphere have also been studied. For example, a transient effect on the bacterial rhizosphere microbiome was observed after the treatment with *Brevibacillus laterosporus* against common scab (Chen et al., 2017). In a study conducted by Roquigny *et al.* (2018) using the BCA *Pseudomonas fluorescens* (strain LBUM223), no significant impact on the bacterial rhizosphere and geucaulosphere (region associated directly with the tuber surface) communities of potatoes was found. On the contrary, Song *et al.* (2021) found an enrichment in bacterial alpha diversity indices after treatment with the BCA *Bacillus subtilis*. Thus, the impact of disease control treatments on the potato rhizosphere microbiome seems to be dependent on many factors, including the type of treatment used. However, even though species such as *P. oligandrum* are documented symbionts that exhibit plant biostimulation properties, the impact of mycoparasitic *Pythium* species on potato, or other crop plant, microbiomes has not yet been well studied.

P. oligandrum is known to be able to colonize the rhizosphere as evidenced by various studies (Al-Rawahi & Hancock, 1998; Le Floch et al., 2003; Takenaka et al., 2008). For effective plant protection, a successful establishment of *P. oligandrum* in the field throughout the cultivation period is crucial. For example, results from Le Floch *et al.* indicate that optimal protection of tomato plants can be achieved when roots are heavily colonized by *P. oligandrum* (Le Floch et al., 2003; Le Floch et al., 2007).

P. oligandrum has been showed to persist on roots for 6 months (Le Floch et al., 2007). However, it is of importance to assess the impact of the BCA on the indigenous microbiome when introducing a new BCA into a cropping system. It has been demonstrated that *P. oligandrum* amendment to the rhizosphere of tomato plants does not impact the fungal microbiome during a long-term (6 month) experiment (Vallance et al., 2009). In another experiment (Vallance et al., 2012) only a transient effect on the bacterial microbiome could be observed after the amendment of *P. oligandrum*, and the effect did not persist up until harvest. It is worth mentioning that these studies were conducted without the use of modern high-throughput sequencing techniques, thus the amount of identified species in the microbiome was limited compared to modern day standards. Regardless, the

impact of other BCAs on soil microbiomes has been studied and several research studies have shown similar results to those seen with *P. oligandrum,* where the impact on the resident microbiomes was somewhat minor. In a study by Cucu *et al.* (2020), a mixture of two BCAs, *Trichoderma* ssp. and *Bacillus subtilis* had no significant impact on the indigenous rhizosphere microbiome of zucchini at the end of the cropping season. *Pseudomonas fluorescens* DR54, a BCA applied to barley plants, was also shown to have a short-lived effect on the soil microbiome in multiple studies (Cabanás et al., 2022; Johansen & Olsson, 2005). Usually, alterations in the rhizosphere microbiomes are short-lived, and the effects are mostly noted immediately after applying a microbial inoculant. After some time, the effect of the microbial inoculant disappears, but several studies have shown that microbial diversity and species evenness increase after inoculation (Berg et al., 2021). On the other hand, a number of studies have shown significant impact on the indigenous rhizosphere microbiome after application of a BCA (Fu et al., 2019; Halifuet al., 2019; Han et al., 2019; Huang et al., 2021). These studies show that there is considerable variation in the impacts of BCAs on rhizosphere microbial communities, presumably related to environmental conditions, the genetics and biology of the plant, the BCA and the surrounding microbes. Thus, it is not easy to predict what the impact of a specific BCA will be on a specific cropping system and there is a need for more thorough investigations. One way of expanding the knowledge in this field of research is using high-throughput amplicon-based sequencing of the potato rhizosphere microbiome under different disease control treatments.

Amplicon sequencing based techniques in microbiome studies

Amplicon sequencing, as described by Caporaso *et al.* (2012), is a prevalent high-throughput method used to determine the composition of microorganisms in various environments. In fact it has been estimated that in 66% of the cases where sequencing techniques were used to study microbial diversity, amplicon sequencing was the method of choice (Brunel et al., 2020). The approach involves PCR amplification of short hypervariable regions within conserved genes, which are then sequenced using high-throughput technologies such as Illumina sequencing. The Illumina sequencing technology, which is based on bridge amplification technology, results in sequences that can be compared to microbial databases

to identify the microorganisms, and thus elucidate diversity, structure, and even sometimes functions in the microbiome. To do so metabarcoded primers are usually used (Brunel et al., 2020). The 16S rRNA gene has been, the most targeted gene for amplicon sequencing of bacteria (Johnson et al., 2019), while the ITS (Internal Transcribed Spacer) region is the most commonly used region for fungi (Tedersoo et al., 2022).

Amplicon sequence variants (ASVs) or sequence clustering-based methods such as operational taxonomical units (OTUs) can be used to compare the amplicon sequences generated. The OTU based method relies on a similarity threshold of 97% sequence identity. However, this method of clustering runs the risk of grouping together different but similar species under the same OTU. This can result in the loss of valuable information. On the other hand, the ASV approach utilizes the exact sequence and counts its occurrence in the data, and thus may be considered a more precise method than sequence clustering by OTUs. The counted sequences are combined with an error model for the sequencing run, which compares similar sequences and calculates the probability of whether their differences are due to sequencing error. The sequences are then filtered by a threshold value of confidence, resulting in several exact sequences with defined statistical confidence. The use of exact sequences without clustering or reference databases is one of the significant advantages of the ASV approach, enabling easy comparison of data with other studies using the same target region. There is also a growing consensus in the community that the ASV approach should be implemented as a standard for amplicon sequencing data analysis (Callahan et al., 2017; Caruso, et al., 2019). After processing of the data, the ASV can be assigned to taxonomy, using for instance the SILVA database (Pruesse et al., 2007) for bacterial ASVs, and the UNITE database for fungal ASVs (Nilsson et al., 2019)

However, consideration must be made to utilize amplicon sequencing approaches to the fullest potential. Metabarcoding of primers is a powerful tool for studying the microbiome of environmental samples, such as the potato rhizosphere. However, the diversity detected in these samples can be affected by both primer choice and the PCR process. Primers can amplify different regions of the target gene, leading to biased results that may exclude certain groups of microorganisms. Meanwhile, PCR is prone to errors that can introduce false positives or negatives and skew the diversity estimates. Thus, optimizing both primer choice and PCR conditions is crucial to

accurately represent the full diversity of microorganisms in the sample. Quality control measures, such as negative controls and replicates, can help identify and correct any biases. In terms of quality control, often a good approach is to use defined mock-communities where the exact composition of species within the samples is known, and thus these can be used to elucidate the accuracy of the sequencing run. Therefore, careful consideration and optimization of both primer choice and PCR conditions are essential to accurately identify the microbiome of potato rhizosphere samples and obtain reliable and representative data.

There are a large number of primers published and available that can be used for studying microbial composition of environmental samples. The choice of primers is dependent on the context of the study and the species that the research is focused on. To study the species within a rhizosphere sample it can be of advantage to use primers with discrepancy against plant related cyanobacteria or mitochondria contaminant amplifications. One good choice is the 16S rRNA primer pair 799 F/1115R which was found useful in this context (Anguita-Maeso et al., 2022). This primer pair has been used successfully to study the bacterial microbiome of the phylosphere, rhizosphere and bulk soil of maize plants (Xiong et al., 2021). The ITS regions are commonly used as the target for fungal metabarcoding. However, the choice of ITS primers can significantly influence the obtained results. The ITS region is highly variable, which means that different primer pairs can amplify different portions of the region. Some primer sets may preferentially amplify certain fungal groups while excluding others, leading to biased results. Additionally, the ITS region is also prone to variations in length and secondary structure, which can affect PCR efficiency and sequencing quality. Thus, choosing ITS primers for fungal metabarcoding should be thought through to maximize diversity of fungal taxa detected and ensuring accuracy in the resulting sequencing data analysis. There is growing consensus that targeting the ITS2 region is advantageous for fungal microbiome studies. An example of a primer pair commonly used to study the fungal microbiome is the fITS7 and ITS4 (Ihrmark et al., 2012; Sarkar et al., 2022) After sequencing, raw reads obtained from the sequencing can be processed using various bioinformatics software, for instance DADA2 (Callahan et al., 2016). Amplicon sequencing through the use of metabarcoding has proven to be a reliable and valuable tool, which is now

widely used to characterise microbial diversity and composition in different environments (Quince et al., 2017).
The amplicon sequencing based method comes with some critical limitations. It offers an insight into bacterial and fungal species within the microbiome, but it does not elucidate whether these microbes are actually alive and actively contributing to microbiome structure and functions. Thus, amplicon sequencing based methods are good for an overview of the microbiome, but for in depth understanding it should be accompanied by either omics, metabolome or culture-based approaches.

2.8 Formulation and application of microbial BCAs in agriculture

In modern agriculture, formulation strategies for microbial BCAs (MBCAs) involve the development of methods to preserve, propagate and apply these organisms in a practical and effective way, within the boundaries of the legislation body.
Commercial formulations of MBCAs have been created for several organisms, including fungi, bacteria and oomycetes (Keswani et al., 2016). The formulation of MBCAs into a product has been recognized as the key to the commercial success of MBCAs used in agriculture, since it affects key aspects in the applicability of the product, such as shelf life and field performance (Fravel, 2005). A good formulation of a MBCA should meet several criteria. Since MBCAs can be affected by various environmental factors, including temperature, humidity, pH, UV-light and even soil type (Hashemi et al., 2022; Keswani et al., 2016), it is important that the MBCA can withstand, thrive and propagate in a range of different environments. Another key aspect is the type of formulation used. The majority of formulated MBCAs have been formulated as wettable powders, liquids or in granular forms (Marian & Shimizu, 2019). Much like wettable powders, another type of formulation strategy is to encapsulate the MBCA in water dispensable granules (WDGs). Before application, both wettable powders and WDGs are solubilized in water, while liquid products are mixed with water until a desired concentration is reached. All these formulations are easy to handle and apply, which is important for the growers/users. A common way to produce these powder or granular formulations is by drying the spores

without killing them (Bejarano & Puopolo, 2020). After this, an amendment of either a powdery substance, such as pectin or starch, bentonite, or chitin is commonly used (Marian & Shimizu, 2019). Wettable powders of *Trichoderma harzianum* were used to control damping off in tomatoes caused by *Pythium aphanidermatum*, with a significant effect, under both controlled greenhouse assays and in open field trials (Jayaraj et al., 2006). Protection against *P. aphanidermatum* and increased plant stand and biomass was also observed with a wettable powder formulation of *Bacillis subtilis* (Jayaraj et al., 2005). The oomycete biocontrol agent, *P. oligandrum*, has also been formulated into wettable powders (Polygandron WP® and Polyversum ®) and these formulations show significant effects in controlling potato late blight (Kurzawińska & Mazur 2007; Wiik et al., 2020).

In a recent study WDGs were developed for both *B. subtilis* and *T. harzianum* and the products showed 48% and 44% reduction of disease incidence caused by *Fusarium oxysporum* f. sp. *Lycopersici* in tomato plants respectively (Jangir et al., 2021). Liquid formulation is also a common strategy. One of the best examples is the commercially available product Serenade ASO®, which is based on *Bacillus amyloliquefaciens*. The product is foliar-applied and has proven efficacy against a variety of plant diseases, including powdery mildew (Matzen et al., 2019), early blight (Egel et al., 2019; Abbasi & Weselowski, 2015) and late blight (Dorn et al., 2007), among many others.

The most common application technique of formulated biocontrol products is through foliar application. Beside foliar application, seed treatment have been used in several crop species against a multitude of plant disease, as reviewed by Callaghan (2016)

A major concern of formulating and using MBCAs in agriculture is the somewhat short shelf life of these products (they contain living organisms that need to proliferate when used). Thus, much research has been performed to increase the shelf life of formulated MBCAs (Bejarano & Puopolo, 2020; Marian & Shimizu, 2019; O'Callaghan, 2016).

A second issue is the difficulties of translating efficacy shown in controlled environments, such as greenhouses, into large-scale open fields; this is a significant issue for both formulated and unformulated MBCAs. As an example, a recent review found almost 100 peer-reviewed papers, all dealing with the biocontrol of late blight caused by *Ph. infestans.* These studies described the use of a wide array of MBCAs, but fairly few of them concluded a significant effect when the MBCA was used in a field setting.

Out of all these experiments, only 8 products had been formulated (Hashemi et al., 2022). This also highlights the importance of formulating MBCAs, for effective use in agriculture.

Another issue that hampers the progress of developing and formulating MBCA products for agriculture is the complex regulations and legalisation procedure set by the EU (Barratt et al., 2018). The EU Commission Regulation No 546/2011 implements Directive (EC) No 1107/2009 and regulates the use of plant protection products, including BCAs in agriculture. A proper regulatory framework is obviously important for safe use of plant protection products, but it can create long and costly procedures in the approval process. To get a MBCA approved in the EU, it must go through two legislative steps. The first evaluates the active substances, and consists of three phases: rapporteur member state, risk assessment, and risk management (Frederiks & Wesseler, 2019). During the first step, applicants submit a dossier to a member state. After evaluation, the European Food Safety Authority assesses food safety risks, and the European Commission carries out the risk management. The second step is to approve and register the product at the member state level (Hashemi et al., 2022). An important part of the approval process is the environmental fate of the MBCA released into the cropping system. However, as important as they are, these are seldom studied in detail (Köhl et al., 2019b) Thus, the process to get a MBCA formulated and approved for use in agriculture is a time consuming and usually a costly affair. Overall, this possibly explains the limited number of BCAs commercially formulated and purchasable in the European farming context, in comparison to the extent of research done on BCAs the last decades.

2.9 The Potato crop, a valuable but vulnerable food source

Potato is among the most important and largest food crops in the world. In fact it is ranked as number four worldwide, straight after rice, wheat, and maize (Lovat et al., 2016). Potato is a valuable source of essential vitamins, minerals, quality protein, and starch (Burgos et al., 2020). Modern potato varieties have a very high yield, reaching up to at least 40 000 kg/ha.

However, to achieve such high yield, intensive agricultural management practices are required, including tillage, and the use of agrochemicals, such as synthetic fertilizers, herbicides, and pesticides. These practices aim to both fertilize the field and/or to control pests and diseases (Goffart et al., 2022).The extensive and continuous use of agrochemicals has been found to pose a threat to both human health and the environment (Hashemi et al., 2022). Furthermore, the adoption of modern agriculture has led to severe declines in agroecosystem biodiversity (Raven & Wagner, 2021) and adversely impacted the physicochemical properties of agricultural soils, resulting in significant losses of soil biodiversity, as well as reduced soil fertility and overall health (Hartman et al., 2018; Tsiafouli et al., 2015). Thus, efforts must be made towards a more sustainable potato production system. One step is the development and implementation of BCAs against notoriously destructive potato diseases, such as late and early blight, with the overall scope to reduce the amount of fungicide use in potato production, whilst still retaining healthy, high yielding crops.

The notorious blight diseases of potatoes

Potato early blight

The fungal genus *Alternaria* is large and contains both notorious plant pathogens *e.g.*, *Alternaria solani*, *Alternaria alternata* and *Alternaria grandis* (Landschoot et al., 2017), and human pathogens (Pastor & Guarro, 2008). In a Swedish context, *A. solani* is believed to be the main causal agent for early blight in potatoes (Edin and Andersson, 2014). On a global scale, early blight is an increasing problem that can causeup to 50% yield loss and affect the quality of potato tubers (Leiminger & Hausladen, 2012; Odilbekov et al., 2020). Early blight in potatoes is a soil-borne, polycyclic disease that is spread via wind and rain splash. This usually results in necrosis and eventual death of the above ground plant material, but the disease can also spread to the tubers, thus affecting both quality and overall yield of potato.

Potato late blight

Late blight is also a disease of the *Solanaceae* family, caused by the hemibiotrophic oomycete pathogen *Phytophthora infestans,* and responsible for loss of food, thus, affecting 81 to 1.270 million people globally on an annual basis (Fisher et al., 2012). Despite extensive control measures, it still causes an average global yield loss of 6% (Savary et al., 2019). It has been estimated that the annual cost of late blight yield losses and management reaches up to at least 7 billion euros (Haverkort et al., 2016). The manifestation of symptoms associated with late blight are characterized by the emergence of brown, necrotic lesions on plant foliage. These lesions initially present as water-soaked and chlorotic, but rapidly progress to extensive tissue necrosis and plant death. Tubers are also affected, and the aggressive polycyclic nature of the disease can make it challenging to control in the field. Approximately 300 000 asexual spores (sporangia) can be produced from a single lesion on potato, and each of these can cleave to produce at least 10 motile zoospores, from which the disease cycle can be reinitiated (Fry, 2008). Thus, epidemics can be swift and brutal; a susceptible field may be destroyed within a week. Infections originating from sexually derived oospores are particularly common in the Nordic region. Brurberg *et al.,* (2011) found that out of 200 field isolates 75% were unique, meaning that they resulted from sexual recombination. This has been correlated with an earlier start of the epidemic, resulting in larger amounts of chemicals used (Fry et al., 2015). In fact, residential sexual populations of *Ph. infestans* have been found to have 17-fold greater risk of causing an epidemic and the disease outbreaks to occur 2-4 weeks earlier (Hannukkala et al., 2007). Moreover, the occurrence of sexual reproduction in *Ph. infestans* results in a significant increase in genotypic diversity and an enhancement in the pathogen's ability to adapt to changing environmental conditions (Andersson et al., 2009).

Management strategies of early and late blight disease

Although Integrated Pest Management (IPM) has been mandated for all crops within the EU since 2014, it is less well developed in potato than in other cropping systems. Control of both early and late blight in potato is

heavily reliant on the use of synthetic chemical pesticides. In fact, 2 million tonnes, or 7% of all the total usage of fungicides, are used every year in the EU alone in potato, largely to control these two diseases (Grenville-Briggs, 2022). In Sweden, potato production takes up a small fraction of the cultivated land, about 1%, but accounts for a large proportion of fungicides used in the country's agriculture (around 21%) (Eriksson et al., 2016). This may reflect the frequency at which fungicides are applied, often at least once a week, resulting in 10-20 spray applications per growing season (Cooke et al., 2011). Two types of fungicides are used to control early blight; single-site specific and broad-spectrum mode of action fungicides (Yellareddygari et al., 2019). Single-site specific fungicides, such as Quinone outside Inhibitors (QoIs), containing the active ingredients (a.i.) azoxystrobin and pyraclostrobin, have been proven highly effective against early blight, as have the SHDI class of fungicides. However, *A. solani* populations have developed resistance to many of the fungicides currently in use (Bauskeet al., 2017; Odilbekov et al., 2019; Pasche et al., 2004). In addition, there have been reports on the negative effects of some of these fungicides on non-target organisms or the plant microbiome in general (Santísima-Trinidad et al., 2018; Zhang et al., 2021; Zhang et al., 2014). Synthetic fungicide applications are also the most commonly used method to control late blight (Leesutthiphonchai et al., 2018), and as with early blight, resistance development in populations of *Ph. infestans* is also commonly observed (Matson et al., 2015; Schepers et al., 2018). Moreover, it has been estimated that the differences in net income resulting from the use of fungicides against late blight range from €167 to €656/ha (Wiik et al., 2018).

A rationale for biocontrol of blight diseases in potato

Evidently, there is a need for a more sustainable production of potatoes, especially in regard to control measures used against early and late blight disease. Even with the development and deployment of resistance towards the diseases, the progress for breeding resistant cultivars is difficult and slow, and the plasticity and adaptability of *A. solani* and *Ph. infestans* make them able to overcome resistance gene(s) bred into new cultivars. One of the options that could be implemented in the management strategies to combat these diseases is the cornerstone of IPM, biological control.

Biocontrol of early blight

Attempts to control early blight or antagonize the pathogen *A. solani* with biological control have already been made, although not to the same extent as with *Ph. infestans.* As is the case for the BCAs against *Ph. infestans*, BCAs against early blight include organisms from different kingdoms, *e.g.* fungi (Boyno et al., 2022; Metz & Hausladen, 2022), bacteria (Attia et al., 2020), and chromista (Stridh et al., 2022). Combinations of biocontrol agents against *A. solani* have also been reported (Boyno et al., 2022; Stridh et al., 2022). Mycoparasitism is one of the mechanisms that has been reported in different species of *Clonostachys,* which succeeded in significantly reducing *Alternaria grandis* sporulation in potato plants, leading to reduced disease incidence in pot grown potato plants (da Silva et al., 2021). A dual culture assay with microscopic observation of the individual interaction between *Trichoderma* spp. and *A. solani* revealed that both *T. viride* and *T. harzianum* were able to inhibit *A. solani* growth *in vitro* by 91.88 % and 80.11 %, respectively (Kumar et al., 2022). Volatiles from *Bacillus subtilis* have been found to influence both spore production and growth of *A. solani* (Zhang et al., 2020). Lipopeptides and volatiles from the strain *Bacillus velezensis* C16, showed inhibitory effects on the mycelia growth *in vitro* and in detached leaf assays infected with *A. solani.* In addition, these compounds also inhibited conidia germination (Zhang et al. 2021). Competition for space and nutrients has also been documented for the BCA *B. subtilis*. Abbasi & Weselowski (2014) found that *B. subtilis* was able to colonize the leaf surface of tomato plants and outcompete *A. solani*, which resulted in a physical prevention of penetration of the pathogen into the leaves.

Biocontrol of late blight

Multiple attempts have been made to develop BCAs to control late blight disease. Recently we reviewed these attempts and found almost 100 articles dealing with the specific subject (Hashemi et al., 2022). In these studies, the majority of the experiments were performed *in vitro* or *in planta* and the organisms tested for their ability to control *Ph. infestans* came from diverse kingdoms [*e.g.* fungi (Shanthiyaa et al., 2013; Wharton et al., 2012), bacteria (Caulier et al., 2018; De Vrieze et al., 2018), and chromista (Liang et al., 2020)]. Besides living organisms, derivatives, metabolites or other

compounds originating from BCAs have also been used (De Vrieze et al., 2015; Lal et al., 2021). Mycoparasitism was observed in the fungal BCAs of the genus *Trichoderma*. *Trichoderma ssp.* displayed mycoparasitism by directly feeding on *Ph. infestans*. Specifically, coiling around prey hyphae was observed along with a secretion of lysing enzymes, helping the BCA to obtain nutrients released upon feeding (Yao et al., 2016). The biocontrol agent *Pythium oligandrum* has also been shown to display direct mycoparasitism, that is direct feeding on *Ph. infestans* (Horner et al., 2012; Liang et al., 2020). Direct antagonism has been reported for the bacterial biocontrol agents *Bacillus subtilis* and *Pseudomonas ssp.*, which were able to reduce *Ph. infestans* growth by 50% *in vitro* (Caulier et al., 2018). Antibiosis, by the release of secondary metabolites or toxins, was reported against *Ph. infestans* from several BCAs. For instance, *Trichoderma ssp.* inhibited *Ph. infestans* mycelia growth by 36.8% through the secretion of volatile compounds (Yao et al., 2016). Moreover, antibiosis from bacterial BCAs against *Ph. infestans* has also been reported. Several fluorescent *Pseudomonas* species produce cyclic lipopeptides and siderophores with anti-oomycete properties (De Vrieze et al., 2018). In addition, pyoverdine-mediated iron acquisition mechanism in many *Pseudomonas* species has been linked to the direct inhibition of *Ph. infestans* development, suggesting that iron competition may be one additional mechanism used to inhibit *Ph. infestans* (De Vrieze et al., 2020). Finally, in a recent study induced resistance caused by BCAs was also speculated to play a part in the reduction of late blight symptoms on potato plants cultivated *in vitro* (Lalaymia et al., 2022).

3. Thesis aims

The overall aim of this thesis is to gain a better understanding of the molecular biology and genetic traits of mycoparasitic *Pythium* species, *P. oligandrum* and *P. periplocum*, and evaluate whether and how these organisms can be utilized to improve current potato cropping systems. In detail, my research aims:
I) To increase the fundamental understanding of the mechanisms related to mycoparasitism in *P. oligandrum* and *P. periplocum* using a combination of comparative genomic analysis and transcriptomics (Paper I and II).
II) To elucidate the extent of the influence of *P. oligandrum* on plant growth and early blight disease control in potato (Paper III to V)
III) To unravel the environmental impact of *P. oligandrum* or synthetic fungicides on the potato rhizosphere microbiome (Paper III and IV).

The specific aims of the individual papers included:

1. The aim of paper one is to broaden our knowledge and understanding of the mycoparasitic *Pythium* species, *P. oligandrum* and *P. periplocum*, and how these oomycete mycoparasites differentiate from their phytopathogenic counterparts at the genomic level.
2. The aim of the second paper is to explore and analyse the CAZYome of the mycoparasitic *Pythium* species. In addition, we aim to understand how these mycoparasites acquired their unique CAZYome complement. Lastly, we aim to understand whether these complex carbohydrates can serve as carbon sources for growth of the mycoparasitic *Pythium* species.

3. The aim of the third paper is to understand whether biostimulation occurs in potato plants as an effect of *P. oligandrum* treatment. Further, to explore whether biostimulation is plant-genotype specific and lastly to evaluate whether treatment with *P. oligandrum* impacts the rhizosphere microbiome of starch potatoes, in terms of effects on microbial diversity and community structure.
4. The aim of the fourth paper is to analyse whether *P. oligandrum* can reduce disease severity of early blight in field grown starch potatoes. In addition, we aim to evaluate the impact on the potato rhizosphere after the treatments with either synthetic fungicide or *P. oligandrum* against early blight.
5. The aim of the fifth paper is to unravel the efficacy of several BCAs and plant resistance inducers (PRIs), including *P. oligandrum,* in comparison to synthetic fungicides against early blight in starch potato

4. Methodology

4.1 Insight into the genomes of mycoparasitic *Pythium* species

Comparative genomics

Comparative genomics (used in Paper I) is a powerful technique that provides information on the similarities and differences in the genomes among related or unrelated organisms. To utilize a comparative genomics approach raw genome sequence data must be acquired, either through genome sequencing from a pure sample or from a reference database, where the genome of a species is readily available. In Paper I we used the genome assembly of *P. oligandrum* and *P. periplocum*, already performed by Kushwaha *et al*. (2017a), to re-predict genes *ab initio*. Gene calling was performed using Augustus (Stanke et al., 2006), GenemarkES (Ter-Hovhannisyan et al., 2008), and SNAP (Korf, 2004) in MAKER2 (Holt & Yandell, 2011). The annotation of the genomes was carried out using EggNOG v5.0 (http://eggnog5.embl.de/#/app/home) and the ortholog analysis between *P. oligandrum* and *P. periplocum* was performed using OrthoVenn2 (https://orthovenn2.bioinfotoolkits.net/home) with an E-value of 1e-05 and an inflation value of 2.0. The pipeline used for the comparative genomic study in Paper I allowed us to identify and compare mycoparasitic determinants in the two *Pythium* species, along with a determination of their phylogenetic relationship to plant pathogenic oomycetes, for instance *Phytophthora infestans.* However, comparative genomics, as powerful as it might seem, has some drawbacks. For example, if the genome assembly of

an organism is incomplete, it can lead to incorrect data interpretation. Sometimes there is a lack of functional annotation, which means that it can be tough to determine the exact genomic differences. Moreover, the analyses are time consuming and computationally heavy, which can also be costly, although the latter might change in the near future.

Transcriptomic analysis

Transcriptomic analysis is a useful tool that can provide information on expressed genes in a sample under certain conditions. Historically, gene expression analyses were performed using microarrays or RT-qPCR studies. Now, with the advancement of NGS techniques, transcriptomic studies are usually performed using high-throughput RNA sequencing (RNAseq). In Paper II we performed RNAseq on the interactions between the two mycoparasites *P. oligandrum* and *P. periplocum* and their prey species, *Ph. infestans* and *Botrytis cinerea.* The mycoparasitic interactions between either *P. oligandrum* and *Ph. infestans* or *P. periplocum* and *B. cinerea* were monitored microscopically and mycelium from the interaction zone was harvested at 12 and 24 hours post interaction (hpi). Total RNA was extracted from three biological replicates per interaction and time point. Libraries were constructed and subsequently sequenced (150 bp paired-end) on the Illumina NovaSeq6000 S4 platform (SciLifeLab, Stockholm). Approximately 30 to 69 million pairs of reads were obtained in raw data for each sample, from which low-quality reads were filtered out before mapping against corresponding reference genomes of *P. oligandrum* and *P. periplocum*. Differential gene expression analysis was then performed as described in Papers II and II.

Validation of gene expression using RT-qPCR

In Paper I and II, differential gene expression seen in the RNAseq experiment was validated using RT-qPCR. Primers were designed in Primer3 and the NCBI BLASTn web platform was used to check the specificity of the sequences for the genes of interest, with the low complexity filter turned off. The interaction zones from confrontation assays between *P. oligandrum* and

P. periplocum and their prey species *Ph. infestans* and *B. cinerea* were monitored microscopically and mycelium from the interaction zone was harvested at 12 and 24 hpi. Total RNA from the confrontation assays was extracted using the RNeasy Plant Mini Kit according to manufacturer´s guidelines (Qiagen, Germany). The RNA was then treated with DNase using the TURBO DNA-free Kit (Invitrogen, USA). The extracted RNA was assessed for quality, and the total amount of RNA was measured using a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific, USA). cDNA was generated using a cDNA first strand synthesis Kit (RevertAid First Strand cDNA Synthesis, Thermo Scientific) The gene expression levels were evaluated using quantitative RT-qPCR using BioRad real-time PCR cycler and Power Up SYBR Green (Thermo Fischer Scientific) as the fluorescent dye according to manufacturer´s instructions.

4.2 Plant growth promotion by *P. oligandrum*

To investigate the biostimulation induced by *P. oligandrum* amendment, we first conducted a series of growth assays in a highly controlled environment. We used three commercially available potato cultivars, which are different genotypes. The table cultivar Desirée was developed in the Netherlands in 1962, it is drought tolerant and fairly resistant to diseases (Martínez et al., 2021). However, it is often used as control in potato late blight trials due to its high susceptibility towards *Ph. infestans* (Haesaert et al., 2015). The second variety cv. King Edward is a very popular consumer variety, with a cherished floury texture. It originated from England more than 100 years ago. Unfortunately cv. King Edward is highly susceptible to *Ph. infestans* (Eriksson et al., 2016). The last, and perhaps most important cultivar for the present thesis, is the starch cultivar Kuras. Starch cultivars are treasured, since they produce larger quantities of starch compared to table potatoes. Starch is an important raw material that is widely used for food and non-food applications. The main sources of starch for industrial applications are potato, maize, rice, wheat and cassava (Zhu, 2018). In northern Europe, the Kuras cultivar is the major starch potato due to a high yield and superior pest resistance (Jørgensen et al., 2006). Furthermore, Kuras is a late maturing cultivar, allowing for a longer period of tuber filling. The cv. Desirée and cv. King Edward were used in the greenhouse trials as indicated in **Table 1**, whereas the cv. Kuras was used both in greenhouse and field trials (**Table 1**).

Table 1. Overview of biostimulation in greenhouse and field trials with *P. oligandrum* or Polygandron WP®. All treatments were carried out with a high-pressure handheld sprayer at 300 L/ha

Location	Cultivar	Application method	Treatment	Concentration	Year
Greenhouse	Desirée	Foliar spray + soil drench	PO (Oospores)	1.25×10^4/ml	Na
Greenhouse	King Edward	Foliar spray + soil drench	PO (Oospores)	1.25×10^4/ml	Na
Greenhouse	Kuras	Foliar spray + soil drench	PO (Oospores)	1.25×10^4/ml	Na
Field trial	Kuras	Foliar spray	PO (Oospores)	2.5×10^4/ml	2019
Field trial	Kuras	Foliar spray	PO (Oospores)	2.5×10^4/ml	2021
Field trial	Kuras	Foliar Spray	Polygandron (WP)	5×10^5/g	2021

Greenhouse experiments

In order to cultivate potato plants for the greenhouse experiments, the potato plants were propagated in MS-media and transferred to pots containing peat soil. Plants were allowed to grow for 10-14 days before any treatments with *P. oligandrum*. The treatment with *P. oligandrum* was carried out using an oospore solution with a concentration of 1.25×10^4 oospore/mL. Each treatment consisted of 10 mL applied as a foliar spray, using a high-pressure hand-held sprayer plus an additional 10 ml of *P. oligandrum* oospore solution applied as a soil drench at the base of the plants. In total, 6 plants from each cultivar were treated and 6 plants were used as controls. Plant growth promotion was determined by harvesting the plants in the fourth week after the first treatment was applied. Total plant height was determined by measuring the longest shoot from the base of the hypocotyl of the plant. Plant biomass measurements consisted of fresh and dry weight of the plant shoots at harvest. For the dry weight measurements, the harvested shoots were dried at 60°C for 24 hours and the weight was then recorded. The experiments under controlled conditions were repeated three independent times.

Field trials

In the field trials (Paper III and IV) biostimulation by *P. oligandrum* was tested on the starch potato variety cv. Kuras. Biostimulation was defined as growth of the longest shoot measured from the base of the plant until the apex. The first application of *P. oligandrum* or Polygandron (WP) was done in July and carried out biweekly until mid-September in both years of field trials. The plants were cultivated from seed tubers planted in May, and cultivated and measured until harvest, i.e. September 2019 or October 2021. For a complete overview of location, trial year, BCA used, application method, and concentration of the BCA applied see **Table 1**.

Statistical analysis of phenotypic data

T-test, Wilcoxon test, ANOVA, Kruskal-Wallis test, and correlation analyses were performed using R-studio (version 4.1.2 2022, RStudio, Inc) and the rstatix package (Kassambara, 2020). The ggplot2 package (Wickham, 2009) was used to generate boxplots. In the greenhouse experiment the effect of treatments was evaluated using a standard one-way ANOVA, including treatment and experiment as predicting factors. For the different cultivars tested, ANOVA models were run by individual genotypes to achieve a normal distribution of residuals. Logarithmic transformation was applied to the shoot dry weight to obtain a normal distribution. In the field trials of 2019 and 2021, a standard one-way ANOVA with treatment as a factor was used to analyse plant growth promotion.

4.3 Early blight field and greenhouse trials

Greenhouse experiments with *P. oligandrum* and Serenade as antagonist against early blight were conducted. An overview of the experiments is found in **Table 2**. For a description of the methods used, and the statistical comparisons used to evaluate the efficacy of treatments against early blight please see Paper V. The remaining section describes the method used to evaluate efficacy of the BCAs against early blight in field trials. In Paper IV the scope of the study was partly to study treatment efficacy against early blight caused by *A. solani* on the starch potato variety cv. Kuras. Seed tubers

were kindly provided by Lyckeby Starch AB. The field trial was conducted in the common garden in Alnarp in 2021. To study the interaction between early blight disease pressure and treatment effects, the plants had to be artificially infected with *A. solani*. Thus, barley kernels were infected with *A. solani*, and evenly spread in the field following the method of Hans Hausladen (personal communication). The *A. solani* strain used was AS112, originally isolated from potato fields in Southern Sweden by Odilbekov *et al.* (2014). The disease symptoms were visually scored weekly from the onset of infection (July 2021) until harvest (October 2021), following the scoring system developed by Duarte *et al.* (2013). The disease progression was calculated as Area Under the Disease Progression Curve (AUDPC) (Shaner, 1977). In study described in Paper V the early blight infection occurred naturally. Disease was scored weekly from the appearance of the first symptoms to the end of the field trial (Duarte et al. 2013). The relative AUDPC was used to calculate the disease severity, following the formula of Shaner (1977). Since the area where the field trials were conducted was heavily cultivated with potatoes, all plants were subjected to *Ph. infestans* fungicide treatment, making it easier to evaluate early blight disease progression, as no late blight symptoms developed that could potentially obscure the results. The field trials from 2018 to 2020 were managed according to the Swedish Rural Economy and Agricultural Societies procedures and repeated for three consecutive years. The field trial in 2021 followed the same recommendations. For an overview of the field trials see **Table 2** and Papers IV and V

Table 2. Overview of greenhouse trials and field trials on the effect of BCA and synthetic fungicides against early blight in potatoes. All treatments were carried out with a high-pressure handheld sprayer.

Location	Treatments	Cultivar	Application method	Formulation	Year
Alnarp: Greenhouse	1.Controls *2.*PO *3.* PO + Serenade	Desirée	Combination of Foliar spray and soil drench	Oospores and or liquid (Serenade)	2019 + 2020
Location	**Treatments**	**Cultivar**	**Application method**	**Formulation**	**Year**
Field trials in Helgegården	1.Controls 2. PO 3.PO + Serenade 4.Polygandron 5.Polygandron + Serenade	Kuras	NA Foliar spray Foliar spray Foliar spray Foliar spray	Oospores	2018-2020
Location	**Treatments**	**Cultivar**	**Application method**	**Formulation**	**Year**
Field trials in Alnarp	1. Controls 2. PO3.Polygandron 4. Fungicides	Kuras	Foliar spray Foliar spray Foliar spray Foliar spray	Na Oospores WP Liquid	2021 2021 2021 2021

Pythium oligandrum oospore production and application

P. oligandrum was used both as an antagonist against early blight (Papers IV and V) and as a biostimulant (Paper III). To do so, *P. oligandrum* was cultivated in liquid V8 medium, at 20 °C and shaking at 120 RPM. When the mycelia had matured and formed spiny oogonia (approximately 7-10 days after inoculation), the mycelia were macerated using a high-speed blender and filtered through Miracloth to release the mature oospores. The final oospore concentration was determined using a haemocytometer and diluted to the desired concentration. The commercially formulated version of *P. oligandrum* (Polygandron WP®) was used in Papers IV and V. The specific

formulation is a wettable powder that was solubilized in distilled water. The commercial formulation was also applied as a foliar spray, following the manufacturer´s recommended dose.

4.4 Soil sampling and amplicon sequencing

To decipher the impact of *P. oligandrum* on the starch potato rhizosphere microbiome, rhizosphere soil was sampled in a non-destructive manner at different time points during the growing season (Papers III and IV). Samples were taken before any treatment was applied, these samples served as the baseline samples of the fungal and bacterial microbiomes in the rhizosphere of the potato plants. In Paper III the samples were: baseline samples (08-07-2019), flowering (06-08-2019) and senescence (03-09-2019). In Paper III we sampled 6 individual untreated control plants and 6 individual *P. oligandrum* treated plants at each time point. In Paper IV the baseline samples were taken at before *A. solani* inoculation (01-07-2021). The next samples were taking a week later, before the treatments was applied (08-07-2021) and thereafter, at the flowering time point of the plants (05-08-2021), and finally at the senescent stage of the plants (02-09-2021). In Paper IV we sampled a total of 9 individual plants of untreated control plants and for each treatment used, at every time point.

Non-destructive rhizophere sampling (NDRS) of the potato plants

Sampling was carried out with a standard soil core sampler. Soil cores were taken directly adjacent the to the plant root base at a depth of 0.2-0.3 m. The soil core sampler was surface sterilized between individual samplings to avoid environmental and cross contamination. Each sample contained both soil and fine roots and thus represents a non-destructive rhizosphere sample. This unconventional sampling strategy was used because it allowed us to follow the individual plant phenotypes (Paper III) and disease development (Paper IV) throughout the cultivation period, without destroying the host plant. In Paper IV we also included bulk soil samples at the baseline time point and at the end of the experiment and compared these samples to the ones from the potato rhizosphere. Furthermore, in both papers (IV and V) we included predefined microbial community samples comprising consortia of

both bacterial and fungal strains. These consortia were ZymoBIOMICS Microbial Community Standard (Biosite-D6310) and ZymoBIOMICS Microbial Community DNA Standard (BioSite-D6306). The DNA from these consortia were extracted using the same kit and protocols as for the potato rhizosphere soil samples and served as a standard check for the sequencing runs. Finally, blank controls from the extraction kit were used to check for unwanted background contaminants.

Metabarcoding and amplicon sequencing

High quality DNA was extracted from the NDRS using the DNeasy PowerSoil Pro Kit (Qiagen, Germany), following the manufacturer´s protocol. The quality and quantity of DNA was initially assessed on NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific, USA) and additional quality checks were performed at the sequencing facility. Two pairs of primers were used to assess the bacterial and fungal microbiomes in the samples. The primer pair used for bacteria was 799F and 1115R and the fungal primer pair was FITS7 and ITS4. The primers were bound to a barcode, thus metabarcoded. The samples were sequenced using an Illumina MiSeq V3 with 300 bp paired-end reads, at LGC Genomics in Germany. The number of raw reads obtained from the sequencing facility was roughly 50 000 raw sequence reads per sample. To process the raw reads from the bacterial and fungal samples, the DADA2 pipeline was used (Callahan et al., 2016) in R version 4.2. The default settings throughout the pipeline were used for filtering and trimming. Identical sequencing reads were combined using the dereplication function. Paired-end reads were merged, chimeras were removed, the amplicon sequence variant (ASV) table was constructed, and taxonomy was assigned using the SILVA database (Pruesse et al., 2007) Version 138.1 for bacteria ASV taxonomy assignment, and the UNITE database (Nilsson et al., 2019) for the fungal taxonomical annotation (https://unite.ut.ee/). DADA2 data outputs were combined into a phyloseq object using the phyloseq-package by McMurdie and Holmes (2013). The phyloseq object was converted into a MicroECO-object using the Microeco-package by Liu *et al.* (2021) for postprocessing data analysis.

Microbiome data visualization, analysis and statistical analysis

Data visualization and presentation was mainly done using the microeco-package (Liu et al. 2021). For the microbiome data a one-way ANOVA combined with the Tukey's honest significance test was conducted to reveal the effects of treatment versus control plants on the alpha-diversity index computed as Shannon-indexes (Shannon, 1948). The PERMONOVA tests with 999 permutations were used to reveal the differences in microbiome community structure between control and treated plants, following the method described in Anderson (2001), and based on the beta-diversity computation (Whittaker, 1972). Principle Coordinate Analysis (PCoA) along with Non-Metric Multidimensional Scaling (NMDS) on the ordinations based on the Bray-Curtis dissimilarity matrix was performed to visualize the stresses of treatment and control plants. All microbiome data was analysed and visualized in R-studio, using a combination of phyloseq-package (McMurdie and Holmes 2013) and the Microeco-package (Liu et al., 2021).

5. Results and discussion

5.1 Insights into the genomes of mycoparasitic *Pythium* species, and differentiation from phytopathogens

Comparative genomics (Paper I) was combined with RNAseq experiments (Papers I and II) to identify genomic features and gene expression patterns related to mycoparasitism in oomycetes. In detail, we used interaction assays between either *Pythium oligandrum* or *Pythium periplocum* and an oomycete prey species (*Phytophthora infestans*) or a fungal prey species (*Botrytis cinerea*). The scope of Paper I was to broaden our understanding of the functionality of gene families important for mycoparasitism, and in Paper II we focused specifically on the CAZyome. Specifically, we investigated the role of CAZymes in the evolution of the mycoparasitic *Pythium* species. Although there are numerous studies on *P. oligandrum* and *P. periplocum* in terms of their biocontrol properties and efficacy (reviewed in Bělonožníková et al., 2022, and presented in Ribeiro & Butler, 1995), we still possess limited knowledge of the genetic features that define the oomycete mycoparasites and distinguish them from their phytopathogenic relatives.

Comparative genomics

As presented in Paper I the mycoparasitic *Pythium* species have smaller genomes and possess higher average ratios of repeat elements compared to the phytopathogenic *Pythium* species. Exploration of the gene density revealed a density of approximately 0.39 genes per kilobase in the mycoparasitic *Pythium* genomes, which is significantly more than that of

plant pathogenic *Pythium* species genomes. This probably means that a major difference between the mycoparasitic and phytopathogenic *Pythium* species is that the mycoparasites do not exhibit the classical "two-speed genomes" that have been described in plant pathogenic oomycetes. To further explore and identify mycoparasitic determinants of *P. oligandrum* and *P. periplocum*, a genome-wide orthologous clustering between their predicted proteins was performed using the ortho venn platform. The analysis showed that 22 expanded orthogroups were identified in the Last common ancestor (LCA) of *P. oligandrum* and *P. periplocum*. Thus, this infers that 22 gene copies from the LCA might have undergone duplication events in the mycoparasites, indicating a unique adaptation of their genomes, and that possibly these genes are important traits and determinants of the mycoparasitic lifestyle. The domain annotation of the *Pythium* genes assigned 467 *P. oligandrum* genes and 346 in *P. periplocum* in the LCA-specific expanded orthogroups (**Fig. 5B**). Most of the genes, found to be present in the LCA-specific expanded orthogroups, were assigned to domains related to growth or metabolism, but 18 of them were genes encoding for ABC transporters (**Fig. 5B**).

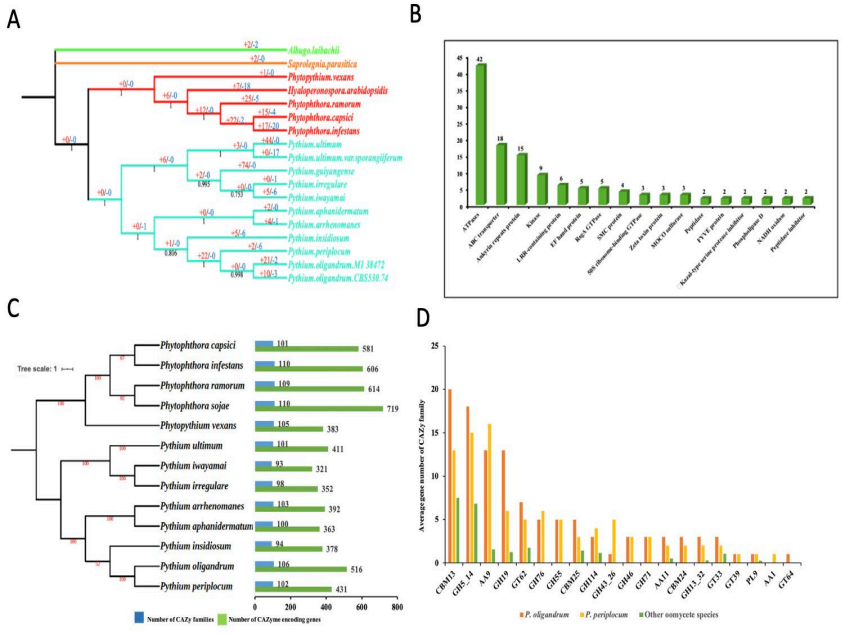

Figure 5. (**A** & **B**) Distribution of expanding or contracting orthogroups in the genomes of oomycetes used in this study. (**A**) Phylogenetic relationships among the oomycete species used in the current analysis. Red numbers represent count of expanded orthogroups in extant species for most recent common ancestor (MRCA); blue numbers represent count of contracted orthogroups in extant species most recent common ancestor (MRCA). (**B**) PFAM domain distribution of mycoparasitic *Pythium* genes assigned in latest common ancestor (LCA)-specific expanded orthogroups. Only domains with two hits or more were included in the analysis. (**C & D**) The CAZyme of *Pythium* species. (**C**) Species tree of oomycetes used in this study and distribution of CAZy proteins. The species tree was constructed using the maximum likelihood method with 1000 bootstraps based on a concatenated alignment of housekeeping genes identified by CEGMA analysis. Green bars indicate the total number of CAZyme encoding genes; blue bars indicate the number of CAZy families present. (**D**) Average gene number of CAZy families unique or expanding in mycoparasitic *Pythium* species. CAZy families were identified as unique or expanding in mycoparasitic *Pythium* species compared to other oomycetes by a T-test analysis (Adapted from Paper I and II).

5.2 Mycoparasitic determinants of *Pythium* species

Alongside the comparative genomics, RNA sequencing was performed on the interactions between the mycoparasites *P. oligandrum* and *P. periplocum* with the phytopathogens *Ph. infestans* and *B. cinerea.* The data was mined to check the expression of mycoparasitic determinants (*e.g.* the CAZYome in Paper II or ABC transporters and effector-like genes in Paper I,) in *P. oligandrum* and *P. periplocum.* This allowed us to identify common and unique differentially expressed genes (DEGs) in the two mycoparasites, when they interact with the prey, and thus further revealed potential conserved mechanisms of mycoparasitism in the two species (**Fig. 6A and B**).

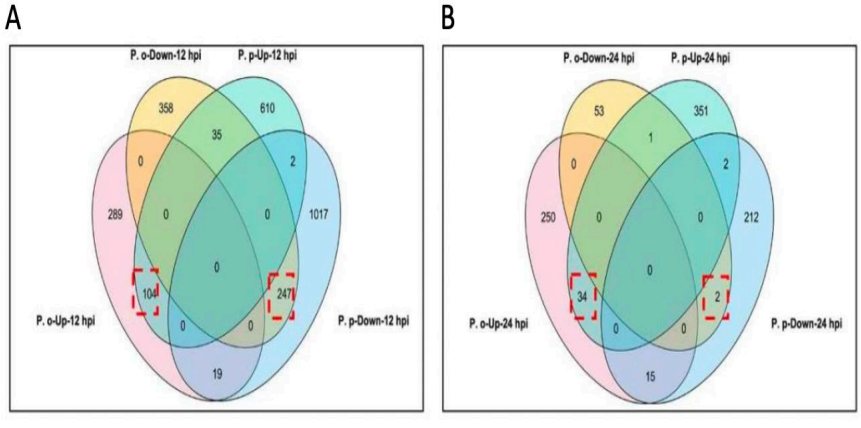

Figure 6. (**A**) Venn diagram showing up- and down-regulated differentially expressed genes of *P. oligandrum* and *P. periplocum* at 12phi. Dashed red boxes indicate common up/down-regulated DEGs at 12 hpi, which means *P. oligandrum* DEGs and their orthologs in *P. periplocum* were both significantly up or down-regulated during interaction with *Ph. infestans* (**B**) Venn diagram showing up- and down-regulated differentially expressed genes of *P. oligandrum* and *P. periplocum* at 24 hpi. Dashed red boxes indicate common up and down-regulated differentially expressed genes at 24 hpi. (Modified from Paper I).

Protein families database (PFAM) domain annotations revealed that some of the DEGs belong to gene families that could harbour mycoparasitic functions. Those were elicitors, AVH-like RXLR effectors, trypsin proteases, and novel AVH-like RXLR effectors (Paper I). Since the ABC transporters were an expanded orthogroup in the LCA of both *Pythium* species (**Fig. 5B**), we also included these in the more detailed downstream analysis of the mycoparasitic determinants of *Pythium* species, carried out in Paper I. The phylogenetic analysis of the ABC transporters in the mycoparasites revealed that the ABC transporter group could be divided into 5 subgroups (**Fig. 7A**). Among these subgroups the ABC-G subgroup was the largest. The generated expression profiles from the transcriptomic data also revealed significantly expressed ABC transporter encoding genes in the two mycoparasitic *Pythium* species during interactions with *Ph. infestans* (**Fig. 7B).** The expression patterns of three ABC transporter genes, an orthologue pair present in both mycoparasites, and a single gene in *P. periplocum* were validated with RT-qPCR (Paper I, **Fig. 5D**). Of those three genes, the gene PYOLI_00006900 followed the expression pattern seen in the transcriptomic analysis, meaning that it was induced when *P. oligandrum* was feeding on the prey (**Fig. 7C**), thus indicating that the specific gene may be an important feature of mycoparasitism for this species. ABC-transporters have also been found to be important mycoparasitic determinants in other mycoparasitic species, for instance *Clonostachys rosea* (Karlsson et al., 2015).

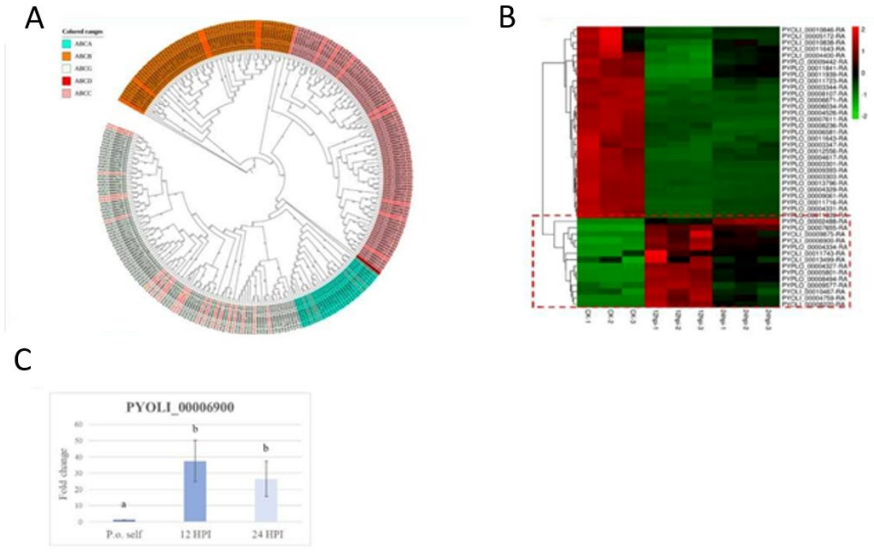

Figure 7. Analysis of ABC transporters in mycoparasitic *Pythium* species. (**A**) Maximum likelihood tree with 1,000 bootstraps (values displayed per branch, displaying a phylogenetic tree of ABC transporters, which were identified in the two *Pythium.* Red lines mark the orthologous pair of ABC transporter genes (red label), which were both significantly expressed during interaction with *Ph. Infestans*. (**B**) Expression profile of significantly expressed ABC transporters encoding genes in two mycoparasitic *Pythium*. (**C**) Quantitative Real-Time PCR verification of an ABC-G gene in *P. oligandrum* (PYOLI_0000690). Gene expression is given as fold change of (n=3) independent biological replicates, error bars represent standard deviation, different letters indicate significant difference, which was tested with one-way ANOVA ($p<0.05$) (The figure is modified from Paper **I**).

Another important gene family that contains determinants of the mycoparasitism by *P. oligandrum* and *P. periplocum* is the AVH gene family of RxLR-like effectors. These effectors have been shown to be important contributors to plant disease in phytopathogens (Jiang et al., 2008). Although previously believed to be absent in the *Pythium* genus, recently they were identified in both *P. oligandrum* and *P. periplocum* (Ai et al., 2020).

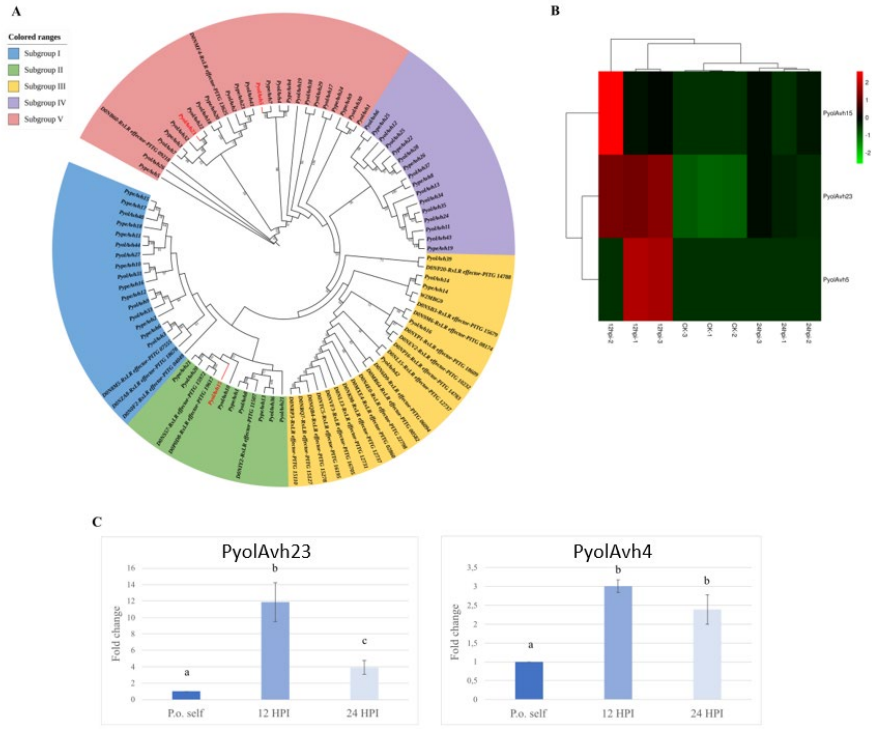

Figure 8. Avh effectors in *P. oligandrum* and *P. periplocum*. (**A**) A phylogenetic tree of Avh effectors identified in *P. oligandrum* and *P. periplocum* compared to the phytopathogenic counterpart *Ph. infestans*. Maximum likelihood tree with 1,000 bootstraps (values displayed per branch). Red lines denote orthologous pairs of Avh effectors encoding genes (red label), which were both significantly expressed during interactions with *Ph. infestans*. (**B**) Expression profile of significantly expressed Avh effectors encoding genes in the two mycoparasitic *Pythium* species. (**C**). RT-qPCR verification for AVH-effector genes expression in *P. oligandrum* (PyolAVH23 and PyolAVH4) during the interactions with *Ph. Infestans*. Gene expression is given as fold change of (n=3) independent biological replicates, error bars represent standard deviation, significant difference is indicated with different letters, tested with one way ANOVA ($p<0.05$) (Adapted from paper **I**).

Using the same bioinformatic pipeline as proposed by Ai *et al.* (2020) several AVH-effector-like proteins were identified in both mycoparasitic *Pythium* species. **Figure 8A** shows the phylogenetic analysis of three orthologous genes in *P. oligandrum* (PyolAvh4, PyolAvh15 and PyolAvh23; marked in red).

These three genes were differentially expressed during the interaction with the oomycete prey *Ph. infestans*. For all three genes, expression was induced during the early stages (12hpi) of the interactions with the prey (**Fig. 8B**). Interestingly, despite *in silico* identification of these RXLR-type effectors in both *Pythium* mycoparasites, the transcriptomic analysis revealed that only *P. oligandrum* RXLR-effector-like genes were upregulated during interaction with the prey. This indicates that *P. periplocum* may differentiate from *P. oligandrum* and may not deploy RXLR-type effectors during mycoparasitism of *Ph. infestans*. However, it could also suggest that these specific effectors are not expressed during the particular *in vitro* confrontation assay, but they might contribute to mycoparasitism *in vivo*.
A validation of the expression pattern of the two most upregulated Avh genes (*PyolAvh4* and P*yolAvh23*) was performed (**Fig. 8C**) and the results showed that the genes *PyolAvh4* and P*yolAvh23* are upregulated compared to self-interacting conditions during the early stages of mycoparasitism on *Ph. infestans* (**Fig. 8C**). Thus, RXLR-type effectors such as AVH-genes are possible mycoparasitism determinants in these *Pythium* species. Finally, trypsin proteases and elicitin-like proteins were also found to be important determinants of the mycoparasitic *Pythium* species as described in Paper I.

5.3 An expanded CAZyome in mycoparasitic oomycetes, is important for mycoparasitism

In Paper II the focus was on identifying CAZy families that potentially play a role in the degradation of fungal or oomycete cell walls, particularly those that are expanded in mycoparasitic oomycetes. We examined the metabolism of major cell wall constituents, including cellulose, glucan, chitin, and hemicelluloses in the mycoparasitic *Pythium* species *P. oligandrum* and *P. periplocum*, as well as in the plant pathogens, including *Ph. infestans*. Our analyses predicted 516 proteins in the CAZyome of *P. oligandrum*, 431 proteins in *P. periplocum*, and 321-719 proteins in other oomycete species (**Fig. 5C**). In *P. oligandrum* the total number of predicted CAZy families was 106. In *P. periplocum* it was almost identical with a total number of CAZy families predicted to be 102. The total number of CAZy families in the other oomycetes ranged from 93 to 110 (**Fig. 5C**). Our predictions are consistent with previously reported findings on the oomycete CAZyome (Ospina-Giraldo et al., 2010; Adhikari et al., 2013), indicating their reliability. We also found that mycoparasitic *Pythium* species had a higher number of CAZy encoding genes on average than their phytopathogenic relatives. To identify redundancy and compare CAZymes among the different species,

we compared the average gene member count per CAZy family. Our analysis identified 20 CAZy families that were either expanded or unique in the mycoparasitic *Pythium* genomes compared to other species in our analysis (**Fig. 5D**). These families belonged to the auxiliary activity, carbohydrate-binding module, glycoside hydrolase, glycosyl transferase, and polysaccharide lyase families. Many of the expanded or unique CAZy families are involved in the degradation of carbohydrates that are components of fungal and/or oomycete cell walls. Genes from three families involved in the degradation of cellulose or chitin (namely AA9, GH5_14 and GH19) were expanded via tandem duplication. Even though we did not see clear evidence of two-speed genomes in the comparative analysis of the genomes of our mycoparasitic *Pythium* species with other oomycetes, the members of these three families were all located in gene-sparse regions of the genomes. These data therefore suggest that the three enzymes are pathogenicity factors that are undergoing rapid evolution. Based on our analysis, we predict the GH55 and GH71 CAZy gene families (involved in the degradation of glucans) are likely to have been obtained by horizontal gene transfer events from fungi. The GH46 chitinosanase gene family is likely to have been acquired from a viral donor, whilst the GH76 family of mannanases may have been transferred horizontally from bacteria to the LCA of the mycoparasitic *Pythiums*. Since these families are absent from the genomes of the plant and animal pathogens included in our study; we hypothesize that horizontal gene transfer may have played an important part in the transition from plant and animal pathogens to mycoparasites, by giving them means by which to break down the cell walls of prey species to obtain the nutrients within.

Growth assays on complex carbohydrates

To complement the novel findings of the existence and importance of the cell wall degrading enzymes (CWDE) from the CAZyme complement in the mycoparasitic *Pythium* species, a series of growth assays with complex carbohydrates (cellulose, chitin or the monomeric unit of chitin, N-acetylglucosamine) was performed. Yeast extract or glucose were used as a positive control, representing normal growth conditions. A comparison of the mycoparasitic *Pythium* species to the phytopathogenic relative *Ph. infestans* was performed (**Fig. 9**). *P. oligandrum* grew the most with glucose as the primary carbon source. In addition, it could grow on cellulose, chitin and N-acetylglucosamine as primary carbon sources (**Fig. 9A and B)**. *P. periplocum* grew equally well on most carbohydrate sources but had a strong

preference for glucose as a carbon source when yeast extract was not present (**Fig. 9C and D**). *Ph. infestans* grew the most on cellulose and was unable to use chitin or N-acetylglucosamine (**Fig. 9E and F**), indicating adaptation to cellulosic plant hosts. This suggests that degradation of complex carbohydrates in fungal and oomycete cell walls is not the major driver for the mycoparasitic oomycetes, and instead they primarily focus on degrading components of prey cell walls to gain access to simple sugars. This is similar to the strategy used by phytopathogenic *Pythium* species which macerate plant cell walls to gain access to nutrients such as simple sugars (Zerillo et al., 2013). However, the expansion of CWDEs within mycoparasitic oomycete genomes shows that they can utilize more complex carbohydrates, when simple sugars are scarce. They also exhibit a fast growth *in vitro*, in all conditions tested, which potentially indicates that they can outcompete other species in their ecological niche.

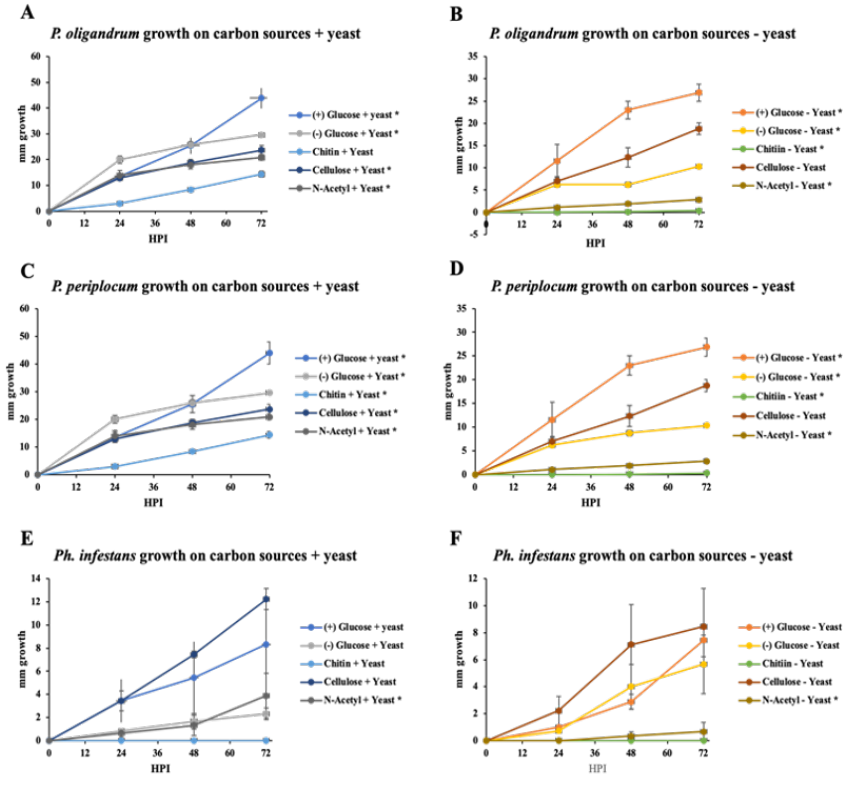

Figure 9. Growth of *P. oligandrum*, *P. periplocum*, and *Ph. infestants* on various carbon sources which are a representation of the major structural carbohydrates in oomycete or fungal cell walls. X-axis shows hours post inoculation (hpi). Y-axis displays colony diameter in mm. (**A** and **B**) Growth rate of *P. oligandrum*. (**C** and **D**) Growth rate of *P. periplocum*. (**E** and **F**) Growth rate of *Ph. infestans*. Asterisk indicates statistically significant difference in comparison to the control (-glucose, -yeast), tested with t-test of the area under the growth curve ($p < 0.05$) (Adapted from Paper II).

5.4 Potato biostimulation by *P. oligandrum*

Biostimulation in potato has been reported after the application of BCAs. For example, application of *Trichoderma harzianum* has resulted in an increase of both shoot length and tuber yield (Rakibuzzaman et al., 2021). Similar observations of increased growth and tuber yield in potatoes were reported after amendment with the bacterial biocontrol agent *Bacillus subtilis* (Kumbar et al., 2019). However, in both studies, only one potato genotype

was used. Plant genotype effects are suggested to play an important role in the growth promoting effects of *Trichoderma* species in sugar beet (Schmidt et al., 2020). Thus, it was hypothesized that different genotypes of potatoes may or may not benefit from *P. oligandrum* amendment, and in addition that phenotypic changes, due to *P. oligandrum* treatments, may vary. This was investigated using three independent bioassays in a controlled, greenhouse environment. Three different potato varieties cv. Kuras, cv. Desirée and cv. King Edward were used. The results showed that the potato genotype cv. Kuras responded to *P. oligandrum* treatment with both a significantly larger plant height and an increased fresh and dry weight of the shoots ($p < 0.001$ and $p < 0.05$, respectively), in comparison to the untreated control plants **(Fig. 10A, D, G).**

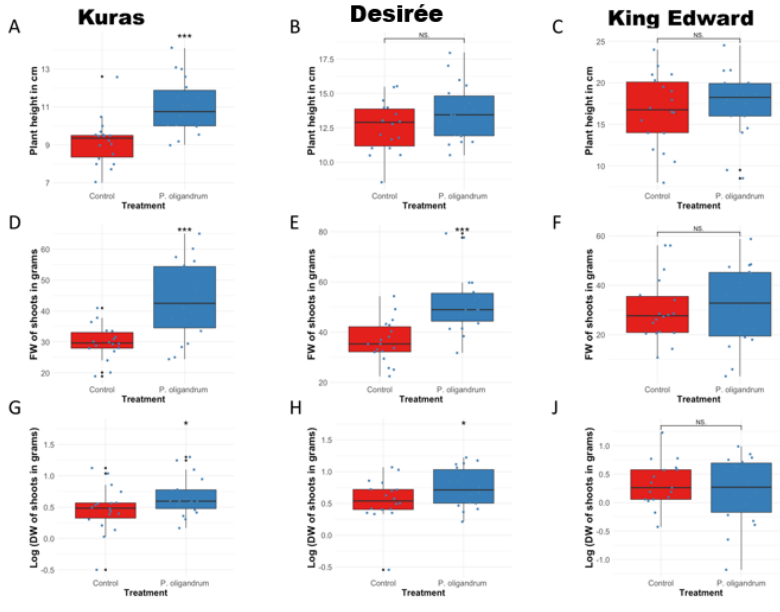

Figure 10. Boxplots of biostimulation of potato plants treated with *P. oligandrum* (blue boxes) or untreated control plants (red boxes), in the three different genotypes Kuras, Desirée and King Edward in greenhouse bioassays. (**A-C**) Plant height of the longest shoot in cm. (**D-F**) Fresh weight of the shoots in grams. (**G-J**) Dry weight of shoots in logarithmic transformed weight in grams. NS $p > 0.05$, * $p < 0.05$, *** $p < 0.001$ The total number of plants treated, or untreated controls were (n=6) per experiment per genotype. The experiment was repeated 3 times independently (Adapted from Paper III).

The genotype cv. Desirée showed an increased fresh and dry weight of shoots (**Fig. 10E and H**), however. no enlargement of shoot length was observed. No significant effect of *P. oligandrum* treatment versus the control plants was observed on the genotype cv. King Edward (**Fig. 10C, F, J**). Thus, the results from controlled environment experiments indicate that the plant growth promoting effects of treatment with the BCA *P. oligandrum* is genotype dependent. Therefore, for field testing of this effect, we selected the potato genotype cv. Kuras, since this genotype responded most significantly to *P. oligandrum* treatment. The experiment was carried out in two field trials in 2019 and 2021 at SLU, Alnarp.

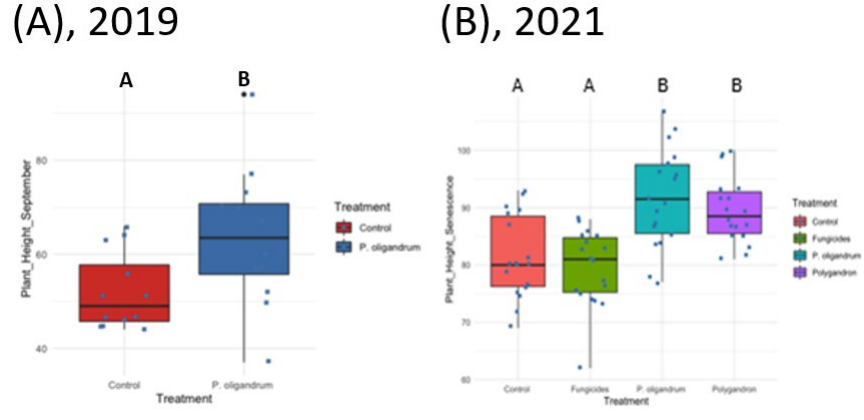

Figure 11. Boxplot of plant height (measured as centimetres of the longest shoot) at the end of the experiment in the field trials where potato plants of the cultivar Kuras were treated with *P. oligandrum*. (**A**) field experiment 2019, and (**B**) field experiment 2021. Significant difference ($p < 0.05$) is indicated with different letters. The total number of plants used per treatment in 2019 was (n=12) and in 2021 (n=18).

In **Figure 11A**, results from the field experiment in 2019 are shown. In 2019, *P. oligandrum* treatment resulted in significantly longer shoots, as indicated with different letters. Similar observations were recorded in 2021, where *P. oligandrum* treatment resulted in significantly longer shoots compared to both fungicide treated and untreated control plants (**Fig. 11B**). Interestingly, treatment with the commercial formulation of *P. oligandrum* (Polygandron WP®) also resulted in longer shoots (**Fig. 11B**), indicating the robustness of the growth-promotion effects induced by *P. oligandrum*. In Papers III and IV the impact of this *P. oligandrum* treatment on the potato cv. Kuras rhizosphere microbiome was also investigated, as described below.

5.5 *Pythium oligandrum* decreases early blight, caused by *A. solani,* in potato plants

In Papers IV and V it was hypothesized that *P. oligandrum* can be used as an antagonist of *A. solani,* in order to control early blight in potato. Therefore, we tested the efficacy of *P. oligandrum* against early blight in potato plants in both controlled, greenhouse environments and in field trials. In addition, we also tested *P. oligandrum* in combination with the commercially available BCA Serenade ASO®, which is based on the bacterium *Bacillus subtilis.* Volatiles produced by *B. subtilis* have been found to strongly reduce both spore production and mycelial growth of *A. solani* (Zhang et al., 2020). The product Serenade was previously used against early blight in greenhouse cultivated tomatoes and a significant decrease in two out three experiments of early blight disease pressure was found (Egel et al, 2019). Also in field grown tomatoes Serenade was found to have a significant effect on both AUDPC and the disease severity (Abbasi & Weselowski, 2015). However, the effect was not consistent in all three years of the field trials, where they tested the product. Thus, *P. oligandrum* and two commercially available biocontrol products, Polygandron and Serenade, were tested in both controlled environments (greenhouse experiments) and in larger field trials (Papers IV and V). For a complete overview of the experiments conducted refer to **Table 2.**

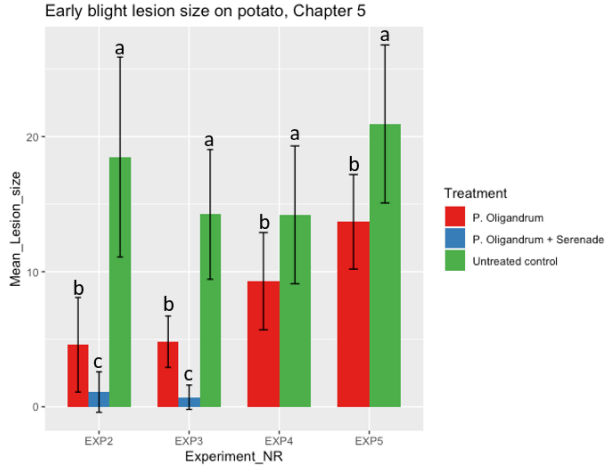

Figure 12. Control of early blight infection, in the potato cv.Desirée, using either *P. oligandrum* or a combination of *P. oligandrum and* Serenade ASO. Treatments were applied 48 h before inoculation of plants with *A. solani*. Untreated control plants were only inoculated with *A. solani.* The differences between treatments in each experiment were calculated according to Tukey's test (p-value < 0.05). Different letters show statistical significance. Vertical bars show standard deviation.

In the greenhouse experiments, the BCA *P. oligandrum* significantly reduced early blight symptoms in three out of four experiments (**Fig. 12,** Exp. 2, 3, 5), when compared to untreated controls plants. A combination of *P. oligandrum* and Serenade resulted in the largest reduction in early blight lesion size on leaves of the potato cultivar Desirée (**Fig. 12**). It was, therefore, tested whether the synergistic effect of the combination of the two BCAs caused the larger early blight reduction. However, the test found no correlation, hence there was no synergistic disease controlling effect (Paper V). The causality of the larger lesion size reduction remains unknown, but it was observed that Serenade alone had a more pronounced effect on early blight in comparison to *P. oligandrum* (Paper V: Fig. 1). Unfortunately, there is still limited knowledge on the efficacy of *P. oligandrum* on the reduction of the early blight disease in field grown starch potatoes, both when used alone or in combination with other BCAs. Based on the significant reduction in early blight lesion size observed in the greenhouse experiments (**Fig. 12** and Paper V: Fig. 2), we decided to test the efficacy of the BCA against early blight in open field trials. In Paper V, we tested several BCAs and plant resistance inducer products against early blight in potatoes in three

constitutive years of field trials. In the present thesis the focus is on *P. oligandrum* and the combination of *P. oligandrum* and Serenade®. In the three years of field trials (reported in Paper V) the disease incidence was scored from mid-August to mid-September, and the relative area under the disease progression curve (rAUDPC) was used to calculate disease progression, and hence, efficacy of disease suppression by the treatments. ANOVA showed that *P. oligandrum* did not significantly reduce the disease progression by itself, in any of the three years of field trials (**Fig. 13**).

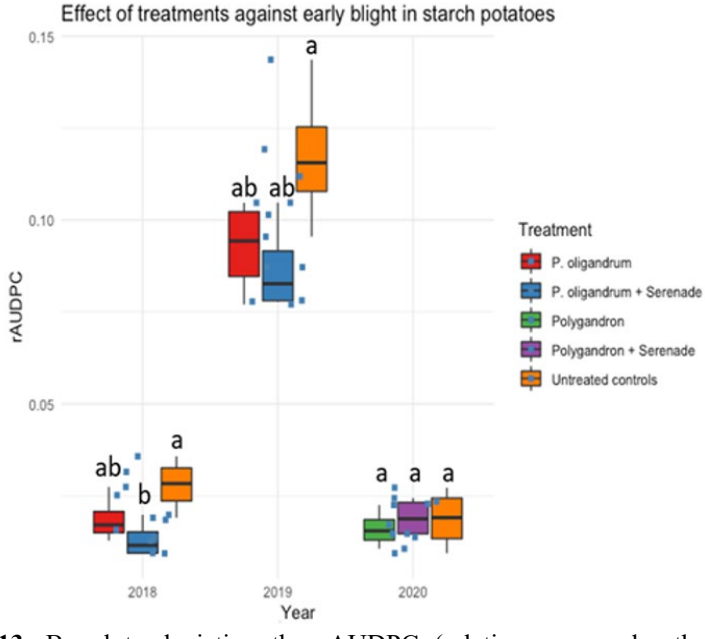

Figure 13. Boxplots depicting the rAUDPC (relative area under the disease progression curve) of early blight infection in the starch potato variety cv. Kuras, in three constitutive years of field trials i.e. 2018, 2019 and 2020. Untreated control plants are compared to treatments with *P. oligandrum*, *P. oligandrum* + Serenade, Polygandron, and Polygandron + Serenade. Different letters indicate significant differences. For a complete overview of statistics refers to Paper V

In 2018 the disease reduction effect was significant for Serenade and for the combination Serenade + *P. oligandrum*, while in 2019 only the effect of the combination Serenade + *P. oligandrum* was significant. In 2020 the commercial formulated product of *P. oligandrum* (Polygandron) substituted the *P. oligandrum* lab strain, which was used in 2018 and 2019. No effect on

disease suppression from any of the BCA treatments was found in 2020 (**Fig. 13**). However, the field trial in 2020 had a very low natural disease pressure. Thus, it was hypothesized that the variation of the effect on the disease suppression caused by the BCAs between the years might be due to the variation of natural infection pressure by *A. solani*.

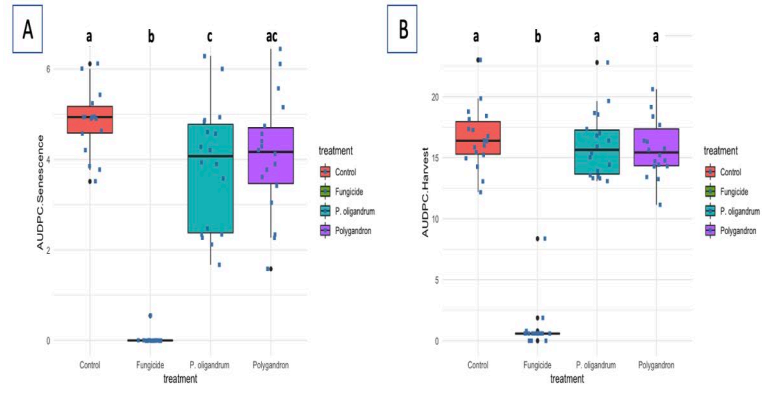

Figure 14. Square root transformed AUDPC (area under the disease progression curve) at (**A)** senescence (02-09-2021) and (**B)** harvest (01-10-2021). Untreated control plants infected with early blight are compared to treatments with fungicide, *P. oligandrum* or Polygandron, a commercially formulated oospore powder from *P. oligandrum*. The number of plants was (n= 18) at each time point per treatment. Different letters indicate significant ($p < 0.05$) differences (Adapted from Paper IV).

To rule out the possibility of low disease pressure, the field trial conducted in 2021 was artificially inoculated with *A. solani.* The inoculation resulted in early blight disease in all the starch potato plants within the field trial (data not shown). In the 2021 field trial, the treatments against early blight consisted of fungicides Narita and Propulse, *P. oligandrum* lab preparation, and the formulated *P. oligandrum* product (Polygandron), all of which were compared to untreated control plants. The disease progression was calculated as area under the disease progression curve (AUDPC), and estimated at two time points i.e. at the senescent stage of the plants (September 2021) and at harvest (October 2021). The results showed that *P. oligandrum* (lab-strain) treatment had a significant disease suppressive effect at the senescent stage of the plants (**Fig. 14A**), but the efficacy disappeared at the harvest stage (**Fig. 14B**). Thus, this may be an indication that this BCA is effective only

when disease pressure is low, or at the early stages of the disease, since the data suggest that when the disease pressure became too high the BCA lost the capability to suppress it. Polygandron did not reduce the disease at any time point (**Fig. 14A and B**).

In contrast to the *P. oligandrum* and Polygandron treatments, the fungicide treatments achieved complete disease suppression at the senescent stage of the plants and the disease suppression persisted up until the harvest (**Fig. 14A and B**). The active ingredient of the fungicide Narita – difenoconazole, has been demonstrated to inhibit mycelia growth of *A. solani* (Issiakhem & Bouznad, 2010), and reduce disease severity, while it also increases tuber yield (Horsfield et al, 2010). The other fungicide used was Propulse, which has the active ingredients floupyram and protioconazol that have been widely used to control early blight in potatoes (Bauske et al., 2017). In Paper V we showed that a combination of the two fungicides controlled early blight in field grown starch potatoes (Paper V: Table. 3A and B), a similar observation was also made in Paper IV.

Currently, it appears that *P. oligandrum* is insufficient as a BCA treatment for early blight in open field starch potato production, especially in comparison to modern fungicides. Inhibition of early blight in greenhouse and field grown potatoes has been investigated using commercially formulated biocontrol products from *Trichoderma* ssp. and *B. subtilis,* with a positive effect on disease suppression (Metz, 2017). However, the author of this study concluded that the BCAs could not substitute chemical fungicides but might help in reducing the number or intensity of chemical treatments. Thus, combining fungicide treatments or replacing some fungicide applications in the treatment schedule with *P. oligandrum* might give a sufficient protection against early blight in potatoes. Indeed, tank mixtures of *P. oligandrum* and fungicides have proven effectiveness against other phytopathogens (Ikeda et al., 2012; Kurzawińska & Mazur, 2007; Kurzawińska & Mazur, 2008).

5.6 Applications of *P. oligandrum* or conventional fungicides have minor impacts on the diversity of the potato rhizosphere microbiome.

In Papers III and IV, we investigated the ecotoxicological effect of the treatments with *P. oligandrum* (Paper III only), Polygandron and fungicides, when used against *A. solani*, on the rhizosphere microbiome of starch potatoes. In Paper III), plants were treated with a *P. oligandrum* oospore solution 5 times throughout the cultivation period, starting in July and ending in September. The plants were sprayed biweekly and the impact on the rhizosphere microbiome was investigated by comparison to untreated control plants. In the same paper (Paper III: Fig. **6 A-D**), the alpha-diversity was compared in August and in September 2019, with no significant difference observed on the fungal or bacterial microbiome between untreated control samples and *P. oligandrum*-treated plants.

The rhizosphere microbiome community structure is visualized in **Figure 15A-D**. No clear separation between untreated control plants and plants treated with *P. oligandrum* was observed in either August or September 2019, for either fungal or bacterial communities, as shown in the Principle Coordinate Analysis (PCoA) plots. Analysis of the beta-diversity using the PERMANOVA test on the permutations of the bray Curtis distance matrices revealed that no significant difference in beta-diversity was observed between *P. oligandrum* treated plants and the untreated control plants.

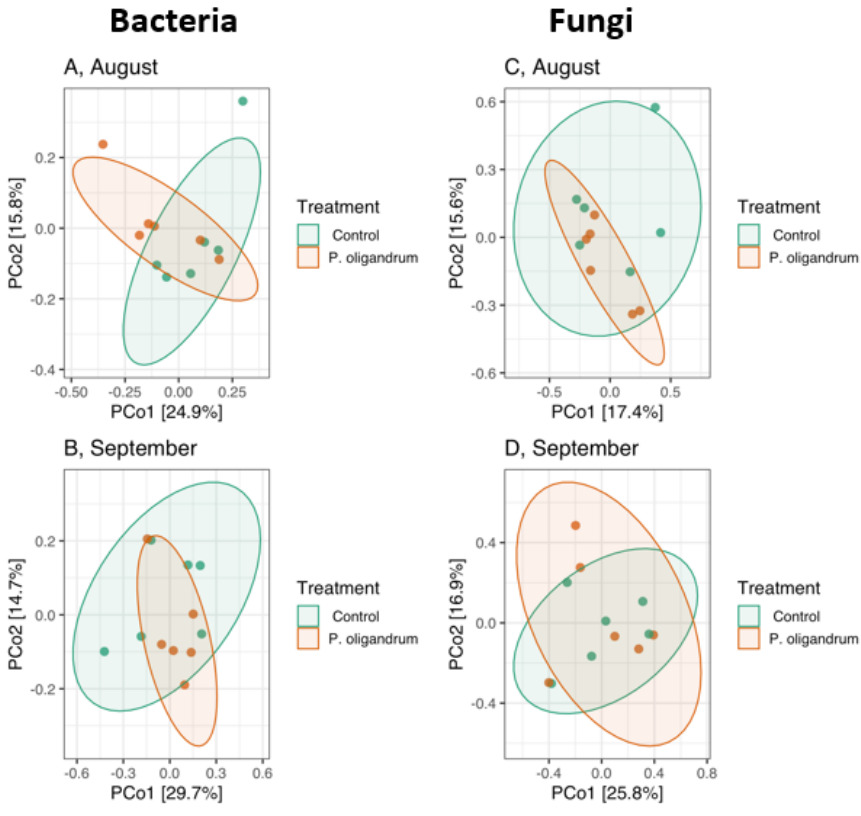

Figure 15. Beta-diversity shown as Principle Coordinate Analysis (PCoA) plots between the rhizosphere microbial communities from either control plants or plants treated with *P. oligandrum*. (**A**) Bacteria community in August. (**B**) Bacteria community in September. (**C**) Fungal community in August. (**D**) Fungal community in September. Asterisk indicates significant differences, PERMANOVA ($p < 0.05$). (Adapted from Paper III)

In Paper IV, the impact of *P. oligandrum* on the alpha and beta diversity of the potato rhizosphere microbiome was also investigated. In addition, two other treatments were included, namely Polygandron WP® and conventional fungicides- Narita® and Propulse ®, commonly used against early blight. For an overview of the field trial refer to **Table 2**. We made an in-depth comparison of the microbiome changes for the *P. oligandrum*-treated plants and untreated control plants at the flowering time point. The results showed that no statistical differences were observed in alpha-diversity measured with Shannon-index at the flowering time point, for either bacterial or fungal communities (Paper IV: Fig. 3C and D and **Fig. 16A**).

We did observe, however, a significant change in beta diversity, specifically in the fungal community structure, between *P. oligandrum* treated plants and untreated control plants, as seen in **Figure 16B**.

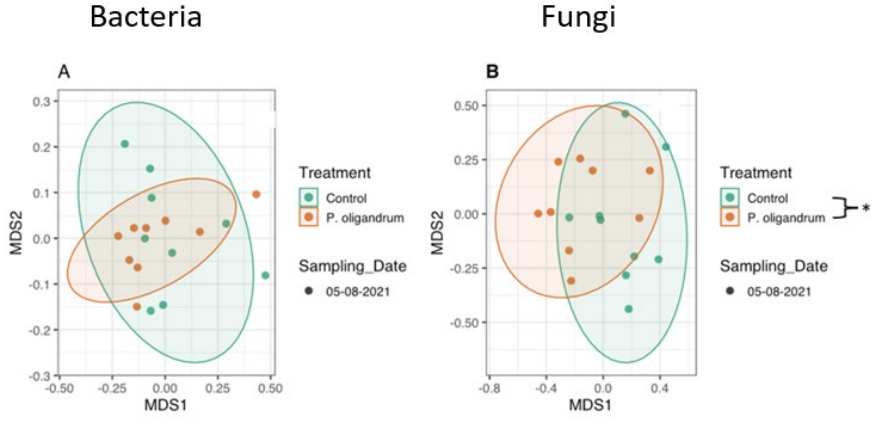

Figure 16. Non-Metric Multidimensional Scaling (NMDS) plots of (**A**) bacterial community and (**B**) fungal community, between untreated control plants and *P. oligandrum* treated plants at the flowering time of the experiment (05-08-2021) under *Alternaria solani* infection. Significant difference is indicated with an asterisk ($p < 0.05$) (Adapted from Paper IV).

At the senescence stage of the plants, we did not observe any significant changes in alpha-diversity between untreated control plants and any of the treatments, in neither the bacterial nor fungal microbiome community (Paper IV: **Fig. 6C-D**). In addition, the analysis of the rhizosphere microbiome community structure at the senescence stage of the treated plants, when compared to the untreated control plants, revealed no clear separation in the NMDS plots, in neither the bacterial nor fungal communities (**Fig. 17A and B**).

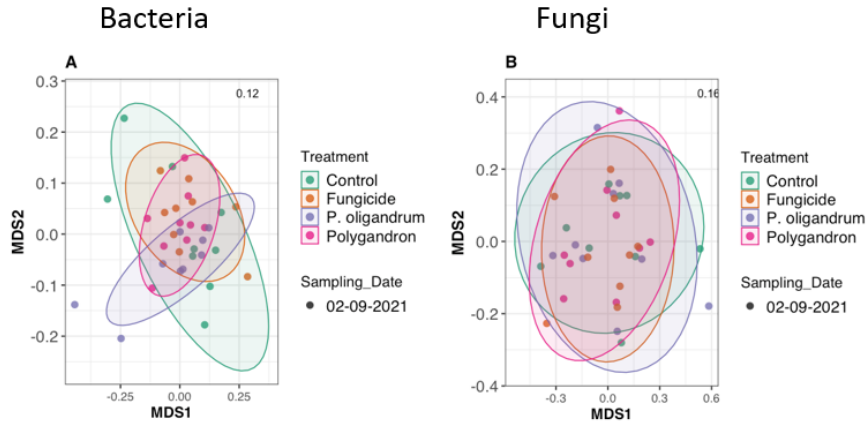

Figure 17. Non-Metric Multidimensional Scaling (NMDS) plots of (**A**) bacterial and (**B**) fungal community changes between nontreated control plants, fungicide-, *P. oligandrum*- and Polygandon-treated plants at the senescence time of the experiment (02-09-2021) under *Alternaria solani* infection. PERMANOVA results of bray curtis distances revealed that, no significant changes in the beta diversity were detected between control plants and any of the treatments (Adapted from Paper IV).

The subsequent PERMANOVA test on the permutation stresses of the bray Curtis distance matrixes showed no significant differences in beta-diversity between untreated control samples or any of the treatments, in neither bacterial nor fungal rhizosphere microbiomes. Thus, the data presented in Papers III and IV indicates that *P. oligandrum* does not have major impacts on the alpha and beta diversity of the rhizosphere microbiome of starch potato (cv. Kuras), at least as observed in our cropping system. Conventional fungicides used against early blight did not significantly impact alpha or beta diversity of the rhizosphere microbiome either. This is, however, surprising, since the active ingredients in the specific fungicides have previously been shown to interfere with the diversity and structure of the rhizosphere microbial community (Santísima-Trinidad et al., 2018; Zhang et al., 2021; Zhang et al., 2014).

5.7 A realistic outlook on *P. oligandrum* environmental impact in the potato cropping system

The insignificant impact of *P. oligandrum* on the potato rhizosphere microbiome was somewhat unexpected. Therefore, we decided to further investigate if similar observations could be seen in a larger-scale potato cropping system. To do so, we collected non-destructive rhizosphere samples from the field trial conducted in Helgegården in Sweden in 2019, and specifically analysed the untreated control plants and the *P. oligandrum* treated plants. However, as this was a larger field trial, we bulked five individuals from each block, with four blocks per treatments, and included samples taken in July, where no treatment had been applied, as a baseline of the rhizosphere microbiome. We also included samples from September, when plants were entering the senescence stage. The soil type here was a sandy soil, and modern agricultural machinery was used to manage the field trial throughout the season. As the scope of that field trial was to investigate treatment efficacy against early blight, the field received the common fungicide treatment regime against late blight, both for untreated and *P. oligandrum*-treated plants. A complete overview of treatments and management strategies can be found in Paper V. No significant difference was observed in alpha-diversity measured as Shannon index between *P. oligandrum* treated plants and the untreated control plants (**Fig. 18A-D**). In addition, species richness measured as "Observed" ASV, also showed no significant difference.

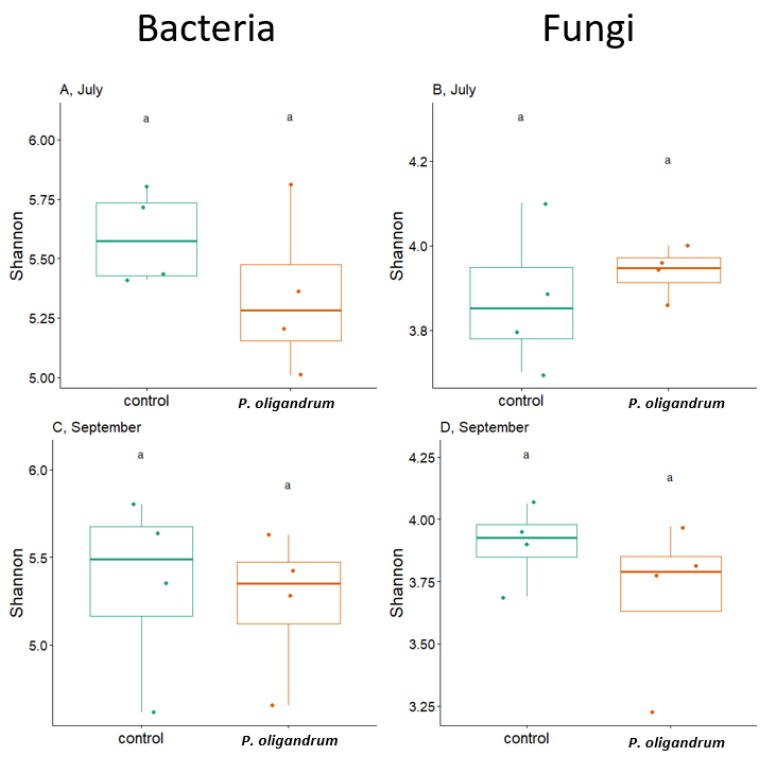

Figure 18. Alpha-diversity, computed as Shannon-index of the rhizosphere microbiome of cv. Kuras, between control plants and *P. oligandrum* treated plants. (**A**) bacteria in July ,(**B**) fungi in July, (**C**) bacteria in September and (**D**) fungi in September. Different letters would indicant significant difference, calculated with ANOVA, with n = 4 samples, per treatment per timepoints.

The analysis of the rhizosphere microbiome community structure in July and September, when untreated control plants were compared to the *P. oligandrum* treated plants, also revealed no clear separation in the PCoA-plots , in neither the bacterial (**Fig. 19A** and **C**) nor the fungal communities (**Fig. 19B** and **D**).

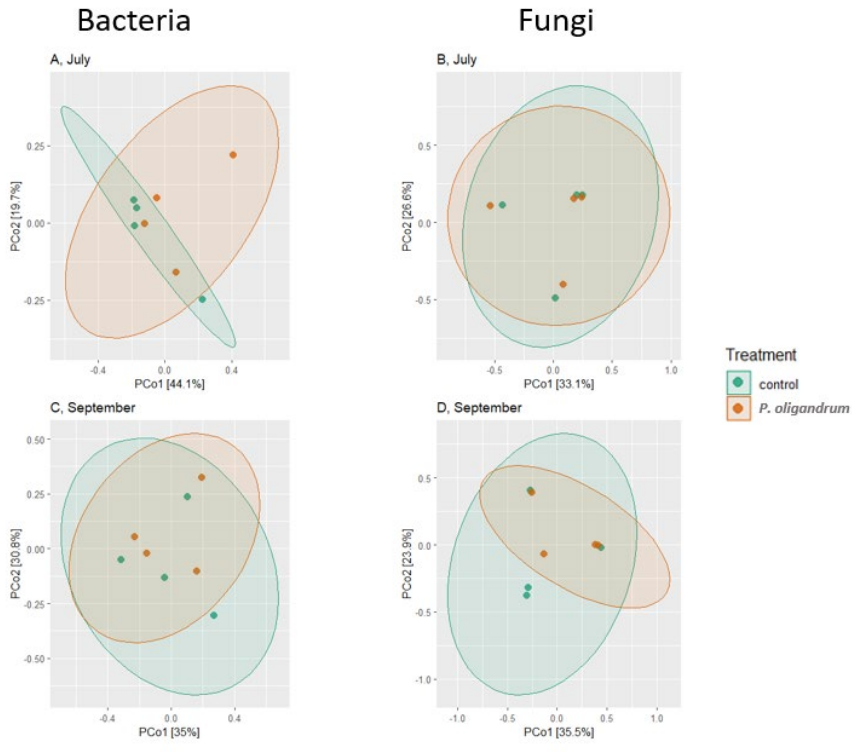

Figure 19. PCoA plots of the beta community structure of the rhizosphere microbiome of cv. Kuras, between control plants and *P. oligandrum)* treated plants. (**A**) bacterial community in July ,(**B**) fungal community in July, (**C**) bacterial community in September and (**D**) fungal community in September. Asterisk would indicant significant difference, calculated with PERMONAVA, with n = 4 samples, per treatment per timepoints.

The subsequent PERMANOVA test on the permutation stresses of the bray Curtis distance matrixes showed no significant differences in beta-diversity of untreated control samples and any of the treated samples, for both bacterial and fungal rhizosphere microbiome. Therefore, it appears that in a large-scale field trial, *P. oligandrum* does not impact the rhizosphere community significantly, when looking into the above diversity measures. Collectively, the observations from Papers III and IV and the results presented here, indicate that the rhizosphere microbiome of the starch potato variety cv. Kuras, is quite resilient to foliar treatments with both the BCA, *P. oligandrum*, and conventional fungicides.

6. Conclusions

In order to improve and broaden the use of BCAs in modern agriculture, it is pivotal to understand the genetics and fundamental biology of these organisms. In Papers I and II, we explored the genetic features that characterize the mycoparasitic *Pythium* species, and how these species differ from their plant pathogenic relatives in the oomycete lineage, using a combination of comparative genomics and transcriptomics approaches. The results showed that a substantial number of these genetic features, such as the ABC transporters, elicitors, proteases and expanded CAZyme families, are also common in mycoparasitic fungi, suggesting that these are probably core determinants of mycoparasitism.

Surprisingly, we have identified features in the genomes of mycoparasitic oomycetes that are similar to those from oomycetes that parasitize animals, such as fish and mosquitos. Thus, it seems that that the arsenal required for parasitism of certain animal species may not be so different from that required for mycoparasitism. Collectively, this study broadens our understanding of the evolutionary relationships between oomycetes that parasitize on different hosts, and of mycoparasites from different lineages. Moreover, identifying key mycoparasitic determinants in *P. oligandrum* and *P. perriplocum* will also potentially contribute to enhancing their biocontrol abilities against phytopathogens.

In Papers IV and V, we investigated the potential of different BCAs to combat early blight in starch potatoes. In light of the aim of the present thesis, we found that *P. oligandrum* did not significantly decrease early blight at the end of the field trials, despite significantly reduced early blight lesion sizes in the greenhouse trials. However, in combination with another BCA, *P. oligandrum* showed a significant reduction of early blight, but only in one out of three consecutive years of field trials. It is often observed that

significant effects of BCAs can be found *in vitro* and in greenhouse trials, but these results rarely translate into open field trials. Thus, our results demonstrate that *P. oligandrum,* applied as a foliar spray of an oospore solution, needs to be rethought and possibly reformulated, since both freshly isolated oospores from the lab strain and wettable powders of the commercial product were insufficient in controlling the disease. Despite being a weak antagonist against early blight in open field trials, we observed a positive effect of the application of *P. oligandrum* (foliar and soil drenches), which was the induction of growth in potatoes. We observed biostimulation only in certain potato genotypes though, indicating a plant genetic component to biostimulation by *P. oligandrum*. The most pronounced biostimulation was observed in the starch potato variety cv. Kuras, where we observed longer main shoots in both controlled environments and in open field trials. In the controlled environment, we additionally measured significantly increased biomass of the shoots due to *P. oligandrum* amendment.

To utilize biological control against phytopathogenic-induced diseases in potato plants, we need to shift our idea away from simple interactions between the host, the pathogen, and the BCA, towards a more holistic view. The plant should be seen as a holobiont, interacting with a multitude of both macro-and-microorganisms. It is, therefore, of prime importance to investigate the impact on resident microorganisms in the potato cropping system, especially when we are amending BCAs, or chemical fungicides. Both BCAs and chemical fungicides have been shown to alter the rhizosphere microbiome of crop species, including potato plants. Our investigation in three different field trials revealed that *P. oligandrum* has a very limited impact on the alpha and beta-diversity of bacteria and fungi in the rhizosphere microbiome of potato cv. Kuras. However, we did observe some differentially abundant fungal and bacterial genera after *P. oligandrum* treatment, but a detailed analysis showed that these were marginal genera in the microbiome. Based on our studies, we cannot fully conclude how these genera impact the soil functionality or fertility. To our surprise, fungicides commonly used against early blight, did not significantly impact the rhizosphere microbiome, which is in contrast to results previously shown in the literature.

In conclusion, the results presented within this thesis broaden our fundamental and applied knowledge of mycoparasitic *Pythium* species and further provide new knowledge about the rhizosphere microbiome

communities of starch potatoes, which is of great importance for both the research community and the farmers.

7. Future perspectives - *P. oligandrum* the friend or the foe?

Historically, plant pathologists have investigated plant diseases as binary systems. We now recognize that plants, including crop species, are instead holobionts. Within the holobiome exists a pathobiome and a symbiome, which are both subjects to external biotic and abiotic stresses, as visualized in the schematic representation in **Figure 4**.

The IPM approach, mandated by the EU, dictates the reduction of agrochemical inputs into these systems. One solution is the use of BCAs to combat plant diseases. However, limited knowledge exists on how BCAs, such as *P. oligandrum*, interfere with the plant holobiome. This is probably due to the complexity of these systems (**Fig. 4)**, and for now we are just scratching the surface of these complex interactions. In the present thesis, the impact of *P. oligandrum* on potato plants was investigated. The results presented in Papers I-V reveal the potential of *P. oligandrum* as both a plant pathogen-suppressor along with its biostimulative properties in starch potatoes. Since *P. oligandrum,* did not significantly impact the rhizosphere microbiome in our cropping system, it should be viewed as an ecologically friendly BCA. However, we identified that the treatment with *P. oligandrum* affected the abundance of several genera, including fungal and bacterial taxa from both the rhizosphere symbiome and the pathobiome. Future research should investigate whether these genera are important determinants of soil fertility (involved into shaping soil physicochemical properties), and overall plant health of the potato crops. Metabarcoded amplicon sequencing has increased our fundamental knowledge of the microbiome composition of a multitude of crop species, including potatoes. However, in combination with metagenomics, metatranscriptomics and metabolomics, we could gain

further insights into the specific functions and properties of microbial taxa within the cropping system, including those in the present study. Further research on identifying the genes that are present in the rhizosphere and the ones that are active under certain conditions, treatments or stresses will contribute to our understanding of the precise roles of individual microbes and their communities in the rhizosphere. For example, it would be interesting to determine whether these differentially abundant taxa identified in our study are active soil dwellers or just occupy a part of the niche, whether they are closely associated with the plant-host and thus, important for plant health and performance, and whether they form interactions with other important microbial taxa and potentially shape the rhizosphere microbial communities. Another important aspect for the future would be to breed potato genotypes that allow long term establishment of BCAs.

In Paper V, we observed a lack of translation of early blight disease suppression, from controlled environments to field trials, when *P. oligandrum* was used as a treatment. This phenomenon is commonly observed for several other BCAs used against other diseases, such as potato late blight. Perhaps, future research on *P. oligandrum* should include rethinking the application method in an open field setting. In addition, research should re-evaluate the formulation, the frequency of application and the timing of application. In comparison to synthetic chemicals used to treat plant diseases, BCAs need to establish, thrive and proliferate within the cropping system in order to be effective in plant disease suppression. Future research should also investigate whether an early establishment of *P. oligandrum* in the field would provide an advantage to the BCA, and ultimately enhance efficacy. In addition, considering that environmental factors such as humidity, temperature, and UV-radiation, can be controlled in enclosed environments, versus the open field, future research should investigate whether the application of a BCA in a favourable environment, for growth and establishment, increases the BCA efficacy in the cropping system.

With the advances in our understanding of what determines the mycoparasitic *Pythium* species, exciting new prospects for developing better and more effective biocontrol agents have opened. Analysis of the genomes of the two *Pythium* species revealed several mycoparasitic key traits, which could be exploited more efficiently to develop hyper-virulent mycoparasites with greater efficacy against fungal or oomycete phytopathogens. The new

knowledge on how these mycoparasites utilize complex carbohydrates as nutrient sources might also provide new ideas for product formulation, by encapsulating, for example, the oospores with an additive that serves as a starter package for growth on the plants or in the rhizosphere. An improved formulation could result in an increased sporulation and colonization competency of the mycoparasites. In addition, further screening of important genetic traits should be conducted to identify unexplored modes of action against pathogens, *e.g.*, searching for biosynthetic clusters for secondary metabolites that have antimicrobial properties.

Fungicide-resistance development is a major threat to modern day cropping practices, due to the nature of conventional monoculture farming systems and the frequency of fungicide applications. Pathogens, such as *A. solani,* can rapidly overcome new active ingredients in modern fungicides. However, there is limited knowledge on the potato pathogens, including *A. solani* and *Ph. infestans*, developing resistance towards BCAs. Regarding *P. oligandrum*, it is unlikely that resistance will develop due to the different modes of action that the mycoparasite harbours. However, it is a valid concern and should be monitored. Transcriptomic analysis of interactions between phytopathogens and BCAs might unravel how the phytopathogens defend themselves against the mycoparasites, and provide clues as to whether they are likely to rapidly develop resistance to these BCAs.

Moreover, future breeding approaches should acknowledge the importance of the microbiome on potato crop productivity, and breeding lines should favour the colonization of symbiotic microbes, within the rhizosphere of the plants. Varieties that form symbiotic relationships with known and used BCAs, such as *P. oligandrum*, should be favoured as breeding lines, since our results suggest that *P. oligandrum* induced biostimulation is somewhat plant genotype-dependent.

Finally, mycoparasitic species such as *P. oligandrum* have a promising role in organic, and especially greenhouse production, due to their ability to serve as BCAs. In conclusion, our increasing understanding of mycoparasitic *Pythium* species and their potential applications present a bright future for sustainable plant production. In my opinion the BCA *Pythium oligandrum* should be considered a friend and not a foe within the potato cropping system.

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Popular science summary

Potatoes are a beloved and important crop, but they are also vulnerable to diseases like early and late blight. To control these diseases, farmers have traditionally relied on synthetic fungicides. However, these chemicals can have negative effects on the environment and human health. Thus, we are in need of more sustainable approaches to control the diseases. One approach could be to use biological control agents (BCAs). Examples of promising BCAs that could be used in the potato cropping system are the oomycetes of the *Pythium* genus that feed on fungal or oomycete pathogens (mycoparasitism). However, to use them effectively, we need to understand more about their biology and interactions within potato crops. That's where my thesis comes in. The goal of this thesis was to broaden our understanding of two *Pythium* species, *Pythium oligandrum* and *Pythium periplocum,* and how they differ from their disease-causing counterparts. The thesis also aimed to identify the characteristics that make these *Pythiums* effective at controlling disease, at the molecular level. The research found that certain genetic traits, were important for the mycoparasitism by both *P. oligandrum* and *P. periplocum*. These traits help the mycoparasitic *Pythiums* prey on plant pathogens. The study also looked at whether *P. oligandrum* could be used to control early blight disease in potato plants. In controlled greenhouse environments, a disease suppression was found. However, in field trials with high disease pressure, the effects were less pronounced. This thesis also found that *P. oligandrum* had a positive effect on potato crops, inducing biostimulation (growth enhancement) in both controlled environments and field studies. However, the biostimulation was dependent on the potato genotype. Finally, this thesis investigated whether *P. oligandrum* had any impact on the resident microbiome in the rhizosphere (the soil around or near the roots) of field-grown potato plants. However, research presented in this

thesis indicates that that the impact was minimal. Overall, this thesis provides valuable insights into the fundamental biology of mycoparasitic *Pythium* species and how they differ from their disease-causing counterparts. It also sheds light on the potential environmental consequences of using *P. oligandrum* as a BCA in potato crops. The results could help inform the development of integrated pest management strategies in the potato cropping system, which aim to minimize the use of synthetic chemicals and promote sustainable farming practices.

Populärvetenskaplig sammanfattning

Potatis är en älskad och viktig gröda. Den är dock också sårbar för sjukdomar som torrfläckssjuka och potatisbladmögel. För att kontrollera dessa sjukdomar har bönder traditionellt förlitat sig på syntetiska fungicider. Men dessa kemikalier kan ha negativa effekter på miljön och människors hälsa. Därför behöver vi mer hållbara tillvägagångssätt för att kontrollera dessa sjukdomar. Ett tillvägagångssätt kan vara att använda biologiska bekämpningsmedel (”biological control agents”, BCA). Exempel på lovande BCA som kan användas i potatisodlingen är mikroorganismer, som algsvampar ur släktet *Pythium*. Dessa algsvampar kan konsumera sjukdomsalstrande svampar och algsvampar (så kallad mykoparasitism). Men för att använda dessa effektivt behöver vi förstå mer om deras biologi och interaktioner med potatisgrödor. Det är där min avhandling kommer in i bilden. Målet med denna avhandling är att bredda vår förståelse för två *Pythium*-arter, *Pythium oligandrum* och *P. periplocum*, som tex hur de skiljer sig från sina sjukdomsframkallande släktingar. Avhandlingen syftar också till att identifiera de egenskaper som gör dessa *Pythium* effektiva vid bekämpning av sjukdomar på molekylär nivå. Forskningen visar att vissa genetiska egenskaper är viktiga för mykoparasitismen hos både *P. oligandrum* och *P. periplocum*. Dessa egenskaper hjälper de mykoparasitiska *Pythium*-arterna att angripa andra växtsjukdomar. Flera delstudier undersökte också om *P. oligandrum* kan användas för att bekämpa torrfläckssjuka hos potatis. I kontrollerade växthusmiljöer upptäcktes en sjukdomsbekämpande effekt. Men i fältförsök med högt sjukdomstryck var effekterna mindre påtagliga. Denna avhandling visade också att *P. oligandrum* hade en positiv effekt på potatis genom att inducera snabbare tillväxt (så kallad biostimulering) både i kontrollerade miljöer och i

fältstudier. Biostimuleringen var dock beroende av potatisens genotyp. Slutligen undersökte denna avhandling om *P. oligandrum* hade någon påverkan på rotmikrobiomet, dvs de mikroorganismer som lever i jorden runt eller nära rötterna (rhizosfären) hos fältodlade potatisplantor. Forskning som presenteras i denna avhandling visar dock att påverkan var minimal. Sammanfattningsvis ger denna avhandling värdefulla insikter om den grundläggande biologin hos mykoparasitiska *Pythium*-arter och hur de skiljer sig från sina sjukdomsframkallande motsvarigheter. Den belyser också de potentiella miljökonsekvenserna av att använda *P. oligandrum* som BCA hos potatis. Resultaten kan bidra till utvecklingen av mer hållbara bekämpningsmetoder.

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Appendix

All supplementary material for the Papers enclosed in this PhD Thesis can be found via the following hyperlink:
https://drive.google.com/drive/folders/1moV2d-SSURJNCaHk-WzVLFD6aXZDKzSE?usp=share_link

II

frontiers in Microbiology

ORIGINAL RESEARCH
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Horizontal Gene Transfer and Tandem Duplication Shape the Unique CAZyme Complement of the Mycoparasitic Oomycetes *Pythium oligandrum* and *Pythium periplocum*

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Crop protection strategies that are effective but that reduce our reliance on chemical pesticides are urgently needed to meet the UN sustainable development goals for global food security. Mycoparasitic oomycetes such *as Pythium* oligandrum and *Pythium periplocum*, have potential for the biological control of plant diseases that threaten crops and have attracted much attention due to their abilities to antagonize plant pathogens and modulate plant immunity. Studies of the molecular and genetic determinants of mycoparasitism in these species have been less well developed than those of their fungal counterparts. Carbohydrate-active enzymes (CAZymes) from *P. oligandrum* and *P. periplocum* are predicted to be important components of mycoparasitism, being involved in the degradation of the cell wall of their oomycete and fungal prey species. To explore the evolution of CAZymes of these species we performed an *in silico* identification and comparison of the full CAZyme complement (CAZyome) of the two mycoparasitic *Pythium* species (*P. oligandrum* and *P. periplocum*), with seven other *Pythium* species, and four *Phytophthora* species. Twenty CAZy gene families involved in the degradation of cellulose, hemicellulose, glucan, and chitin were expanded in, or unique to, mycoparasitic *Pythium* species and several of these genes were expressed during mycoparasitic interactions with either oomycete or fungal prey, as revealed by RNA sequencing and quantitative qRT-PCR. Genes from three of the cellulose and chitin degrading CAZy families (namely AA9, GH5_14, and GH19) were expanded via tandem duplication and predominantly located in gene sparse regions of the genome, suggesting these enzymes are putative pathogenicity factors able to undergo rapid evolution. In addition, five of the CAZy gene families were likely to have been obtained from other microbes by horizontal gene transfer events. The mycoparasitic species are able to utilize complex carbohydrates present in fungal cell walls, namely chitin and N-acetylglucosamine for growth, in contrast to their phytopathogenic counterparts. Nonetheless, a preference for the utilization of simple sugars for growth appears to be a common trait within the oomycete lineage.

Keywords: mycoparasitism, CAZy, carbohydrate active enzymes, cell wall degrading enzymes, biological control, comparative genomics, oomycete genomics

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INTRODUCTION

The oomycetes are notorious as plant pathogens that cause devastating diseases in crop plants and our natural landscapes. Efficient control of many oomycete diseases relies on the usage of synthetic pesticides, which may have detrimental effects on the environment. The Genus *Pythium* contains predominantly saprotrophs and necrotrophic pathogens that occupy diverse ecological niches and infect various plant, arthropod, and even, human hosts (Plaats-Niterink, 1981). Some members of this fungal-like eukaryotic lineage are mycoparasites, obtaining nutrients from living fungal or oomycete hosts and are thus of great interest as potential biological control agents as part of an Integrated Pest Management (IPM) system for the control of crop diseases.

Mycoparasitic oomycetes have been less-well studied than their plant pathogenic counterparts, nevertheless, two species, *Pythium oligandrum* and *Pythium periplocum* have been investigated in this context. *P. oligandrum* has been documented as an antagonist and/or mycoparasite of a wide range of hosts including plant pathogenic *Pythium* spp. (Benhamou and Chet, 1997), *Phytophthora* spp. (Picard et al., 2000a; Horner et al., 2012), ascomycetes and basidiomycetes (Bradshaw-Smith et al., 1991; Benhamou et al., 1997). As well as an ability to antagonize fungi and oomycetes, these species display several other key features important for biological control of plant diseases. They typically grow faster than their plant pathogenic counterparts, meaning that they can outcompete other species for rhizosphere space and nutrition and they are able to promote both plant growth (Le Floch et al., 2003), and induced resistance in host plants (Takenaka et al., 2011; Ouyang et al., 2015). Whilst these experiments demonstrate their potential as biological control agents and detailed microscopic analysis has revealed the nature of their mycoparasitic interactions with various prey species (Benhamou et al., 1999; Picard et al., 2000b), there have been fewer mechanistic studies investigating the molecular or genetic determinants of their mycoparasitic lifestyle. Several cell wall-degrading enzymes and putative effectors were previously revealed to be expressed by *P. oligandrum* in the presence of oomycete tissue (Horner et al., 2012) and microarray analysis was recently used to investigate *P. oligandrum*-plant interactions (Yacoub et al., 2018), which showed significant reprogramming of the *Vitus virifera* root transcriptome in the presence of *P. oligandrum*. However, these studies were limited either in their methodology (a small sequencing study of 3,000 cDNA clones) or scope (focus on changes in plant roots), respectively, and thus we still lack a detailed mechanistic understanding of mycoparasitism in the oomycete lineage.

To provide a more complete basis for detailed molecular and genetic analysis of mycoparasitic *Pythium* species, we have sequenced and assembled the genomes of both *P. oligandrum* (Kushwaha et al., 2017a) and *P. periplocum* (Kushwaha et al., 2017b). Two other isolates of *P. oligandrum* have also been sequenced (Berger et al., 2016; Faure et al., 2020). We have also performed RNA sequencing of selected *Pythium oligandrum*-prey and *Pythium periplocum*-prey interactions, a detailed analysis of which will be published elsewhere.

The Carbohydrate-active enzyme complement (CAZyome) is the repertoire of predicted genes coding for enzymes involved in carbohydrate metabolism in an organism (CAZymes), including the synthesis, degradation, and modification of structural components of the cell wall. The CAZymes can be divided into five superfamilies, glycoside hydrolases (GH), glycosyl transferases (GT), polysaccharide lyases (PL), and carbohydrate esterases (CE) based on their activity and sequence similarity (Lombard et al., 2013). Comparative analysis of the mycoparasitic fungi *Trichoderma atrovidirde* and *Trichoderma virens* with other closely related fungal species, reveals the expansion of the CAZyome, particularly of genes from the family GH18, which comprises proteins with putative functions as chitinases in the mycoparasitic *Trichoderma* species. Other CAZy families expanded were those encoding endo-β-N acetylglucosimindases and β-1,3-glucanases from the families GH17, GH55, GH64, and GH81 (Kubicek et al., 2011). Chitin is a major component of the fungal cell wall, and therefore an obvious target for mycoparasitic lytic attack. Carbohydrate binding domains (CBMs) are also more abundant in the chitinase sequences from mycoparasitic *Trichoderma* species, compared to other fungi. As well as an expansion in the number of genes encoding chitinases, these *Trichoderma* genomes also contain an expanded number of GH75 chitosanases. It has long been known that the binding and degradation of chitin is important for successful mycoparasitism in these *Trichoderma* species. *T. virens* strains with chitinase knock-out mutants show reduced mycoparasitic ability, whilst strains that constitutively overexpress the same gene show enhanced biocontrol capabilities (Baek et al., 1999). The addition of CBMs to chitinases from *Trichoderma harzianum* has been shown to increase the antifungal activity of this species (Limón et al., 2004). Interestingly, Druzhinina et al. (2018) found nearly half of CAZy families in the mycoparasitic *Trichoderma* species were obtained by lateral gene transfer from plant-associated filamentous Ascomycete fungi, which has allowed *Trichoderma* species to expand their nutritional base (Druzhinina et al., 2018).

In the present study, we report the detailed mining of the *P. oligandrum* and *P. periplocum* genomes to investigate the presence and role of genes encoding CAZymes. Expression was investigated through analysis of RNA sequencing data from *Pythium oligandrum-Phytopthora infestans* and *Pythium periplocum-Ph. infestans* as well as *P. periplocum-Botryis cinerea* interactions. Transcript abundance of selected genes was confirmed through qRT-PCR analysis of the same parasite-prey interactions. The genomes of our mycoparasitic *Pythium* species were compared to those of nine other oomycete pathogens with different host and lifestyle ranges, to test the hypothesis that like their fungal counterparts, mycoparasitic oomycetes have expanded CAZyomes and that deployment of these cell wall degrading enzymes is also important for mycoparasitic oomycete-oomycete or oomycete-fungal interactions.

Our findings suggest that an expanded CAZyome may be one hallmark of mycoparasitism in eukaryotic microbes. Several of the CAZy encoding gene families appear to have been acquired by in mycoparasitic oomycetes through horizontal gene transfer. Our data also suggests that some CAZy-encoding genes act as pathogenicity factors, residing in similar genomic locations and

with the potential to undergo rapid evolution in a similar manner to effector genes from phytopathogenic oomycetes.

RESULTS AND DISCUSSION

The CAZyme of Mycoparasitic *Pythium* Species Contains Unique Features

Whilst it is well known that mycoparasitic fungi such as species within the *Trichoderma* Genus secrete an array of cell wall degrading enzymes during mycoparasitism of their prey, there is more limited information on the molecular mechanisms of mycoparasitism in the oomycete lineage. Previously several CAZyme-encoding genes were found to be expressed by *P. oligandrum* when interacting with tissue from the oomycete prey species, *Ph. infestans* (Horner et al., 2012). However, no comprehensive analysis of the CAZyome of *P. oligandrum* or *P. periplocum* has so far been carried out. In order to identify the key features and evolution of the mycoparasitic *Pythium* CAZyome, putative CAZyme encoding genes from *P. oligandrum*, *P. periplocum* and 11 other oomycete species were predicted using the dbCAN CAZy annotation pipeline (Yin et al., 2012). We first evaluated genome completeness in terms of the expected gene content of the thirteen species used for the comparison by carrying out a Benchmarking Universal Single-Copy Orthologs (BUSCO) analysis (Seppey et al., 2019). Complete and single copy BUSCO groups account for at least 80% of each genome (**Supplementary Figure S1**), and thus we concluded that the genome completeness of each organism was comparable. The CAZyme complement of the genomes of the mycoparasitic species *Pythium oligandrum* (Kushwaha et al., 2017a) and *Pythium periplocum* (Kushwaha et al., 2017b), was compared to those from the plant pathogens: *Pythium ultimum* (Lévesque et al., 2010), *Pythium aphanodermatum*, *Pythium arrhenomanes*, *Pythium iwayamai*, *Pythium irregulare*, *Phytopythium vexans* (Adhikari et al., 2013), *Phytophthora ramorum* (Tyler et al., 2006), *Phytophthora infestans* (Haas et al., 2009), and *Phytophthora capsica* (Lamour et al., 2012), and the human pathogen *Pythium insidiosum* (Rujirawat et al., 2015).

We predicted 516 proteins in the CAZyome of *P. oligandrum*, 431 proteins in that of *P. periplocum*, and 321–719 proteins in the other oomycete species (**Figure 1A**). The total number of CAZy families predicted in *P. oligandrum* was 106, with 102 predicted in *P. periplocum*. The total number of CAZy families in the other oomycetes ranged from 93 to 110 (**Figure 1A**). These numbers are in line with previously reported predictions of the oomycete CAZyome (Ospina-Giraldo et al., 2010; Adhikari et al., 2013), indicating that our predictions are reliable. Within the *Pythium* genus, the total number of CAZy encoding genes was higher on average in the mycoparasitic *Pythium* species compared to the plant pathogenic *Pythium* species. However, since phytopathogenic *Phytophthora* species exhibit higher numbers of total CAZy genes, there is no significant difference within the oomycete lineage in the total numbers of CAZy encoding genes or in the total number of CAZy families per species.

To identify redundancy within the dataset and to compare CAZymes between the species, a comparison of the average gene member count per CAZy family was performed. Twenty CAZy families were identified as either expanded or unique within the mycoparasitic *Pythium* genomes when compared to the other species used in the analysis ($p < 0.05$, *T*-test; **Figure 1B**). These families include: three Auxiliary Activity families (AA1, AA11, AA9), three Carbohydrate-Binding Module families (CBM13, CBM24, CBM25), nine Glycoside Hydrolase families (GH114, GH13_32, GH19, GH43_26, GH46, GH5_14, GH55, GH71, GH76), four Glycosyl Transferase families (GT33, GT39, GT62, GT64), and one Polysaccharide Lyase family (PL9).

Alongside these analyses, we also mined RNA sequencing (RNA-Seq) data from the interactions between *P. oligandrum* with *Ph. infestans*. *P. periplocum* with *Ph. infestans* and from *P. periplocum* with *Botrytis cinerea,* to check the expression of these and other CAZy families. As well as the detailed analysis

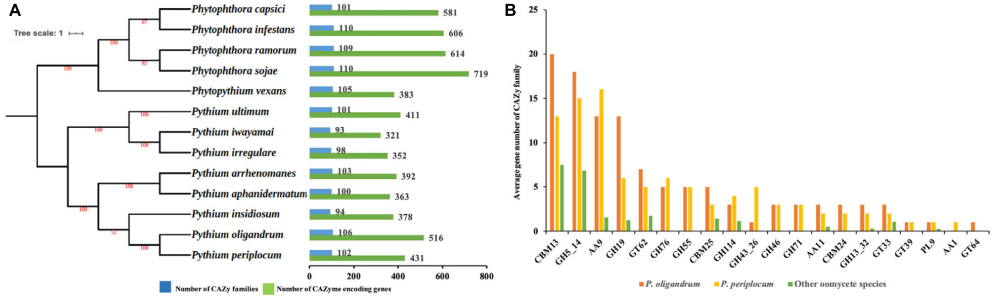

FIGURE 1 | The CAZyme of two mycoparasitic *Pythium* species. **(A)** Species tree of oomycetes in this study and distribution of CAZy proteins. The species tree was constructed using the maximum likelihood method with 1,000 bootstraps based on a concatenated alignment of housekeeping genes identified by CEGMA analysis. Green bars indicate the total number of CAZyme encoding genes. Blue bars indicate the number of CAZy families present. **(B)** Average gene number of CAZy families unique or expanding in mycoparasitic *Pythium*. CAZy families were identified as unique or expanding in mycoparasitic *Pythium* species compare to other oomycetes by a *T*-test analysis.

of the CAZy families described below, this also allowed us to identify differentially expressed genes during mycoparasitism of oomycete prey that were not encoded by families expanded in the mycoparasites, but that nonetheless may play a role in parasite-prey interactions (**Supplementary Figure S2**).

Since CAZy families with a putative role in the degradation of fungal or oomycete cell walls were predominantly expanded in the mycoparasitic oomycetes, we therefore focused the rest of our studies on carbohydrate binding and the metabolism of, cellulose, glucan, chitin and hemicelluloses, the major constituents of the cell walls of these prey species. Thus, eight CAZy families namely, AA9, GH5_14, GH55, GH71, GH19, GH46, GH76, and GH43_26 were chosen for detailed analysis. Among them, representatives of the CAZy families GH55, GH71, GH46, GH76, and GH43_26 were only detected in mycoparasitic *Pythium* species and were absent in the other oomycete genomes tested (**Figure 1B** and **Supplementary Table S2**) and thus appear to be unique components of the mycoparasitic oomycete CAZyme. Moreover, we also performed ortholog clustering using OrthoFinder (Emms and Kelly, 2019), which provides ortholog relationship analysis within each of the CAZy families mentioned above (**Supplementary Table S3**).

Cellulose Metabolism

The major structural component of the oomycete cell wall is cellulose (Sietsma et al., 1969) and cellulose has been implicated in the maintenance of correct cell morphology and the production of appressoria (Grenville-Briggs et al., 2008). Penetration of plant tissue also requires active cellulose synthesis in the phytopathogenic oomycete, *Ph. infestans* (Grenville-Briggs et al., 2008). Homologs of the predicted cellulose synthase encoding genes [the CesA genes from the glycoside hydrolase family 2 (GT2)], previously identified in *Ph. infestans* (Grenville-Briggs et al., 2008) were identified in both *P. oligandrum* and *P. periplocum,* and this family was not expanded in the mycoparasites compared to the phytopathogens, although *P. oligandrum* contains one extra copy of the gene encoding CesA3 compared to *P. periplocum,* and the gene encoding CesA1 appears to be absent from *P. periplocum* (**Supplementary Figure S7A**). Although the CesA family is not expanded in the mycoparasites, genes encoding all eight CesA proteins identified in the mycoparasitic *Pythium* species, and a further GT2 protein from *P. periplocum,* were differentially expressed during interactions of the mycoparasite with *Ph. infestans* (**Supplementary Figures S2A, S7B**). Thus, although, it is not known whether appressorium production is also a significant component of oomycete mycoparasitism, the synthesis of cellulose may of general importance for growth during mycoparasitic interactions.

We hypothesize that cellulose degradation enzymes may be important pathogenicity determinants in the mycoparasitic *Pythium* species, since they are part of the cell wall of oomycete prey. Two families of genes encoding enzymes predicted to be involved in the degradation of cellulose were expanded in the mycoparasitic species, namely AA9 and GH5_14.

The auxiliary activity family 9 (AA9), proteins are lytic polysaccharide monooxygenases that are predominantly found in fungi. The AA9 family was significantly expanded in the mycoparasitic *Pythium* genomes compared to the other oomycete species studied. 16 AA9 proteins were predicted in *P. oligandrum* and 17 in *P. periplocum*. A range of one to five AA9 proteins were identified in plant pathogenic *Pythium* species, one AA9 protein was identified in the human infecting *Pythium*, and six AA9 proteins in *Phytophthora* species. Q86K62, an Auxiliary Activities 10 (AA10) protein in *Dictyostelium discoideum*, was used as outgroup to produce an AA9 phylogenetic tree, which consists of four clades (**Figure 2B**). Among clades, 17 mycoparasitic *Pythium* AA9 proteins are present in clade 1 (eight *P. oligandrum* and nine *P. periplocum*). Eight AA9 proteins are present in clade 3 (four from *P. oligandrum* and four from *P. periplocum*). Therefore, expansion of AA9 family in mycoparasitic *Pythium* appears to occur primarily in clade 1 and clade 3.

Previous studies have identified a "two-speed" architecture in the genomes of plant pathogenic oomycetes, in which the gene sparse regions exhibit higher plasticity, and hence drive adaptive evolution, that the gene dense regions (Raffaele and Kamoun, 2012). Pathogenicity determinants such as the RxLR effectors from phytopathogenic oomycetes tend to occupy regions that are gene poor, but repeat rich, facilitating rapid evolution of those regions (Dong et al., 2015). Detailed analysis of the *Ph. infestans* genome (Haas et al., 2009) postulated that accelerated effector evolution is driven by tandem gene duplication and homologous recombination. Based on gene density analysis, the majority of the *P. oligandrum* AA9 genes, encoding AA9 proteins assigned to clade 1, are located within the gene sparse regions (**Supplementary Figure S3A**). AA9 genes encoding clade 3 AA9 proteins, however, are present in the gene dense regions. Genome location analysis of the AA9 genes within the *P. oligandrum* genome shows that they cluster in two tandem arrays, consisting of AA9 genes encoding AA9 proteins of clade 1 and clade 3, respectively (**Supplementary Figure S4A**), suggesting that members of the AA9 family may be under rapid evolution in mycoparasitic oomycetes and further, may be important pathogenicity determinants for this group of organisms.

Using normalized TPM read counts from RNA-Seq data of the interaction between either *P. oligandrum* or *P. periplocum* and the prey *Ph. infestans*, we found seven AA9 genes were highly expressed specifically in the interactions between host and prey (**Figure 2B**). Among them, *PyolAA9-2*, *PyolAA9-3,* and *PyolAA9-4* were significantly up-regulated at 12 and 24 hpi of the interaction and may be of general importance during parasite-prey interactions. *PypeAA9-6* was only significantly up-regulated at 12 hpi, so may act predominantly in the early stages of mycoparasitism and thus could be described as a putative pathogenicity factor. *PypeAA9-5* and *PyolAA9-1*were significantly up-regulated at 24 hpi and therefore play an important role in the later stage of mycoparasitism. *PyolAA9-13* was comparatively highly expressed during both *in vitro* growth of both *Pythium* species as well as during interactions with oomycete prey and thus may be important for remodeling of the parasite cell wall during both vegetative growth and feeding.

We were able to design specific primers for qRT-PCR amplification of three of the AA9 genes, namely *PyolAA9-3*, *PyolAA9-13,* and *PyolAA9-16* (**Figure 10A**). Both genes showed

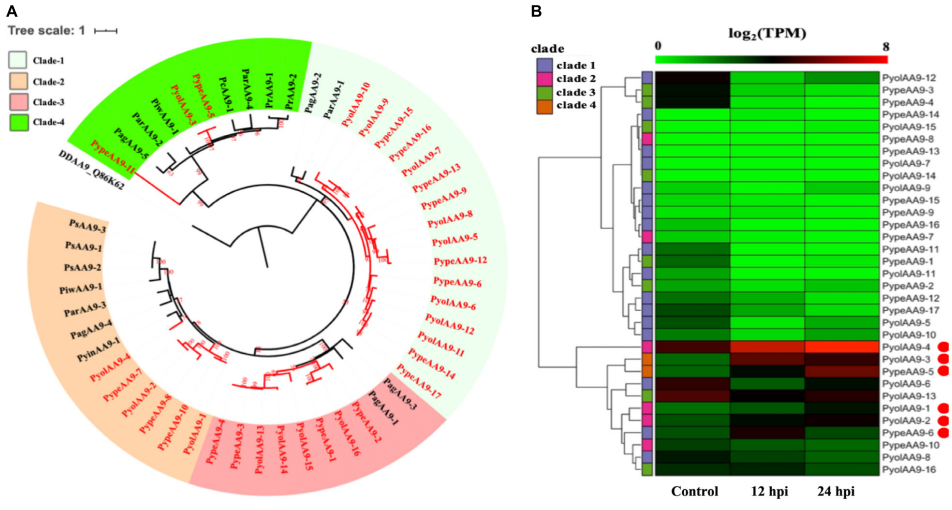

FIGURE 2 | Analysis of auxiliary activity family 9 (AA9). **(A)** Phylogenetic tree of AA9 proteins identified in oomycete species. Maximum likelihood tree, with 1,000 bootstraps (values displayed per branch). DDAA9_Q86K62 (an AA10 protein verified in *Dictyostelium discoideum*) was used as an outgroup. AA9 proteins identified in *P. oligandrum* (Pyol) and *P. periplocum* (Pype)are marked in red. **(B)** RNAseq Expression profiles of AA9 genes from *P. oligandrum* and *P. periplocum* during interactions with *Ph. infestans* during *in vitro* growth (control) or at 12 or 24 h post interaction (hpi). The phylogeny of each protein is marked using colored bars to represent each clade. The genes with the highest expression level during the interactions with *Ph. infestans* are marked with red circles. Expression levels are expressed as the $\log_2$ fold change of transcripts per million (TPM), per gene.

an induction during mycoparasitism of *Ph. infestans,* with *PyolAA9-3* displaying the highest relative expression at 6 h, during the early colonization of *Ph. infestans* tissue. *PyolAA9-13* was induced at 6 h and continued to show an elevated expression relative to *in vitro* growth levels from 6 to 48 h, with a peak at 36 h. *PyolAA9-16* displayed constitutive levels of expression during both vegetative growth and the first 6 hpi which then reduced during the later stages of the interaction. These results suggest that AA9 family proteins have a role in mycoparasitism of *Ph. infestans* by mycoparasitic *Pythium* species.

An AA9 gene from the saprotrophic fungus *Chaetomium globosum* has previously been shown to promote cellulose hydrolysis by GH5_14 family cellulases and also exhibits the same synergistic effect in xylan hydrolysis (Kim et al., 2015, 2016). Since we also found the GH5_14 (predicted enzyme activity as endo-1,4-β-D-glucanases/cellulases (EC3.2.1.4), to be significantly expanded in the mycoparasitic *Pythium* genomes, compared to their phytopathogenic counterparts, we also checked for the presence of the genes encoding the GH5_14 family in gene sparse regions of the genome and for duplication events. The GH5_14 family comprises 18 proteins in *P. oligandrum* and 15 in *P. periplocum*. The phylogenic tree of GH5_14 proteins from all oomycetes in this study can be divided into seven clades (**Figure 3A**). Two plant GH5_14 proteins, ZmGH5_B6TTA1 and OjGH5_Q8RU06, were used as outgroups. GH5_14 proteins from the mycoparasitic *Pythium* species are present in clade 1, clade 2, clade 4, and clade 6. Notably, most of the GH5_14 proteins from the mycoparasitic *Pythiums* are present in clade 1 (10 from *P. oligandrum* and seven from *P. periplocum*). Thus, the expansion of the GH5_14 family occurred primarily in clade 1. Furthermore, genes encoding those proteins in clade 1 reside in the gene sparse regions of the genome and are located in tandem arrays, suggesting that these two families of associated genes are undergoing rapid evolution (**Supplementary Figures S3B, S4B**) and thus may also be important as pathogenicity determinants.

Using normalized TPM read counts from RNA-Seq data of the interaction between either *P. oligandrum* or *P. periplocum* and the prey, *Ph. infestans,* we could determine that eight putative cellulase, GH5_14 genes encoding proteins assigned to clade 1, were highly expressed during mycoparasite-prey interactions, or during *in vitro* growth (**Figure 3B**), among them, two genes, *PyolGH5_14-11* and *PypeGH5_14-5*, showed near constitutive expression levels in all conditions tested, suggesting a role in the remodeling of the mycoparasitc cell wall during all phases of growth. Four genes, *PypeGH5_14-11*, *PyolGH5_14-12*, *PypeGH5_14-14*, *PypeGH5_14-16*, were highly expressed during *in vitro* growth and slightly down-regulated during interactions with the prey species. *PyolGH5_14-1* and *PypeGH5_14-13*, were highly expressed mainly during *in vitro* growth. This suggests the latter two genes have a role in the modification of the cell wall of the mycoparasite during vegetative growth, and little to no role in mycoparasitism. The genes encoding GH5_14 proteins in clade 2, clade 4, and clade 6 were all expressed at relatively low levels during *in vitro* growth, and/or during the interaction with *Ph. infestans*. However, transcripts from *PyolGH5_14-10*,

assigned to clade 6, were highly abundant in the *Ph. infestans* interaction at 24 hpi.

Detailed expression analysis of a time course of *Ph. infestans* infection by *P. oligandrum* revealed that, of the genes tested, *PyolGH5_14-16* was highly expressed during mycoparasitism, peaking at 36 h with a relative expression level greater than 300 times that of the *in vitro* expression (**Figure 10A**). Furthermore, data from three independent biological replicates shows that the expression of this gene oscillates between higher and lower expression over the interaction time course. A temporal switching of gene expression may be a response to the availability of new host hyphae, reflecting enzyme production only as and when needed, e.g., through digestion of a single hypha at a time. Alternatively, this could be part of a mechanism to avoid accidental self-damage. The fungus *Trichoderma reesei,* is a prolific producer of cellulases, and as such is a model organism for industrial production of several CAZy enzymes involved in cell wall degradation. Major cellulase transcription factor genes have been identified that show differential regulation under light or dark conditions and photoreceptors play an important role in regulation of nutritional uptake in this organism (Schmoll, 2018). *PyolGH5_14-16* transcript abundance peaks occurred during the night sampling (12 and 36 h) of the interaction time course and this pattern is reminiscent of a circadian rhythm. However, this may also reflect gene induction under the somewhat artificial *in vitro* conditions under which the experiments were performed and thus investigation of the expression of CWDEs under natural or field conditions would be interesting for the future. *PyolGH5_14-1* and *PyolGH5_14-12* show a decreased expression during mycoparasitism, relative to *in vitro* levels, indicating a potential role predominantly during vegetative growth. Neither the GH6 family of putative cellobiohydrolases (EC 3.2.1.91) or the GH5_20 family of endo-β-1,4-glucanases/cellulases (EC 3.2.1.4) were expanded in the mycoparasitic oomycetes, however, five GH6 family genes from *P. oligandrum* were differentially expressed (significantly upregulated) during the early stages of interactions with *Ph. infestans*. whilst five GH5_20 genes were down-regulated (**Supplementary Figure S2A**). Four GH5_20 genes from *P. periplocum* were also down-regulated at the same time point, as were three GH3 β-glucosidases (EC 3.2.1.21) (**Supplementary Figure S2B**), indicating these may be used to cleave celluloses from other substrates, or involved in other phases of the mycoparasitic lifecycle. Genes encoding 10 putative GH17 family endo-1,3-β-glucosidases (EC 3.2.1.39) from *P. oligandrum* were upregulated during the early stages of interactions with *Ph. infestans* five from *P. periplocum* were upregulated in the later stages of mycoparasitism (**Supplementary Figure S2**), indicating that the different mycoparasites may use a progression of different cellulose degrading enzymes at different time points in the mycoparasitic life cycle.

Thus, the metabolism of cellulose is likely to be important for both vegetative oomycete growth and for mycoparasitism of oomycete prey and several of the genes involved in this process display the genomic hallmarks of pathogenicity factors undergoing rapid evolution.

Glucan Metabolism

Glucans are structurally related to cellulose and are another important component of the oomycete cell wall. In contrast to cellulose, glucans are largely absent from plant cell walls and thus dissecting the occurrence and evolution of enzymes involved in the degradation of glucans may provide unique insights into the evolution of the mycoparasitic oomycete species. GH55 CAZy family members are predicted to function as exo-β-1,3-glucanases (EC 3.2.1.58 degrading glucans containing β-1,3-linkages as well as participating in the hydrolysis of laminarin, a component of fungal cell walls (Bara et al., 2003). Thus, genes in this class may potentially target both oomycete and fungal prey. Genes assigned to the CAZy family GH55 were found only in the mycoparasitic *Pythium* and were absent in the other oomycete genomes tested, indicating that they might be important for mycoparasitism. We found genes encoding five GH55 proteins each in *P. oligandrum* and *P. periplocum*. Phylogenetic analysis shows that oomycete GH55 proteins are most closely related to those from a variety of fungal species (**Figure 4A**). It has recently been shown that almost half of the cell wall degrading carbohydrate active enzymes found in mycoparasitic *Trichoderma* species were obtained via horizontal gene transfer, a process by which genetic material may be transferred between distinct evolutionary lineages, either cross-Kingdom or within Kingdom (Doolittle, 1999; Andersson, 2009), from plant associated filamentous fungi (Druzhinina et al., 2018). Our data supports the hypothesis that *P. oligandrum* and *P. periplocum* have developed the ability to be mycoparasitic, in part, through the acquisition of CWDEs from the GH55 family, via horizontal gene transfer from filamentous fungi. The flanking regions of mycoparasitic *Pythium* GH55 genes show conserved collinearity with regions of the *P. ultimum* genome (**Supplementary Figure S5A**), although GH55 genes are absent in *P. ultimum*. Therefore, mycoparasitic *Pythium* GH55 genes appear to reside within conserved regions of their genomes and may potentially have been inserted there via horizontal gene transfer from fungi.

Analysis of normalized TPM read counts from the RNA-Seq interaction libraries, revealed two different expression patterns. Six GH55 genes, (*PyolGH55_4*, *PypeGH55_5*, *PypeGH55_1*, *PypeGH55_3*, *PyolGH55_2*, *PyolGH55_5*), were highly expressed during *in vitro* growth of and significantly down-regulated in the interaction stages (**Figure 4B**). Notably, *PyolGH55_4* was highly expressed both during *in vitro* growth and at the later stage of the interaction between *P. oligandrum Ph. infestans*. Four GH55 genes (*PyolGH55_3*, *PypeGH55_4*, *PyolGH55_1*, *PypeGH55_2*), show a low level of expression in all stages tested, suggesting that they do not have a major role during *in vitro* growth or in the interaction with *Ph. infestans* as a prey species. They may, however, be expressed more highly in other growth stages or in the interaction with other prey species.

Like the GH55 family, members of GH71 family were only identified in mycoparasitic *Pythium* among oomycete species in this study. GH71 proteins have a predicted enzymatic activity as α-1,3-glucanases (EC 3.2.1.59). We found three GH71 proteins in each of the mycoparasitic species *P. oligandrum* and

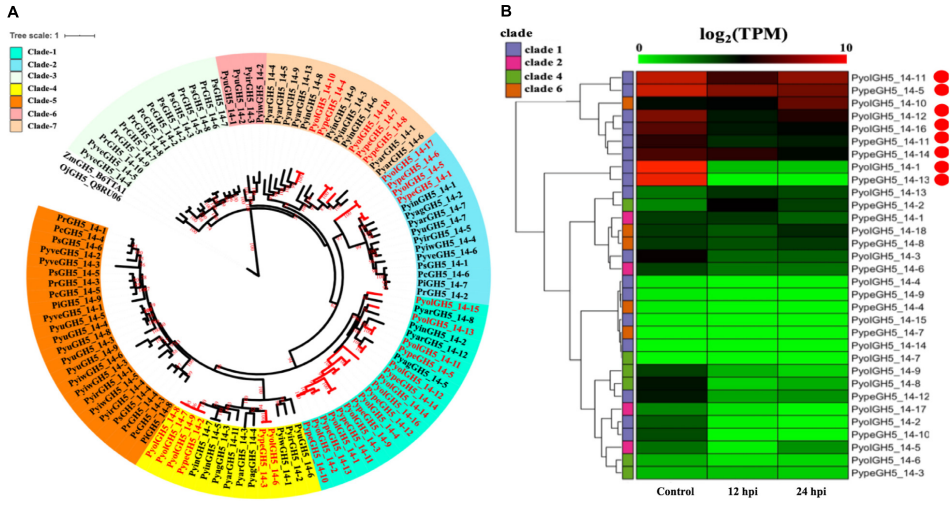

FIGURE 3 | Analysis of glycoside hydrolase family 5_14 (GH5_14). **(A)** Phylogenetic tree of GH5_14 proteins identified in oomycete species. Maximum likelihood tree, with 1,000 bootstraps (values displayed per branch). ZmGH5_B6TTA1 and OjGH5_Q8RU06 (GH5_14 proteins verified in plant species) were used as outgroups. GH5_14 proteins identified in *P. oligandrum* (Pyol) and *P. periplocum* (Pype) are marked in red. **(B)** RNAseq Expression profiles of GH5_14 genes from *P. oligandrum* and *P. periplocum* during *in vitro* growth or interactions with *Ph. infestans* and 12 or 24 h post interaction. The phylogeny of each protein is marked using colored bars to represent each clade. The genes with the highest expression level during the interactions with *Ph. infestans* are marked with red circles. Expression levels are expressed as the $\log_2$ fold change of transcripts per million (TPM), per gene.

P. periplocum, respectively. Phylogenetic analysis groups these genes within a branch of fungal GH71 proteins (**Figure 5A**). Thus, this suggests that the GH71 genes from the mycoparasitic *Pythium* species may also have been obtained via horizontal transfer from fungi. Two of the genes, *PyolGH71-1* and *PypeGH71-1*, are highly expressed during *in vitro* growth and are significantly down-regulated during the interaction with *Ph. infestans* (**Figure 5B**). The remaining members of this family show low levels of expression in the stages tested in this study, which is a similar expression profile to the GH55 glucanase family. *PyolGH71*-1 showed high relative expression levels at 36 and 48 h of the interaction with *Ph. infestans*, suggesting either a role in degradation of prey glucans that are exposed later during mycoparasitism or a role in *P. oligandrum* cell wall rearrangement and/or growth, after nutrient uptake from the prey.

We next used the GH55 and GH71 protein sequences detected in *P. oligandrum* and *P. periplocum* in blastp searches against the NR database, excluding sequences from *P. oligandrum* and *P. periplocum* themselves. Compiling the resultant data as a phylogenetic tree, further confirms that GH55 and GH71 proteins appear to have been transferred horizontally from Ascomycete fungi to the oomycetes (**Supplementary Figure S8**). Moreover, we also made Hidden Markov Model (HMM) searches against the genomes of other available oomycetes, namely, *Hyaloperonospora arabidopsidis*, *Albugo laibachii*, *Saprolegnia parasitica*, *Saprolegnia diclina*, *Phytophthora parasitica,* and *Pythium brassicum*. However, we could not find any GH55 and GH71-like genes in these species, and thus conclude that they are absent in other oomycetes, for which we have genome data. Therefore, it appears that horizontal gene transfer of GH55 and GH71 genes occurred within the latest common ancestor of *P. oligandrum*.

Chitin Metabolism

Members of the GH19 family, with predicted activity as chitinases (EC 3.2.1.14), are abundant in mycoparasitic *Pythium* species (**Figure 6A**). Here we found 13 GH19 proteins *in P. oligandrum* and six in *P. periplocum*. A range of one to four GH19 proteins were found in other oomycetes in this study. As shown in **Figure 6A**, GH19 proteins can be divided into only two clades. GH19 proteins from the mycoparasitic *Pythium* species were only present in clade 2, which also includes one GH19 proteins from each of the plant pathogens *P. aphanidermatum*, and *Ph. capsici* and three genes from the human and animal pathogen *P. insidiosum*. Based on gene density analysis, most of the GH19 genes identified, are predicted to be present in the gene sparse regions of the genomes of both *P. oligandrum* and *P. periplocum* (**Supplementary Figures S3C,D**). Moreover, one tandem array, consisting of genes *PyolGH19-3* to *PyolGH19-10*, is present in the genome of *P. oligandrum* (**Supplementary Figure S4C**), suggesting that these genes are able to undergo rapid evolutionary change, and may be important pathogenicity factors in fungal mycoparasitism.

Analysis of normalized TPM read counts from our RNA-Seq interaction libraries reveals that GH19 genes (*PypeGH19-2*, *PyolGH19-2*, *PypeGH19-5*, *PyolGH19-8*, *PyolGH19-13*), were

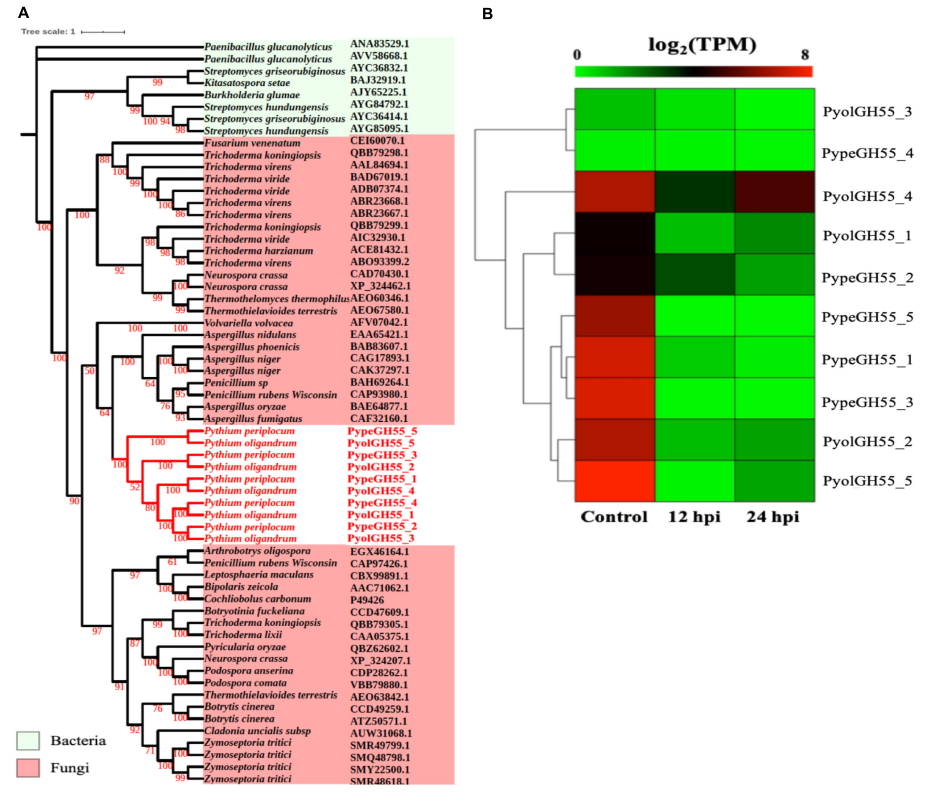

FIGURE 4 | Analysis of glycoside hydrolase family 55 (GH55). **(A)** Phylogenic tree of GH55 proteins identified in *P. oligandrum* and *P. periplocum*. Maximum likelihood tree, with 1,000 bootstraps (values displayed per branch). GH55 proteins identified in *P. oligandrum* (Pyol) and *P. periplocum* (Pype) are shown in red. **(B)** RNAseq Expression profiles of GH55 genes detected in *P. oligandrum* and *P. periplocum* during *in vitro* growth or interactions with *Ph. infestans* at 12 or 24 h post interaction. Expression levels are expressed as the $\log_2$ fold change of transcripts per million (TPM), per gene.

highly expressed during *in vitro* growth and significantly down-regulated during the interaction with the prey oomycete *Ph. infestans* (**Figure 6B**). Thus, GH19 genes seem to be dispensable for interactions between mycoparasitic *Pythium* species and *Ph. infestans,* which has a cellulosic cell wall and no detectable chitin (Sietsma et al., 1969; Mélida et al., 2013). To test the hypothesis that GH19 proteins are used for parasitism of fungal prey, we mined our sequenced transcriptomes of interactions between *P. periplocum* and the gray mold fungus, *Botrytis cinerea*. Three *P. periplocum* GH19 genes (*PypeGH19-2*, *PypeGH19-3*, and *PypeGH19-5*), were highly expressed during both *in vitro* growth and during the interactions between *P. periplocum* and *B. cinerea*. Two *P. periplocum* GH19 genes (*PypeGH19-4*, and *PypeGH19-6*), were highly expressed solely during interactions between *P. periplocum* and *B. cinerea* and *PypeGH19-1* induction was not detected in any of the conditions we tested (**Figure 6C**).

To confirm the role of GH19 family members in mycoparasitism of *B. cinerea*, we performed qRT-PCR analysis of selected GH19 genes during the early stages of parasitism by either *P. periplocum* or *P. oligandrum*. Four *P. periplocum* GH19 genes, (*PypeGH19-1*, *PypeGH19-4*, *PypeGH19-5*, *PypeGH19-6*), were significantly upregulated (between two and fifteen -fold change) at 12 and 24 h of interactions with *B. cinerea*. Six *P. oligandrum* GH19 genes (*PyolGH19-1*, *PyolGH19-2*, *PyolGH19-3*, *PyolGH19-8*, *PyolGH19-9*, *PyolGH19-11*), were significantly induced (7–1,300-fold change) during the interaction with *B. cinerea*. Thus, chitinase gene expression is likely to be important for mycoparasitism of fungal prey and the mycoparasitic *Pythium* species are able to sense the cell wall components of their prey and adjust the expression of their CWDEs accordingly, since these genes are not expressed in the presence of a non-chitinous prey species.

The GH46 family (EC.3.2.1.132) is predicted to have chitosanase activity and was detected only in the mycoparasitic *Pythium* and not in the other oomycete species screened in this study. Chitosan is a cationic polysaccharide composed of

β-1,4-linked D-glucosamine (GlcN) that is derived from chitin, with chitosanase being the major substrate-specific enzyme that acts on the β-1,4-glycosidic linkages of chitosan (Sun et al., 2018). We detected three GH46 genes in both *P. oligandrum* and *P. periplocum* respectively. To explore the phylogenic relationship of the mycoparasitic *Pythium* GH46 genes, we retrieved GH46 proteins from a diverse range of species (Viens et al., 2015). As **Figure 7A** shows, the phylogenic tree of the GH46 family is divided into five clades, as previously reported (Viens et al., 2015). Mycoparasitic *Pythium* GH46 proteins are present in clade C, which includes GH46 proteins from *Chlorella virus*. Thus, we inferred that mycoparasitic *Pythium* may have been obtained GH46 genes by horizontal transferred from a viral donor. Genes neighboring the GH46 gene family in the mycoparasitic *Pythium* show collinearity with regions of the *P. ultimum* genome (**Supplementary Figure S5B**). Therefore, it would appear that the mycoparasitic *Pythium* GH46 genes were transferred into conserved regions of their genomes.

Interestingly, transcripts of one GH46 gene (*PypeGH46-1*) were highly abundant, relative to *in vitro* levels, during the parasitism of both *Ph. infestans* and *B. cinerea* by *P. periplocum*. One gene from *P. oligandrumm (PyolGH46-1)* was also expressed during parasitism of *Ph. infestans* (**Figures 7B,C**). Quantitative RT-PCR assays of GH46 genes during the early stages of interactions of either *P. periplocum* or *P. oligandrum* with *B.* cinerea reveal five genes (*PyolGH46-1*, *PyolGH46-2*, *PyolGH46-3*, *PypeGH46-1,* and *PypeGH46-3*), that are induced between 2 and 50-fold during parasitism of this fungus (**Figure 10**).

It has long been thought that the oomycetes are predominantly cellulosic in contrast to the chitinous fungi with whom they share many ecological niches. However detailed analysis of the structural carbohydrates in the cell wall of several plant and fish pathogenic oomycetes has identified three types of cell wall-based differences in structural carbohydrates within different oomycete Genera (Mélida et al., 2013). Type I contains glucuronic acid and mannose and no N-acetylglucosamine (GlcNAc), type II is characterized by cross linking between cellulose and 1,3,β glucans and up to 5% GlcNAc and the third type contains the highest GlcNAc content along with unusual carbohydrates (Mélida et al., 2013). This analysis did not include any of the mycoparasitic oomycetes or members of the *Pythium* genus though, so it is not clear which cell wall type *P. oligandrum* or *P. periplocum* contain, although based on our data showing that chitin metabolism-linked genes are expressed under *in vitro* conditions, we hypothesize that the mycoparasitic oomycetes may be modulating related carbohydrates or chito-oligosaccharides within their own cell walls. Alternatively, these genes may be expressed *in vitro* in preparation for degradation of prey cell walls, or in response to a depletion of nutrients.

Hemicellulose Metabolism

Four GH76 family members were found each in both *P. oligandrum* and *P. periplocum* although GH76 genes were absent in the other oomycete species screened. GH76 proteins are annotated as α-1,6-mannanases (EC 3.2.1.101), targeting α-1,6-mannosidic linkages and thus participating in the deconstruction of fungal cell walls (Cuskin et al., 2015). The phylogenic analysis, with an exo-β-1,3-glucanase of GH55 proteins (BAB83607.1) as the outgroup, shows that mycoparasitic *Pythium* GH76 proteins group most closely in the GH76 proteins from bacterial species (**Figure 8A**). Thus, these genes may have been horizontally transferred to a common mycoparasitic *Pythium* ancestor from bacteria.

Analysis of normalized TPM read counts from our RNA-Seq libraries revealed that one of the *P. oligandrum* genes (*PyolGH76-2*) and one of the *P. periplocum* genes (*PypeGH76-3*) were highly expressed during *in vitro* growth (**Figure 8B**). However, there was little to no detectable differential expression during interactions with the prey species tested, meaning that these genes are dispensable for antagonism of the fungal and oomycete prey used in this study.

GH43_26 genes were only identified in mycoparasitic *Pythium* among oomycetes in our study. Here we found one GH43_26 protein in *P. oligandrum* and eight GH43_26 proteins in *P. periplocum*. These genes have predicted activity as α-L-arabinofuranosidases (EC 3.2.1.55), which hydrolyze terminal non-reducing alpha-L-arabinofuranoside residues in alpha-L-arabinosides. **Figure 9A** shows that GH43_26 proteins from mycoparasitic *Pythiums* (red branch) group within a fungal GH43_26 clade (green branch). An endo-β-1,4-xylanase from the GH11 family (AHE13930.1) was used as an outgroup. This suggests that this gene family may have been horizontally transferred from fungi to mycoparasitic *Pythium*. Three of the GH43_26 genes (*PypeGH43_26-7*, *PypeGH43_26-5*, *PyolGH43_26-1*) were highly expressed both during *in vitro* growth and 24 hpi of the interaction with *Ph. infestans* indicating that they may be of some importance for growth of the mycoparasitic species themselves and of a lesser importance during mycoparasitism of oomycete prey (**Figure 9B**).

The GT71 family encodes genes with activity as α-mannosyltransferases (EC 2.4.1.-), and whilst this family was not expanded, nine GT71 genes were significantly upregulated in *P. oligandrum* during interactions with *Ph. infestans*. GH16 family members with predicted xyloglucanase activity (EC 3.2.1.151) were not expanded in the mycoparasitic oomycetes, however, three putative GH16 genes from *P. oligandrum* were upregulated during interactions with the prey oomycete *Ph. infestans* (**Supplementary Figure S2**). The family with the highest number of differentially expressed genes when interacting with the oomycete prey in both *P. oligandrum* and *P. periplocum* was CE1. Thirteen genes from *P. oligandrum* and fifteen genes from *P. periplocum* were upregulated during the early interactions with *Ph. infestans* (**Supplementary Figure S2**) and thus these family members may play a role in the degradation of hemicelluloses of oomycete origin. This Carbohydrate esterase family has diverse esterase functions including as acetyl xylan esterases (EC 3.1.1.72). GH30_1 family endo-β-1,4-xylanases from both mycoparasites were downregulated in the same interactions, indicating that they are not important in the interaction with oomycetes.

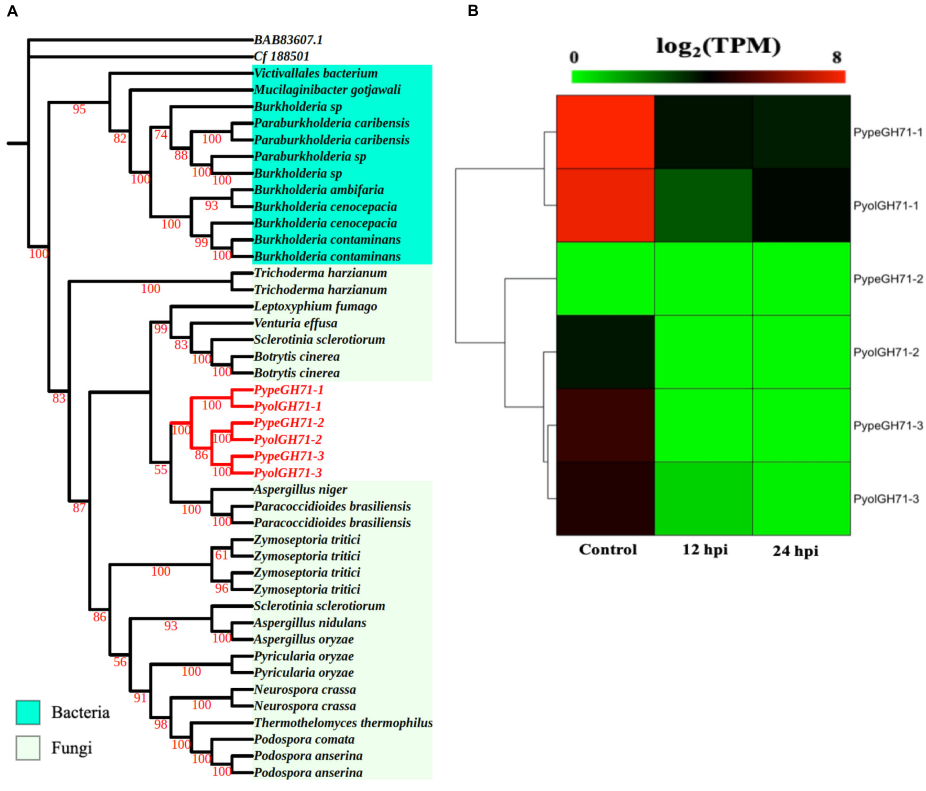

FIGURE 5 | Analysis of glycoside hydrolase family 71 (GH71). **(A)** Phylogenic tree of GH71proteins identified in *P. oligandrum* and *P. periplocum*. Maximum likelihood tree, with 1,000 bootstraps (values displayed per branch). GH71 proteins identified in *P. oligandrum* (Pyol) and *P. periplocum* (Pype) are shown in red. **(B)** RNAseq Expression profiles of GH71 genes detected in *P. oligandrum* and *P. periplocum* during *in vitro* growth or interactions with *Ph. infestans* at 12 or 24 h post interaction. Expression levels are expressed as the $\log_2$ fold change of transcripts per million (TPM), per gene.

Carbohydrate Binding Genes

Since destruction of cellulose appears to be important to oomycete-oomycete mycoparasitic interactions, and these *Pythium* species appear able to sense the major carbohydrate component of the prey cell wall and adapt their CWDE gene expression, we next investigated the presence and expression of cellulose binding module containing proteins, with the hypothesis that these may play a role in the sensing or binding of oomycete prey cells.

The cellulose-binding elicitor lectin (CBEL) family was found to contain two cellulose-binding domains (CBDs), belonging to the family 1 carbohydrate binding modules (CBM1). The CBM1 family is almost exclusively found only in fungi and oomycetes. In the phytopathogenic oomycetes, CBEL family members have been shown to have necrosis-inducing activity in host plants, and to bind host cellulose (Mateos et al., 1997; Gaulin et al., 2006). In a previous study of a small cDNA library of *P. oligandrum* interacting with dead tissue from *Ph. infestans,* a CBEL gene from *P. oligandrum* was shown to be upregulated during the interaction (Horner et al., 2012).

In the current study we found six CBEL proteins each in *P. oligandrum* and *P. periplocum*. A range of 2–10 CBEL proteins were detected in the other oomycete genomes screened. **Supplementary Figure S6A** shows that CBEL proteins were divided into four phylogenetic clades with a *Trichoderma reesei* exoglucanase used as an outgroup. Mycoparasitic *Pythium* CBEL proteins were only present in clade 4 (**Supplementary Figure S6A**). Based on domain architecture analysis, CBD (CBM1) domains are present in all putative CBEL proteins and PAN domains are only present in CBEL proteins grouped within clade 4. PAN domains are found in a diverse array of proteins and have been implicated in protein-protein or protein-carbohydrate interactions. Some of the mycoparasitic *Pythium* CBEL proteins are also predicted to contain other domains, including transglutaminase elicitors (TGase_elicitor), Leucine Rich Repeats (LRR_4 or LRR_8) and elicitin domains.

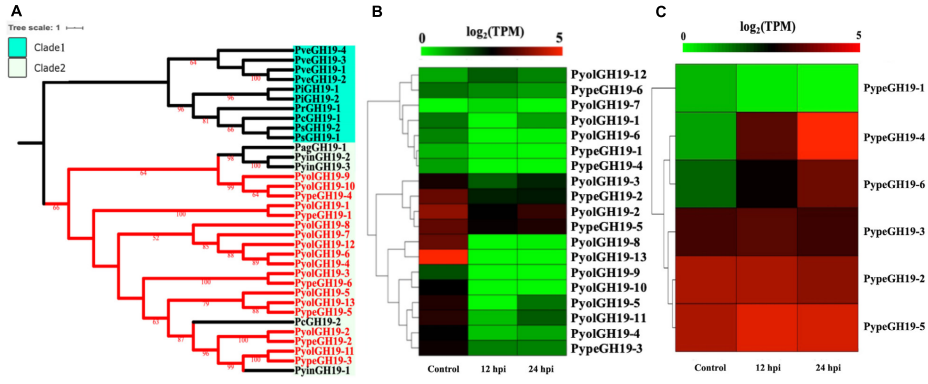

FIGURE 6 | Analysis of glycoside hydrolase family 19 (GH19). **(A)** Phylogenetic tree of GH19 proteins identified in oomycete species. Maximum-likelihood method with 1,000 bootstraps (values displayed per branch) was used to construct tree based on alignment of GH19 proteins. GH19 proteins identified in *P. oligandrum* (Pyol) and *P. periplocum* (Pype) are shown in red. **(B)** RNAseq Expression profile of GH19 genes detected in *P. oligandrum* and *P. periplocum* during *in vitro* growth or interactions with *Ph. infestans* at 12 or 24 h post interaction. Expression levels are expressed as the $\log_2$ fold change of transcripts per million (TPM), per gene. **(C)** RNAseq Expression profile of GH19 genes detected in *P. periplocum* during *in vitro* growth or interactions with *B. cinerea* at 12 or 24 h post interaction. Expression levels are expressed as the $\log_2$ fold change of transcripts per million (TPM), per gene.

Analysis of normalized TPM read counts from our RNA-Seq libraries revealed that putative CBEL genes from *P. oligandrum* (*PyolCBEL_2*, *PyolCBEL_4*, *PyolCBEL_5*), and *P. periplocum* (*PypeCBEL_3*, *PypeCBEL_6*) were highly expressed during *in vitro* growth. These CBEL proteins are predicted to contain only a PAN domain and carbohydrate binding domain along with a signal peptide. Of these, *PyolCBEL_5* and *PypeCBEL_3* were also somewhat expressed during interactions with *Ph. infestans* (**Supplementary Figure S6B**). The Transcripts from mycoparasitic *Pythium* CBEL proteins, predicted to contain either a TGase_elicitor or an Elicitin domain, were not highly expressed during the interaction with *Ph. infestans* under the conditions tested (**Figure 6B**).

Based on qRT-PCR (**Figure 10A**), transcripts of *PyolCBEL-1*, *PyolCBEL-2,* and *PyolCBEL-3* were all more abundant in the interaction with *Ph. infestans* than during *in vitro* growth of *P. oligandrum,* with *PyolCBEL-1* and *PyolCBEL-3* showing the highest expression levels. *PyolCBEL-1* peaked at almost 150-fold the *in vitro* expression level at 6 hpi compared and *PyolCBEL-3* shows a 12-fold expression increase at the same time point. *PyolCBEL-2* was expressed at near *in vitro* levels throughout the infection time course, and *PyolCBEL-4* was expressed at extremely low levels throughout the interaction. Three genes containing CMB63 domains from *P. periplocum* were highly upregulated during the early stages of the interactions with *Ph. infestans* (**Supplementary Figure S2B**), but interestingly CBM63 genes were not differentially expressed in the *P. oligandrum-Ph. infestans* interaction. The CBM63 module from *Bacillus subtilis* expansin protein EXLX1 has been experimentally shown to bind cellulose (Nikolaos et al., 2011).

Overall, we compared our RNA-seq data and qRT-PCR assay results (**Figures 2B**, **3B**, **5B**, **6B,C**, **7B,C**, **10** and **Supplementary Figure S6**). From the RNA-seq analysis we concluded that, *PyolAA9-3*, *PyolAA9-13*, *PyolGH5_14-16*, *PypeGH46-1,* and *PyolCBEL-1* were upregulated during interactions with *Ph. infestans* or *B. cinema*. *PyolAA9-16*, *PyolGH5_14-1*, *PyolCBEL-2*, and *PyolCBEL-4* were downregulated during interaction with *Ph. infestans*. All the above genes mentioned also show similar expression profiles using qRT-PCR assays. *PyolGH5_14-12*, *PypeGH19-1*, *PypeGH19-2*, *PypeGH19-3*, *PypeGH46-2*, *PypeGH46-3*, *PyolCBEL-3* were constitutively expressed. There was generally a good agreement between the RNA-seq and qRT-PCR expression data, showing the reliability of our data. However, *PyolGH5_14-12*, *PypeGH19-1*, and *PyolCBEL-3* expression using RNA-seq is not so consistent with that shown by qRT-PCR.

Utilization of Complex Carbohydrate Sources

To investigate the extent to which the mycoparasitic oomycetes could directly utilize the major complex carbohydrate components of oomycete and fungal cell walls as the sole sources of carbon we performed *in vitro* growth assays in minimal media amended with either cellulose, chitin or the monomeric unit of chitin, N-acetylglucosamine with glucose or yeast extract amendments used as control conditions. We compared the growth of *P. oligandrum* and *P. periplocum* to that of *Ph. infestans* (**Figure 11**).

In either the presence or absence of yeast, *P. oligandrum* grew largest with glucose as a carbon source and was also able to utilize cellulose, chitin and N-acetylglucosamine as carbon sources. In the presence of yeast, *P. periplocum* grew equally well in most treatments, but in the absence of yeast, grew best with glucose as the sole carbon source. In the absence of yeast *P. periplocum* showed a strong preference for growth in glucose media and very reduced growth in the other substrates. In either the presence or absence of yeast, *P. infestans* grew

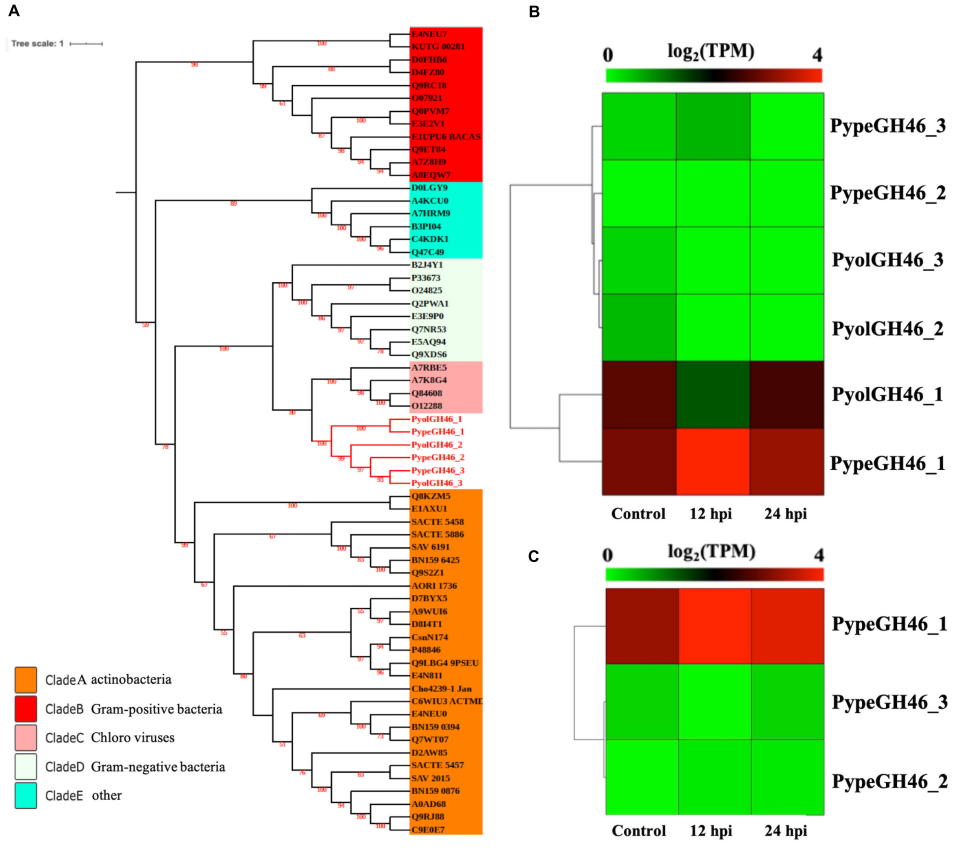

FIGURE 7 | Analysis of glycoside hydrolase family 46 (GH46). **(A)** Phylogenetic tree of GH46 proteins identified in oomycete species. Maximum likelihood tree, with 1,000 bootstraps (values displayed per branch). GH46 proteins identified in *P. oligandrum* (Pyol) and *P. periplocum* (Pype) are marked in red. **(B)** RNAseq expression profile of GH46 genes detected in *P. oligandrum* and *P. periplocum* during *in vitro* growth or during interactions with *Ph. infestans* at 12 or 24 h post interaction. Expression levels are expressed as the $\log_2$ fold change of transcripts per million (TPM), per gene. **(C)** RNAseq Expression profile of GH46 genes detected in *P. periplocum* during *in vitro* growth or interacting with *B. cinerea* at 12 or 24 h post interaction. Expression levels are expressed as the $\log_2$ fold change of transcripts per million (TPM), per gene.

largest with cellulose as the primary carbon source, and was not able to utilize chitin or N-acetylglucosamine reflecting the adaptation of this species to cellulosic plant hosts. Several CAZy encoded genes that function in the release of the simple sugar glucose from cellulose (e.g., GH6 and GH17 family members) were upregulated during mycoparasitism of the cellulosic host *Ph. infestans*, and genes for the degradation of the cell wall carbohydrates from fungal and oomycete prey were abundant and expanded in the mycoparasite genomes.

Taken together, these data suggest that degradation of more complex carbohydrate constituents of fungal and oomycete cell walls is only of limited importance to mycoparasitic oomycetes. Instead, destruction of the major components of prey cell walls in order to gain access to simple sugars within the cells appears to be the predominant strategy of these mycoparasites. This is in line with similar strategies used by phytopathogenic *Pythium* species, where CWDE secretion to allow maceration of the plant cell wall and subsequent uptake of simple sugars from within plant cells appears to be the major mechanism by which these species obtain nutrition (Zerillo et al., 2013). However, due to the expansion of CWDEs within mycoparasitic oomycete genomes they show a clear ability to utilize more complex carbohydrates not found within the plant cell wall when necessary. The mycoparasites also exhibit overall faster growth *in vitro* than the plant pathogen indicating a more efficient usage of these carbon sources for growth, and potentially allowing these species to outcompete their plant pathogenic or saprotrophic counterparts within the agroecosystem.

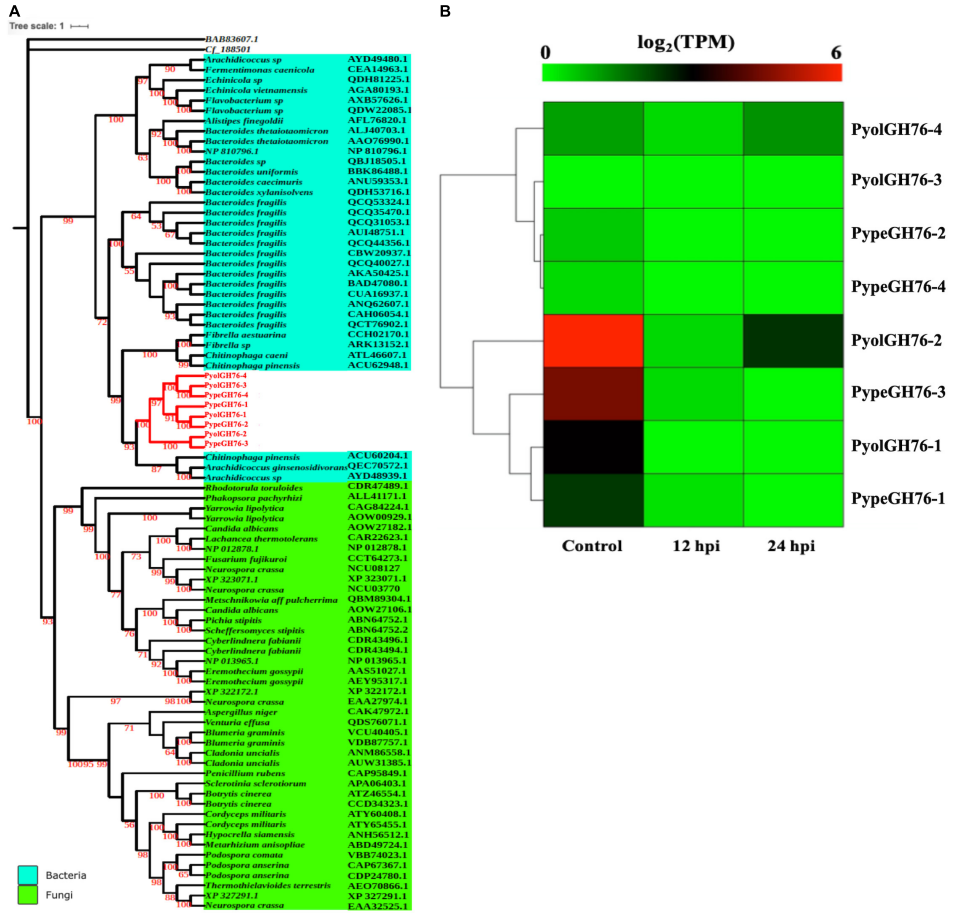

FIGURE 8 | Analysis of glycoside hydrolase family 76 (GH76). **(A)** Phylogenetic tree of GH76 proteins identified in *P. oligandrum* and *P. periplocum* with GH76 proteins of bacteria and fungi. Maximum likelihood tree, with 1,000 bootstraps (values displayed per branch). GH76 proteins identified in *P. oligandrum* (Pyol) and *P. periplocum* (Pype) are marked in red. BAB83607.1 and Cf_188501 (GH55 proteins verified in fungal species) were used as an outgroup. **(B)** RNAseq Expression profile of GH76 genes detected in *P. oligandrum* and *P. periplocum* during *in vitro* growth or interactions with *Ph. infestans* at 12 or 24 h post interaction. Expression levels are expressed as the $\log_2$ fold change of transcripts per million (TPM), per gene.

CONCLUSION

P. oligandrum and *P. periplocum* have long been recognized for their ability to inhibit plant pathogens (Paul, 1999; Rey et al., 2008), although the molecular basis of their interactions remains largely unknown. A small cDNA library sequencing study previously identified the potential role of CWDEs in *P. oligandrum* mycoparasitism (Horner et al., 2012). Through a detailed *in silico* and transcriptomic analysis, we have investigated the CAZyome of both *P. oligandrum* and *P. periplocum* in relation to plant pathogenic oomycetes. In conclusion, we found that tandem duplication and horizontal gene transfer events have been the major drivers for the formation of the distinctive and expanded CAZyome of mycoparasitic *Pythium* species.

We identified three major CAZy families from which the member genes are predominantly located in the gene sparse regions of their respective genomes and that have expanded through tandem duplication. These are hallmarks of genes undergoing rapid evolution such as the RxLR effectors of plant pathogenic oomycetes (Haas et al., 2009; Dong et al., 2015). Based on our data, we postulate, for the first time, that mycoparasitic oomycete genomes also display a "two-speed genome" phenomenon in a similar manner to plant pathogenic oomycetes, but that the key pathogenicity determinants, at

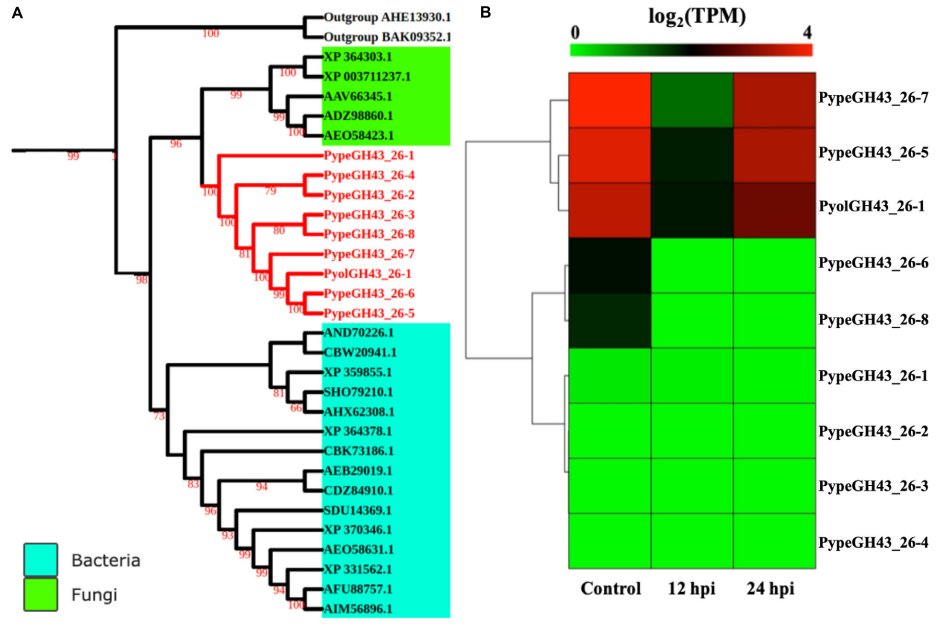

FIGURE 9 | Analysis of glycoside hydrolase family 43_26 (GH43_26). **(A)** Phylogenetic tree of GH43_26 proteins identified in *P. oligandrum* and *P. periplocum* with GH43_26 proteins of bacteria and fungi retrieved from CAZy database. Maximum likelihood tree, with 1,000 bootstraps (values displayed per branch). GH43_26 proteins identified in *P. oligandrum* (Pyol) and *P. periplocum* (Pype) are marked in red. AHE13930.1 and BAK095352.1 (GH11 proteins verified in fungi species) were used as an outgroup. **(B)** RNAseq Expression profile of GH43_26 genes detected in *P. oligandrum* and *P. periplocum* during *in vitro* growth or at 12 or 24 h post interaction with *Ph. infestans*. Expression levels are expressed as the $\log_2$ fold change of transcripts per million (TPM), per gene.

least those responsible for the onset of infection are CAZyme CWDEs that afford the mycoparasitic oomycetes the opportunity to macerate microbial tissue from which they can extract the sugars and other nutrients they require for growth and reproduction.

We have identified that carbohydrate active enzymes such as cellulose degrading enzymes are potentially important pathogenicity determinates in the mycoparasitic oomycetes. Since both mycoparasite and prey oomycetes all contain such enzymes, we hypothesize that these enzymes must be tightly regulated or compartmentalized to limit or prevent self-degradation and thus the regulation of CAZy gene function in mycoparasitic oomycetes is an important topic for further investigation in the future. Such studies will help to answer one of the most important questions in mycoparasitism; how self, and non-self, recognition occurs.

Furthermore, we have provided evidence for the horizontal gene transfer of five CAZy families from bacteria, fungi or viruses to the mycoparasites. In contrast, mycoparasitic fungi of the *Trichoderma* species are reported to have obtained almost 50% of their CWDE complement by horizontal, or lateral, gene transfer (Druzhinina et al., 2018). However, Richards et al. (2011) investigated the occurrence of horizontal gene transfer events from fungi to oomycetes and concluded that only a small number of such events could be found, thus our results are in line with these previous findings (Richards et al., 2011).

Taken together our data suggest a possible phytopathogenic ancestral state for the *Pythium* Genus, with the ability to mycoparasitise other eukaryotic microbes an adaptation derived from expansion of several key CAZyme gene families, and horizontal gene transfer events that occurred in the last common ancestor of *P. oligandrum* and *P. periplocum*.

MATERIALS AND METHODS

Isolates and Sequence Retrieval for Comparative CAZyme Analysis

The published genomes of the following oomycetes were used for the comparative analysis of their CAZyme encoding gene complement: *P. oligandrum* (CBS 530.74) (Kushwaha et al., 2017a), *P. periplocum* (CBS 532.74) (Kushwaha et al., 2017b), *P. ultimum* (DAOM BR144) (Lévesque et al., 2010), *P. irregulare* (DAOM BR486) (Adhikari et al., 2013), *P. iwayamai* (DAOM BR242034) (Adhikari et al., 2013), *P. arrhenomanes* (ATCC 12531) (Adhikari et al., 2013), *P. aphanidermatum* (DAOM BR444) (Adhikari et al., 2013), *P. insidiosum* (Strain Pi-S) (Rujirawat et al., 2015), *Phytopythium. vexans* (DAOM BR484)

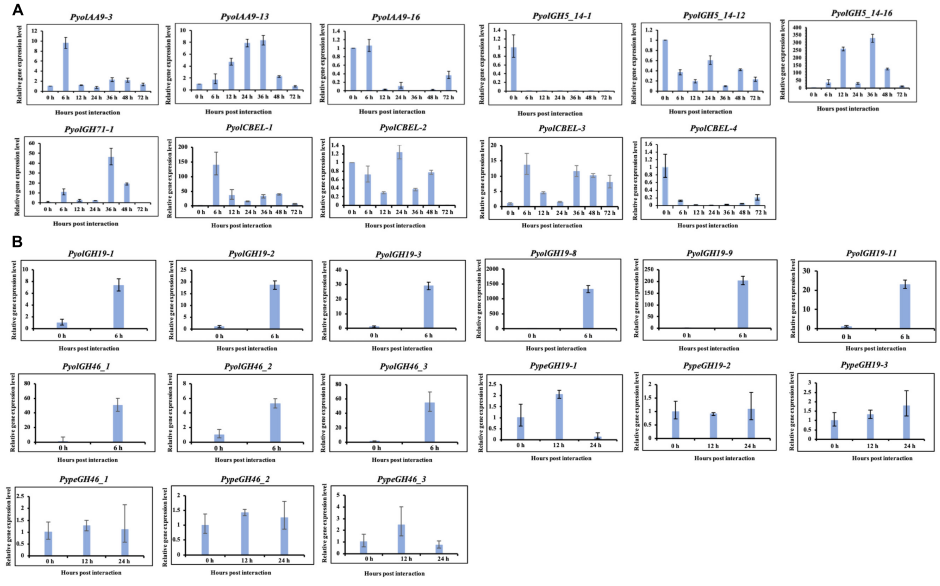

FIGURE 10 | Quantitative Real-Time PCR verification of expression of selected CAZy genes in *P. oligandrum* and *P. periplocum* during *in vitro* growth or during interactions with *Ph. infestans* or *B- cinnerea*. *In vitro* growth at 0 h was used as the reference and normalized to 1.0. The α-tubulin genes of *P. oligandrum* and *P. periplocum* (Pyoltua and Pypetua) were used as internal reference genes. Data displayed as the average of three biological replicates and error bars indicate standard deviations. 6, 12, 24, 36, 48, 72 h represent hours post interaction with *Ph. infestans* or *B. cinerea*. **(A)** Quantitative Real-Time PCR verification for CAZy genes of *P. oligandrum* and *P. periplocum* during interactions with *Ph. infestans*. **(B)** Quantitative Real-Time PCR verification for CAZy genes of *P. oligandrum* and *P. periplocum* during interactions with *B. cinerea*.

(Adhikari et al., 2013), *Ph. infestans* (T30-4) (Haas et al., 2009), *Ph. sojae* (P6497) (Tyler et al., 2006), *Ph. ramorum* (Pr102) (Tyler et al., 2006), *Ph. capsici* (LT1534) (Lamour et al., 2012).

To explore overall distribution of CAZymes among the above 11 oomycetes, the genome assemblies, transcript sequences, and protein sequences were downloaded from the NCBI Genome portal[1] and the Joint Genome Portal[2], along with the gene model annotation files in GFF3 format, which were used for CAZy annotation and synteny analysis.

BUSCO Comparisons

Benchmarking Universal Single-Copy Orthologs BUSCO v4 (Seppey et al., 2019)[3] was used to evaluate the completeness of the genome assemblies of the oomycetes used in this study. Default parameters were used with lineage-specific datasets set to stramenopiles_odb10.

Growth and Maintenance of Oomycete and Fungal Species

P. oligandrum (CBS 530.74) and *P. periplocum* (CBS 532.74) were maintained on V8 media amended with $CaCO_3$ as previously described (Horner et al., 2012; Kushwaha et al., 2017a,b) at 18°C in the dark. *Ph. infestans* (88,069) was maintained on rye sucrose agar at 18°C in the dark as described (Grenville-Briggs et al., 2008). *B. cinerea* (B05) was maintained on either V8 media or corn meal agar (CMA) at 20°C in the dark. Prior to confrontation, *P. oligandrum*, *P. periplocum* and *B. cinnerea* were grown in liquid V8 broth amended with calcium carbonate and *Ph. infestans* were grown in liquid pea broth, at 20°C in the dark.

Annotation of the CAZyome

CAZyme-encoding genes were predicted using the dbCAN pipeline (Yin et al., 2012). Briefly, hidden Markov models of all CAZy families were download from CAZy database (Lombard et al., 2013)[4]. Hidden Markov searches using the predicted proteomes of the oomycete species listed above were performed, with a cut-off value of 1E-03. To predict members of the CBEL family, only candidates containing two CBM_1 domain and a signal peptide were retained.

Transmembrane domain searches were conducted using the TMHMM Server v2.0 (Käll et al., 2005)[5]. SignalP 3.0 was used to predict the presence of a signal peptide for secretion

[1]https://www.ncbi.nlm.nih.gov/genome

[2]https://mycocosm.jgi.doe.gov/mycocosm/home

[3]www-busco.ezlab.org

[4]http://www.cazy.org/

[5]http://www.cbs.dtu.dk/services/TMHMM/

(Bendtsen et al., 2004)[6]. Domain architecture predictions were conducted with by searching the Pfam database (El-Gebali et al., 2018)[7]. For CAZy families with diverse enzyme activity, the exact enzyme activity annotation of members in these families were conducted by using BLASTP search of verified proteins in the ExPASy database (Artimo et al., 2012)[8].

Identification of CAZy Families Specific to, or Expanding in, the Mycoparasitic *Pythiums*

Fisher's exact test, conducted in the R program suite v3.5.3, was used to compare the gene counts of CAZy family members in the mycoparasitic *Pythium* species with those of the plant pathogenic *Pythium*, human *Pythium*, and *Phytophthora* species, respectively. The CAZy families with *p*-values higher than 1E-05 were removed. The CAZy families with higher gene counts in mycoparasitic *Pythium* were thus retained for further analysis.

Phylogenetic Analysis

Protein sequences were aligned by MUSCLE (Edgar, 2004). Phylogenetic analysis of full-length proteins alignments was performed by IQ-TREE (Nguyen et al., 2014), using the Maximum Likelihood approach and 1,000 bootstrap values. The models for phylogenetic analysis were automatically selected by IQ-TREE program. The visualization and modification of phylogenetic trees were performed using the iTOL server (Letunic and Bork, 2006). Branches with the bootstrap values higher than 50 were displayed.

Synteny Analysis

The predicted proteomes of *P. oligandrum* and *P. periplocum* were used to search against the predicted proteome of *P. ultimum* using BLASTP, using the default maximum hits setting with an *E*-value cut-off of 1E-10. Identification of gene collinearity between mycoparasitic *Pythium* and *P. ultimum*

[6]http://www.cbs.dtu.dk/services/SignalP-3.0/

[7]https://pfam.xfam.org/

[8]https://www.expasy.org/

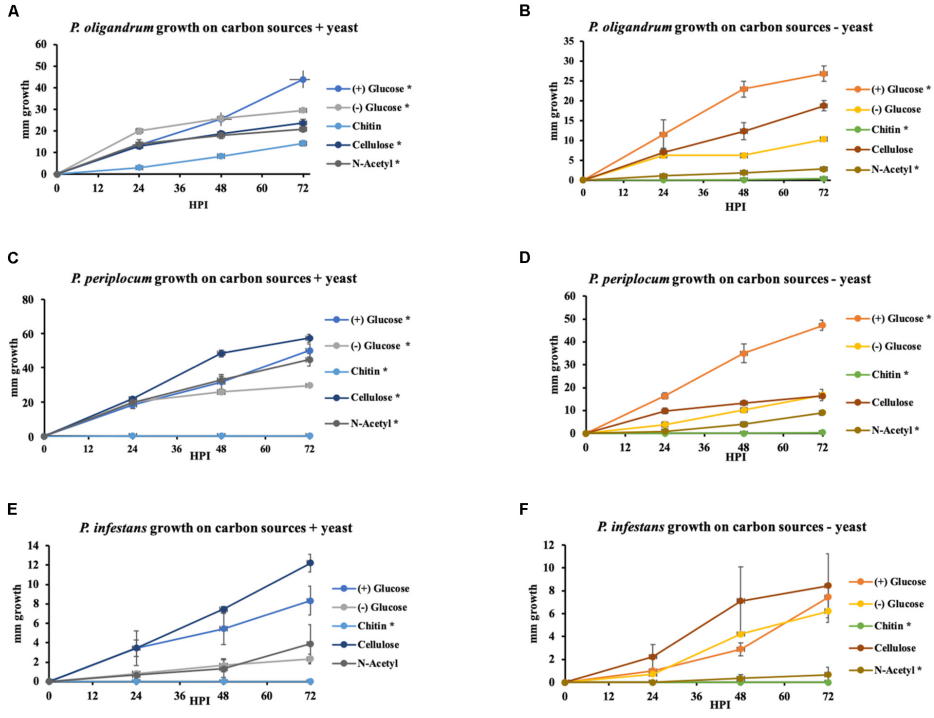

FIGURE 11 | Growth of *P. oligandrum*, *P. periplocum,* and *Ph. infestants* on varying carbon sources representing the major structural carbohydrates in fungal or oomycete cell walls. *P. oligandrum*, *P. periplocum,* and *Ph. infestans* growth on the carbon sources, cellulose, glucose, chitin, and N-Acetyl-glucosamine in modified Plich media plus or minus yeast, denoted by different colored lines. *X*-axis shows hours post inoculation (hpi). *Y*-axis displays colony diameter in mm. **(A,B)** Growth rate of *P. oligandrum*. **(C,D)** Growth rate of *P. periplocum*. **(E,F)** Growth rate of *Ph. infestans*. * denotes statistically significant difference from the control (-glucose, -yeast) data at $p < 0.05$ (*t*-test).

was performed using MCScanX (Wang et al., 2012). TBtools (Chen et al., 2018) was employed for visualization of the synteny analysis.

Evaluation of Average Fold Change of CAZy Families

We screened significantly differentially expressed CAZy genes and the CAZy families they belong to. By calculating the average fold change of these CAZy families, with treatment by log2, we could distinguish overall expression profiles of differentially expressed CAZy genes that were assigned to the same CAZy family. We used a log2-treated average fold change approach, to analyze the changes in expression of genes from each family where values equal to, or less than, −1 (to the left of the dashed black line in **Supplementary Figure S2**) correspond to down regulation of most of the genes within a family and values equal to, or greater than, 1 (to the right of the dashed red line in **Supplementary Figure S2**) correspond to up-regulation of the majority of the genes in that family. Numbers of genes from each gene family, on which this analysis is based are shown in the left of each panel of **Supplementary Figure S2**.

Mycoparasite-Prey Confrontation Assays

For confrontation assays, approximately 5 cm^3 of mycelium, washed with sterile dH_20, from mycoparasite (*P. oligandrum* or *P. periplocum*) and prey (either *Ph. infestans* or *B. cinnerea*) were placed at opposite sides of a polycarbonate membrane on V8 agar. The interaction zone (1 cm) was sampled at the point of contact (0 h post interaction, hpi) and then subsequently at 12 and 24 hpi. Control samples of the mycoparasites were prepared using two mycelial plugs from the same organism interacting with each other for 3 days, and mycoparasitism samples were prepared using either the oomycete *Ph. infestans* or the fungus *B. cinnerea* as the prey, which was prepared from liquid media samples as described above. Collected samples were snap frozen in liquid nitrogen and used for RNA extraction. Five replicates of each interaction were prepared and three of these replicates were randomly chosen for RNA extraction and sequencing. For detailed quantitative RT-PCR of a time course of mycoparasitism, the interacting mycelium from the confrontations between the mycoparasite and the prey in sterile tap water, was excised at 0, 6, 12, 24, 36, 48, and 72 hpi and immediately snap frozen and ground in liquid nitrogen, prior to RNA extraction.

RNA Extraction

Approximately 100 mg RNA from each sample was extracted using the RNeasy Plant Mini Kit (#74904 QIAGEN) according to the manufacturers protocol, and treated with RNase-free DNase for 20 min at 37°C (Ambion, TURBO DNA-free Kit). The extracted RNA was qualitatively visualized by agarose gel electrophoresis and a NanoDrop 1,000 spectrophotometer (Thermo Fisher Scientific) was used to quantify the total amount of RNA. Prior to RNA sequencing, the integrity of the samples was corroborated using the Experion™ Automated Electrophoresis System (Bio-Rad Laboratories, Hercules, United States).

Expression Analysis From RNA Sequencing

Polyadenylated messenger RNA was captured from 200 ng total RNA per sample using magnetic beads and Illumina adaptors with sample specific barcode sequences were ligated before subsequent library amplification using PCR using the Illumina TruSeq RNA poly-A selection kit. Sequencing of 150 bp paired-end libraries was carried out using the Illumina NovaSeq6000 S4 platform (SciLifeLab, Stockholm). All raw sequencing data in this study have been deposited in National Center for Biotechnology Information (NCBI) under BioProject accession number PRJNA637834 and a full analysis of these data will be presented elsewhere. The resulting bcl2fastq demultiplexed FastQ files were *de novo* mapped to our previously published reference genomes (Kushwaha et al., 2017a,b) and Adaptor cleaning was conducted by Trimmomatic (Bolger et al., 2014). Normalization and quantification of expression levels was performed by Salmon (Patro et al., 2015). The full analysis of differential gene expression during mycoparasitic interactions will be published elsewhere, however, in the current study we have mined these data to investigate the expression levels of selected CAZyme-encoding genes as presented in the results. pheatmap package of R project (Kolde, 2012) was used to produce heatmaps showing the log fold change in gene expression values (TPM counts) for selected CAZyme-encoding genes between the transcriptome samples.

Validation of Gene Expression Using Quantitative RT-PCR

cDNA synthesis was carried out using the Superscript IV Reverse transcriptase cDNA synthesis kit (Thermo Fisher Scientific) using 2 μg template RNA. All cDNA samples were diluted to 20 ng^{-1} prior to qRT-PCR. The gene expression levels were evaluated using quantitative RT-PCR (Biorad real-time PCR cycler using SYBG as the fluorescent dye). *P. oligandrum* and *P. periplocum* α-tubulin genes (*Pyoltua*; Genbank accessions MT623563 and MT811915; **Supplementary Table S1**). Primers (listed in **Supplementary Table S1**) were designed in Primer3[9] and the NCBI BLASTN web platform was used to check the specificity of the sequences for the genes in question, with the low complexity filter turned off. The internal reference genes list above were used to normalize expression levels of CAZyme candidates from the corresponding species.

Growth Assays to Investigate Utilization of Complex Carbohydrate Carbon Sources

To study growth of mycoparasitic *Pythium* on different carbon substrates, representing the major structural components of oomycete and fungal cell walls, we compared the growth of *P. oligandrum, P. periplocum,* and *Ph. infestans* in modified Plich media (van West et al., 1999) amended with, cellulose, glucose, chitin and N-Acetylglucosamine individually at 25 mM^*mL^{-1} both with and without yeast extract. Modified plich media

[9]http://primer3.wi.mit.edu

without glucose or yeast extract served as controls. 7.5 mm plugs from 7-day old liquid cultures of *P. oligandrum* CBS 530.74 and *P. periplocum* CBS 532.74 cultivated in V8 media and *Ph. infestans* 88,069 cultivated in pea-broth were used to investigate the growth rate of the oomycetes on different the carbon sources. *Ph. infestans* was used as a comparative control. Growth of each organism was quantified as diagonal growth in mm and measured every 24 hpi for three constitutive days. In total 10 treatments were performed. The experiment was repeated three times with three independent biological replicates. Statistical analysis of the growth assays was calculated as the area under the growth curve using the trapezoidal method. The differences among treatments was assessed using student's *T*-test, on the means of the area under the curve, assuming two-tailed distribution and two-sample with unequal variance. Significant difference was accepted if ($p < 0.05$).

DATA AVAILABILITY STATEMENT

RNA-seq data (NCBI accession: PRJNA637834); *P. oligandrum* internal reference gene (Genbank accession: MT623563); *P. periplocum* internal reference gene (Genbank accession: MT811915).

AUTHOR CONTRIBUTIONS

LG-B designed the experiments and analysis methods. DL designed and performed the bioinformatic analysis. CA and RV designed and performed the wet lab experiments and analyzed the wet lab experimental data. DL, CA, and LG-B wrote the manuscript. LG-B and DD revised the manuscript. All authors reviewed and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2020.581698/full#supplementary-material

Supplementary Figure 1 | BUSCO analysis for evaluation of genome assembly completeness. *X*-axis represent genome assemblies of organisms mentioned in this article. *Y*-axis represent number of each category of BUSCO groups.

Supplementary Figure 2 | Comparison of significantly expressed genes assigned to CAZy families that are not unique or expanded in the mycoparasitic *Pythium* species. Numbers within the heatmaps represent the count of significantly expressed genes assigned to each corresponding CAZy family. Green histograms represent $\log_2$-treated average fold change of Pythium CAZy genes significantly expressed from *in vitro* growth to 12 hpi in the presence of *Ph. infestans*. Light blue histograms represent $\log_2$-treated average fold change of *Pythium* CAZy genes significantly expressed from 12 hpi in the presence of *Ph. infestans* to 24 hpi in the same interaction. Black and red dashed lines represent $\log_2$-treated average fold change equal to -1 and 1 respectively. **(A)** overview of significantly expressed CAZy genes of *P. oligandrum*. **(B)** overview of significantly expressed CAZy genes of *P. periplocum*.

Supplementary Figure 3 | Analysis of gene density for selected CAZy gene families. **(A)** Gene density heatmap of AA9 genes in *P. oligandrum*. **(B)** Gene density heatmap of GH5_14 genes in *P. oligandrum*. Dots with different color represent intergenic distance of encoding genes in different clades according to respective phylogenic tree. **(C)** Gene density heatmap of GH19 genes in *P. oligandrum*. **(D)** Gene density heatmap of GH19 genes in *P. periplocum*. Dots with black color represent intergenic distance of GH19 encoding genes detected in *P. oligandrum* and *P. periplocum*.

Supplementary Figure 4 | Analysis of genome location of selected CAZy genes. **(A)** Genome location of *P. oligandrum* AA9 encoding genes from clades 1 and 3. Blue arrows represent the orientation of *P. oligandrum* AA9 encoding genes from clades 1 and 3. **(B)** Genome location of *P. oligandrum* GH5_14 encoding genes encoding from clade 1. Blue arrows represent the orientation of *P. oligandrum* GH5_14 genes from clade 1. **(C)** Genome location of *P. oligandrum* GH19 genes. Blue arrows represent orientation of *P. oligandrum* GH19 genes.

Supplementary Figure 5 | Synteny analysis. **(A)** Synteny analysis of GH55 genes between mycoparasitic *Pythium* species and *P. ultimum*. The red arrows represent GH55 genes identified in *P. oligandrum* and *P. periplocum*. The blue arrows represent genes near to GH55 genes. The gray lines represent syntenic relationships. **(B)** Synteny analysis of GH46 genes between mycoparasitic *Pythium* and *P. ultimum*. The red arrows represent the orientation of GH46 genes identified in *P. oligandrum* and *P. periplocum*. The blue arrows represent the orientation of genes neighboring the GH46 genes. The gray lines represent syntenic relationships.

Supplementary Figure 6 | Analysis of the cellulose-binding elicitor lectin (CBEL) family. **(A)** Phylogenetic tree of CBEL proteins identified in oomycete species. Maximum likelihood tree, with 1,000 bootstraps (values displayed per branch). CBEL proteins identified in *P. oligandrum* (Pyol) and *P. periplocum* (Pype) are marked in red. TrCBHI_P62694 (an exoglucanase verified in *Trichoderma reesei*) was used as an outgroup. Domain architecture of CBEL proteins is shown on to the left of the phylogenetic tree. **(B)** RNAseq Expression profile of *CBEL* genes detected in *P. oligandrum* and *P. periplocum* during *in vitro* growth or during interactions with *Ph. infestans* at 12 or 24 h post interaction. Expression levels are expressed as the $\log_2$ fold change of transcripts per million (TPM), per gene.

Supplementary Figure 7 | Analysis of the cellulose synthase (CesA) family. **(A)** Phylogenetic tree of CesA proteins identified in oomycete species. Maximum likelihood tree, with 1,000 bootstraps (values displayed per branch). CesA3 proteins identified in *P. oligandrum* (Pyol) and *P. periplocum* (Pype) are marked in red. CesA proteins identified in *Arabidopsis thaliana* (At) were used as outgroup genes. The domain architecture of each of the CesA proteins is shown to the left of the Phylogenic tree. **(B)** RNAseq Expression profile of CesA genes detected in *P. oligandrum* and *P. periplocum* during *in vitro* growth or during interactions with

Ph. infestans at 12 or 24 h post interaction. Expression levels are expressed as the log_2 fold change of transcripts per million (TPM), per gene.

Supplementary Figure 8 | Phylogenic analysis of GH55 and GH71 proteins detected in mycoparasite *Pythium* and their homologous proteins detected by BLAST search. Maximum likelihood tree, with 1,000 bootstraps (values displayed per branch). Proteins identified in *P. oligandrum* (Pyol) and *P. periplocum* (Pype) are shown in red.

Supplementary Table 1 | Primers for CAZyme-coding genes verified by qRT-PCR in this study.

Supplementary Table 2 | ID and gene model sequences of CAZyme-coding genes in this study.

Supplementary Table 3 | Ortholog clustering for each CAZy families mentioned in this study.

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ORIGINAL ARTICLE

Reduced efficacy of biocontrol agents and plant resistance inducers against potato early blight from greenhouse to field

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Abstract
Early blight in potato, caused by *Alternaria solani*, is mainly controlled by frequent applications of synthetic fungicides. Reducing the use of synthetic fungicides in agriculture is desired to reach an overall sustainable development since the active components can be harmful for humans and for the ecosystem. In integrated pest management, IPM, the idea is to combine various measures, including optimized crop management, crop rotation, use of resistant cultivars, biological control agents (BCAs), plant resistance inducers, and fertilizers, to decrease the dependence on traditional chemical fungicides. In this paper, we present the results from greenhouse and field trials where we evaluated the effect of strategies aimed at reducing our reliance on synthetic fungicides including treatments with biological control agents (BCAs) (*Pythium oligandrum*, Polygandron®, and *Bacillus subtilis*, Serenade®) and plant resistance inducers (silicon products HortiStar® and Actisil®) for early blight in potato. The agents were applied separately or in combination with each other or with synthetic fungicides. In the greenhouse, trials application of these agents resulted in 50–95% reduction of infection by *A. solani*, but their combination did not generally improve the outcome. However, the effects were much smaller in the hand-sprayed field trials, 20–25% disease reduction and almost disappeared in full-scale field trials where application was done with tractor sprayers. In this article, we discuss possible reasons behind the drop in efficacy from greenhouse trials to full-size field evaluation.

Keywords *Alternaria solani* · Biological control · Biocontrol agents · Plant resistance inducer · Potato disease · Field trials

Introduction

The fungus *Alternaria solani* is a soil-borne pathogen causing early blight in several Solanum species including potato (*Solanum tuberosum* L.). *A. solani* overwinters in the soil and causes infection when the right climate is obtained for development of disease. Early blight affects tuber yield globally, and yield losses of up to 50% have been reported (Leiminger and Hausladen, 2012). Besides late blight, caused by *Phytophthora infestans,* early blight is one of the most important foliar diseases in potato (Abuley et al., 2019). Early blight affects starch potato yield in southern Sweden, causing earlier defoliation of the plants (Andersson and Wiik, 2008). Starch potato cultivars are harvested later in the season than ware potato cultivars since the starch is stored in the tubers later in the summer. Most of the Swedish table potato is already harvested when the early blight infection strikes in Sweden, while the yield of potato starch can be significantly reduced. Therefore, starch potato cultivars are more affected by the pathogen *A. solani* in southern Sweden.

To control early blight infection, synthetic fungicides are traditionally used, but to reach a more sustainable agriculture it would be beneficial to exchange some of these chemical treatments with biological equivalents. According to the EU Directive (2009/128/EC), the dependence on chemical pesticides should be reduced by combining alternative

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measures. Biological control agents (BCAs) in this case bacteria or oomycetes are natural antagonists to the pathogens and are thus used to control diseases. The BCAs can either parasitize or in other ways, through antibiosis or nutrient competition, outcompete the unwanted pathogen (Gao et al., 2017). Additional nutritional supplements or plant resistance inducers (PRIs) that are not classified as synthetic fungicides may also replace or complement traditional chemical treatment strategies in order to develop more sustainable disease management methods in agriculture. Another important reason to search for alternative disease control methods is that fungicide resistance is developing quickly in the *A. solani* population in response to fungicide applications (Odilbekov et al., 2019; Mostafanezhad et al., 2021) resulting in a vulnerable crop production. There are only a limited number of efficient fungicides against early blight currently available for farmers. This causes a vulnerability in Swedish potato cultivation and increases the risk of fungicide resistance development in the pathogen population.

There are several alternatives to synthetic fungicides that have shown effectiveness against early blight in greenhouse and field trials. The biocontrol agent *Pythium oligandrum* has been shown to have effects on a wide variety of plant pathogens in different crops, like damping-off of sugar beet caused by *Pythium ultimum* (Martin and Hancock, 1987), bacterial wilt of tomato caused by *Ralstonia solanacearum* (Hase et al., 2008), *Verticillium* wilt in pepper caused by *Verticillium* spp. (Rekanovic et al., 2007), and grapevine trunk wood disease caused by *Phaeomoniella chlamydospora* (Yacoub et al., 2016). Ikeda et al. (2012) reported that treatment of potato seed tubers with *P. oligandrum* oospores significantly decreased black scurf disease severity index on stolons caused by *Rhizoctonia solani* in field conditions. This decrease was at a same level as that caused by Flutolanil®, a chemical fungicide commonly used to treat black scurf. Kurzawińska and Mazur (2009) showed that potato tuber dressing and/or plant spraying with Polyversum® (a commercial formulation of *P. oligandrum*) significantly decreased late blight disease infection caused by *P. infestans* in the field, at the same level as the chemical pesticide Vitavax 2000 FS (Active components karboxin and thiuram).

Abbasi and Weselowski (2014) studied the effect of weekly foliar sprays of commercial formulations of *Bacillus subtilis* in the form of dried (Serenade MAX®, 1 kg/ha) and aqueous suspension (Serenade ASO®, 4 L/ha) on foliar early blight disease of tomato during 2008–2010.

Their field trials during 2008-2010 showed that Serenade ASO had a significant effect on early blight development based on both rAUDPC (relative area under disease progress curve) values and final disease severity rating in 2008. Treatments with Serenade MAX also significantly reduced early blight infection in field trials for tomatoes conducted in 2009. Egel et al. (2019) studied the effect of Serenade® in the management of *A. solani* on tomato plants in greenhouse and two field sites, where the field sites had different climatic conditions. In the greenhouse studies, Serenade® was used alone as a treatment and it significantly decreased early blight disease levels in two out of three greenhouse trials. In the field studies, Serenade® was alternated with botanical product Regalia® (a commercial formulation of the plant *Reynoutria sachalinensis*). The treatment regime in which Serenade® and Regalia® were applied alternatively did not significantly decrease disease levels compared to the untreated control (Eget et al., 2019).

In addition to BCAs, there are other low risk alternatives for possible use against plant pathogens like PRIs. Silicon, the second most abundant element on earth (Kumaraswamy et al., 2021), has been used against different pathogens in potato as well as other crops. Silicon may strengthen plant cell walls or induce defense responses in plants (Wang et al., 2017). Gulzar et al. (2021) showed that treating tomato plants with silicon (in the form of potassium silicate, 1.7 mM), increased resistance to *A. solani*. Spraying silicon (in the form of Na_2SiO_3, 100 mM) on potato leaves enhanced potato resistance against another common potato disease, late blight, caused by *Phytopthora infestans,* in a detached leaf assay (Xue et al., 2021). However, the effectiveness of silicon to protect potato in the field from either late blight or early blight has not been thoroughly investigated in the literature.

In the present study, biocontrol agents (BCAs) and plant resistance inducers (PRIs) were tested against early blight in greenhouse and field studies. To be able to practically integrate BCAs and/or PRIs into IPM strategies, reliable data showing efficacy under agricultural relevant field situations are needed. Even though it is known that alternative products have lower efficacy than fungicides, direct comparisons between greenhouse and field settings such studies are rare in the literature. Based on the previous promising studies, several treatments including the BCAs Serenade® (*B. subtilis*), Polygandron® (*P. oligandrum*) and an oospore suspension of *P. oligandrum* (prepared in the laboratory), and the PRIs/silicon fertilizers HortiStar® and Actisil® were used in the present study. The aim was to evaluate their efficacy against early blight disease in greenhouse experiments and field trials to conclude if results from the greenhouse can help predict the efficacy under field conditions. Since knowledge about how to combine or alternate these alternative products with traditional synthetic fungicides is also needed, traditional fungicide treatments and combinations of fungicides and alternative treatments were included in the trials. The experiments were conducted in three phases, greenhouse trials, small scale field trials with manual application of the treatments and large field trials with tractor sprayer applications. The main questions were: 1) Is the efficacy

against early blight disease consistent between the greenhouse and the field, or between field trial plots of different sizes? 2) Do combinations of different alternative treatments improve efficacy against early blight disease? 3) Can traditional fungicide treatments applied in lower amounts be combined with alternative treatments give sufficient disease control?

Materials and methods

In this study different alternative measures are evaluated against early blight infection. Four organisms/products; *P. oligandrum* (also as Polygandron®), Serenade®, Actisil® and HortiStar® are tested alone or in combinations in three different settings; Greenhouse, small plot field trials and large plot field trials (Table 1). In greenhouse experiments, the products were diluted in distilled water, while in all field trials non-chlorinated water from a well at the experimental farm was used.

Preparation of *Alternaria solani* inoculum

Alternaria solani isolate AS112, isolated from a field in Sweden, was used in the greenhouse experiments. To obtain a spore suspension, the fungi was grown on 20% strength potato dextrose agar medium (PDA) supplemented with 12 g L^{-1} Bacto Agar in 9 cm petri dishes and incubated at 25 °C for 7 days in darkness. To increase sporulation, the plates were incubated for another seven days under UV-C light (254 nm dominant wavelength) for 5–6 h per day. Conidia were harvested by flooding the plates with Milli-Q distilled water containing 0.01% (v/v) Tween 20, while conidia were dislodged using a sterile L-shape cell spreader. The final concentration of the conidial suspension was adjusted to 10^4 conidia per mL using a hemocytometer. To ensure the adherence of conidial suspension at the inoculation site on the leaves surface, the conidial suspension was supplemented with 0.1% Bacto Agar (Odilbekov et al., 2014).

Pythium oligandrum preparation

Slightly different formulations of *P. oligandrum* were used in the greenhouse and field trials over the three years, due to new registration of a formulated product to the Swedish market in March 2019. To produce inoculum for the greenhouse and field trials in 2018 and 2019, solid agar plates of V8 media were inoculated with one agar plug of *P. oligandrum* (CBS-strain 530.74) and allowed to grow for seven days at 20 °C. From the solid *P. oligandrum* cultures, five agar plugs were inoculated into 1L bluecap bottles containing 300 mL clarified V8 broth. The bottles were put into a rotary incubator, shaking at 120 rpm at 20 °C for seven days. To harvest the oospores from the liquid cultures, the mycelia were macerated using a high-speed blender and 200 mL of sterile water was amended. The inoculum was then filtered. A final concentration of 2.5×10^4 oospores/mL was obtained. In 2020 field trials,

Table 1 Overview of all the treatments performed in different settings

Treatment	Active ingredient	Type	Green house	Small field	Large field	Field trial year
P.oligandrum lab formulation		BCA	x	x		2018–2019
Polygandron	*P.oligandrum*	BCA	x	x		2020
Serenade	*B.subtilis*	BCA	x	x	x	2018–2020
Actisil	Silicon + Calcium + Cholinechloride	PRI	x		x	2016–2017
HortiStar	Silicon	PRI	x	x	x	2018
Combinations:						
P. oligandrum + Serenade	x	x				2018–2019
Polygandron + Serenade	x	x				2020
P. oligandrum + HortiStar	x	x				2018
Polygandron + HortiStar	x					
HortiStar + Serenade	x	x				2018–2019
Serenade + HortiStar + Polygandron	x	x				2020
Fungicide + Actisil					x	2016–2017
Alterations:						
Serenade/Fungicide					x	2019–2020
HortiStar/Fungicide					x	2018

the registered product from Biopreparaty, Polygandron WP, batch 08,022,020, with a concentration of 5×10^5 oospores per gram, or 200 g/ha, was diluted with well water at the trial site and applied according to the label corresponding to a dose of 300 L liquid/ha. A decision was made in 2020 to use the formulated product and not produce inoculum at SLU, since it would make the field trials easier to reproduce later and to handle during the season.

Serenade® preparation

The registered product Serenade® ASO from Bayer Crop Science containing *Bacillus subtilis*, strain QST 713, containing a minimum of 1.05×10^{12} cfu/L according to the label was used. For the greenhouse trials, 12.5 mL of Serenade® was diluted with tap water resulting in a concentration of 0.5% Serenade®. The same process was done using well water for the small trials. For the large trials, Serenade® was applied in a concentration of 2.0–6.0 L/ha diluted with well water to a total liquid dose of 300 L/ha. Slightly different doses were used for the large field trials. The dose was increased the second season from 2.0 L/ha in 2019 to 4.0 L/ha in 2020. Also, five treatments in 2019 were decreased to three in 2020 for the Serenade® only treatment, and the first application, T0, was done earlier in 2020 to enable earlier colonization of soil and lower leaves (Table 3a and b and supplementary files Table 2). The treatment consisting of reduced fungicide, with two full-dose sprays instead of four, was only present in the trial in 2020.

HortiStar® preparation

HortiStar® is a product containing silicate foliar fertilizer from Hortifeeds with a silicon content of 19%. 2.5 mL of HortiStar® was diluted with tap water resulting in a concentration of 0.10% HortiStar® for using in the greenhouse trials. The same process was done using well water for the small trials. For the large trial in 2018 HortiStar® was added at a dose of 0.5 L/ha.

Actisil® preparation

YaraVita Actisil® is a silicon containing fertilizer from Yara marketed as a plant strengthener. The silicon is present in the available form of stabilized orthosilicic acid. Actisil® also contains choline and calcium. According to the label, Actisil® will increase the cell wall stability and further increase the natural resistance. For greenhouse experiments, Actisil® was evaluated for two different potato cultivars, Désirée and Matilda. Actisil® was sprayed 24 h prior to

Table 2 rAUDPC and rAUC results from year 2018–2020 in the small hand-sprayed trial testing disease reduction from different substances and combinations. The analysis was done both separately for each year and pooled across years. Different letters indicate statistical differences ($p < 0.05$) according to the post hoc Tukey test for all years pooled, except for the treatments only present in some of the years where the analysis was done separately for the years that treatment was present

			rAUDPC				rAUC			
Treatment	Brand name	Active component	2018	2019	2020	mean	2018	2019	2020	mean
Untreated control			0.028 a	0.118 a	0.019 a	0.055 a	0.036 a	0.191 a	0.033 a	0.087 a
P. oligandrum	Polygandron WP (2020)*	*Pythium oligandrum*	0.019 ab	0.093 ab	0.016 a	0.043 b	0.028 a	0.156 ab	0.034 a	0.073 b
Serenade	Serenade ASO	*Bacillus subtilis*	0.015 b	0.093a b	0.011 a	0.040 b	0.027 a	0.156 ab	0.030 a	0.071b
HortiStar	HortiStar	Silicate	0.018 ab	0.093 ab	0.015 a	0.042 b	0.032 a	0.157 ab	0.031 a	0.073b
Serenade + *P. oligandrum*			0.013 b	0.087 ab	0.019 a	0.040 b	0.026 a	0.151 b	0.032 a	0.070b
P.oligandrum + hortistar			0.017 ab				0.030 a			
Serenade + hortistar			0.017 ab	0.090 ab			0.030 a	0.147 b		
Serenade + hortistar + *P.o ligandrum*					0.011 a				0.026 a	

*The formulated product Polygandron WP® was used in 2020. In 2018 and 2019, the inoculum was produced in laboratory

inoculation with *A. solani* (as a 0.1% solution on the foliage). The effect of YaraVita Actisil® was evaluated in 2016 and 2017 large field trials. Actisil® was used in a dose of 0.4 L/ha diluted with well water to a total liquid dose of 300 L/ha.

Greenhouse experimental design

The greenhouse experiments were performed at the Swedish University of Agriculture, in Alnarp, Sweden. Five separate greenhouse trials were conducted to examine the efficacy of different treatments (Table 1). The experiments had a randomized complete block design with 4–6 replicate blocks.

Plant material preparation and growth conditions

Solanum tuberosum cv. Désirée and cv. Matilda was grown by subculturing of 3-week-old stems cutting to around 2 cm with one leaf on Murashige and Skoog (MS) media (30 g/L sucrose, 8 g/L phyto agar, 4.4 g MS, pH 5.8), in tissue culture boxes. The boxes remained in a phyto chamber with 16 h of light (140 µE) per day for 21 days. After that, the in vitro plantlets were transferred to 2.5 L plastic pots in a greenhouse chamber with adjusted temperature to 22 °C with 16 h of natural day light supplemented with artificial light. In all greenhouse experiments, only the cultivar Désirée was used except in the first greenhouse experiments where both Désirée and Matilda cultivars were used.

Greenhouse treatments

Forty-five days after transferring the plants into the greenhouse, plants were sprayed with Serenade®, *P. oligandrum*, Polygandron®, HortiStar® or Actisil® using a 600 mL hand sprayer until run-off. Oospore suspension of *P. oligandrum* lab strain (CBS-strain 530.74) was also added to the soil (20 mL) in the second and third greenhouse experiments as a separate treatment. Combined treatments (Table 1) were sprayed separately with 1–2 h interval for the foliage to dry. After 48 h (24 h for Actisil® experiment), the plants were inoculated by placing a drop of 10 µL *A. solani* conidial suspension on the surface of 10 chosen leaflets (5 leaflets per leaf) in the middle part of the plant. A tent was constructed to maintain high humidity (around 95%) during the first 24 h after inoculation. Then, relative humidity was stabilized at 85% using a misting system within the chamber.

Disease assessment

Ten days after inoculating the plants with *A. solani*, disease development was estimated by measuring the diameter of the lesions in two perpendicular directions using a vernier caliper supposing an oval area. Then the lesion area, LA, was calculated as the following equation:

$$LA = 0.25 \times \pi \times D1 \times D2$$

where D1 and D2 are the diameters in millimeters.

Synergy calculation

In the combined treatments, the synergy factor (SF) was calculated according to the Abbott method (Abbott, 1925):

$$SF = C_{obs}/C_{\exp}$$

where C_{obs} is the observed disease protection ratio and C_{exp} is the expected disease protection ratio. A SF value greater than 1 indicates synergistic interaction, and a SF value smaller than 1 indicates antagonism interaction between the compounds of a treatment. C_{exp} was calculated as:

$$C_{exp} = A + B - A \times B$$

for two-compound treatments, and as:

$$C_{exp} = A + B + C + A \times B \times C - A \times B - B \times C - A \times C$$

for three-compound treatments. A, B and C in the above equations denote the observed disease protection ratios of the single compounds.

Field trial experimental design

Field trials were conducted from 2016 to 2020 in southern Sweden at two different sites. In all the field trials, the starch potato cultivar Kuras was used since it is the most common cultivar used in this part of Sweden for potato starch production. For the trial with Actisil in 2016, there was also an additional starch cultivar, Stayer, included. Kuras and Stayer have been noted to be susceptible for early blight (unpublished data). The seed tubers were obtained from Lyckeby, SSF. Both the large and the small field trials were fertilized, and managed following standards set by the Swedish Rural Economy and Agricultural Societies, as seen in the supplementary material, in Mosslunda, south of Kristianstad, Sweden. The potatoes were planted with a row distance of 75 cm and a planting distance of 38 cm. No inoculations with pathogens were done in any of the trials. Natural early blight infection did occur every year (Fig. 2). Standard treatments to control late blight, insects, and weeds were implemented throughout the season according to the supplementary files. Irrigation was done when needed.

Small plot field trials

The small field trials were placed in Helgegården (56.018696, 14.064942) in 2018, 2019 and 2020. Hand sprayers of the brand Ferrox 5L model 3565 were used for application and the formulations were diluted with well water to correspond to 300 L liquid/ha. The applications were done at a pressure of three bars five times over the season with two-week intervals starting in the beginning of July. The combined treatments were sprayed twice without the solutions being mixed. In between the two treatments, enough time passed for the foliage to dry. The layout consisted of four blocks with a randomized complete block design. Each plot consisted of four 2.0 m long rows of potatoes where the two middle rows were treated and visually scored.

Large plot field trials

The large-scale field trials were carried out in 2016, 2017, 2018, 2019 and 2020 at two locations, Helgegården (56.018696, 14.064942) and Nymö (56.024848, 14.335998), in southern Sweden. The setup was a randomized complete block design with four blocks with plots of 10 m in five rows where the three central rows, 18 m^2, were harvested. The yield and starch content were measured, and total yield and starch yield were converted to yield/ha. The starch content of the tubers was calculated from measurements of specific weight (International Starch Institute Denmark, 1986). A tractor sprayer (Lechler IDKT Purple 0,25) with a flat fan nozzle medium droplet size was used for application at 300 L liquid/ha at a pressure of three bars.

Field trial treatments

Four different treatments were evaluated from which two are classified as BCAs, *P. oligandrum* and *B. subtilis*, and two as fertilizers/PRIs, HortiStar® and Actisil® (Table 1). The date of each treatment is presented as T1, T2, T3, etc., where the exact date for each year's T1 can be found in the supplementary files. The treatments following T1 were applied with 1 week intervals.

Field disease assessment

In the field trial assays, the level of early blight infection and defoliation was visually scored weekly according to Duarte et al. (2013). Infection was defined as the percentage of green leaf area covered by typical dark early blight spots, and defoliation was defined as the percentage of the total canopy that was dead or defoliated. The relative area under the disease progress curve, rAUDPC, as well as the area under the defoliation curve, rAUC, was calculated according to Shaner and Finney (1977) by using the formula:

$$AUDPC = \sum_{i=1}^{n} \left[\left(Y_{i+n1} + Y_i \right) / 2 \left[X_{i+1} - X_i \right] \right]$$

where Y_i is the level of early blight infection in percentage at observation number *i*. X_i is the date of the scoring, and *n* the total number of observations. The scoring was done weekly from the beginning of August to mid-September, and rAUDPC/rAUC was calculated from AUDPC/AUC by dividing the AUDPC/AUC value with the total area of the graph by multiplying the number of days with 100% infection. Leaves with lesions from the field were collected, and the presence of the early blight causal agent *A. solani* was confirmed both in microscope and with PCR (Landschoot et al., 2017).

Statistical Analysis

Differences in lesion sizes for plants in the greenhouse experiments across treatments were tested with ANOVA (PROC GLM) using SAS 9.4 (SAS Institute, Cary, USA). To investigate effects of the treatments in the field trials, R-studio (version 1.1.456–© 2009–2018 RStudio, Inc) ANOVA was also used, with sum of squares type III for both trial settings. For post hoc comparisons of means, Tukey's test (p-value < 0.05) was used.

The ANOVA series for the small field trial consisted of the two response variables rAUDPC and rAUC, as a function of the fixed variables: treatment, year and block (nested within year) and of the interactions of these variables. For the large field trials, the same methods were used with additional response variables for tuber yield, starch content and starch yield.

Results

Greenhouse experiments

All treatments, except for adding *P. oligandrum* to the soil, including foliar sprays with Polygandron®, Serenade®, HortiStar®, Actisil® and combined treatments gave significantly decreased lesion sizes caused by *A. solani* on the greenhouse potato plants (Fig. 1). Treatment with *P. oligandrum* on foliar parts of the plants resulted in a significant decrease in lesion size compared to untreated control in three out of four experiments. In the experiment with Actisil®, the cv. Matilda had larger lesions than cv. Désirée (Fig. 1, exp 1; Anova $F = 5.9$, $p = 0.038$). The experiment with Actisil® was repeated once with cv. Désirée and similar results were obtained.

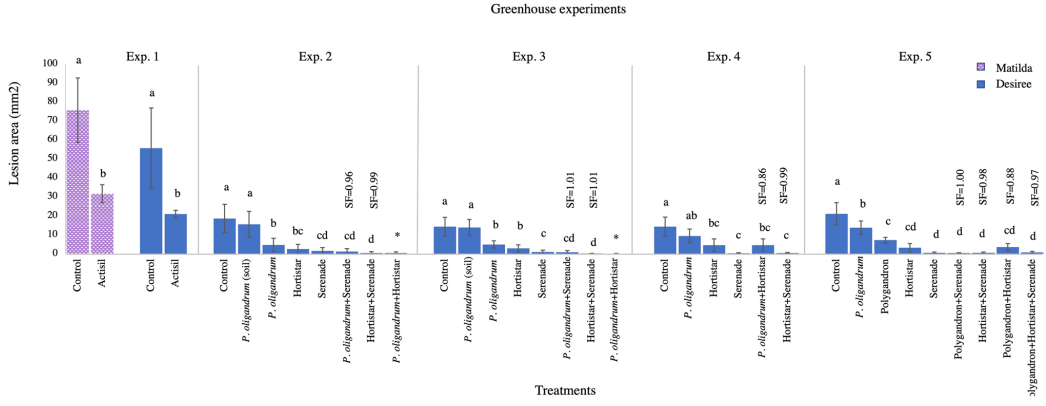

Fig. 1 Control of early blight disease of potato (cultivar Désirée and Matilda) caused by *Alternaria solani* using *Pythium oligandrum*, Polygandron®, Serenade®, Actisil® and HortiStar® in greenhouse experiments. Treatments were applied 48 h (Exp. 2–5) or 24 h (Exp. 1) before inoculation of plants with *A. solani*. All treatments were sprayed on the plants, while *P. oligandrum* was added to the soil (20 mL) in the second and third experiments as a separate treatment, marked as *P. oligandrum* (soil). Different letters show statistically significant differences between treatments in each experiment according to Tukey's test (p-value < 0.05). Vertical bars show standard deviation. SF = Synergy factor calculated according to the Abbott method. Control: plants only inoculated with *A. solani*. *: Excluded from the statistics due to foliage falling off

On average, application of Serenade® (alone or in combination with other treatments) resulted in the largest reduction of lesion sizes. A 90% reduction in lesion size was seen in these treatments (1.5 mm^2 average lesion size) compared to untreated controls (14–21 mm^2 average lesion size). In the second and third experiments, there were a lot of dropped leaves in plants treated with the combination of *P. oligandrum* and HortiStar® (Fig. 1) indicating a possible phytotoxic effect.

As shown in Fig. 1, the Synergy Factor (SF) values were generally close to one ($0.96 \leq SF \leq 1.01$) except for *P. oligandrum* + HortiStar® (SF = 0.86 in exp 4) and Polygandron® + HortiStar® (SF = 0.88 in exp 5). Thus, no synergistic effects between different agents were observed in the greenhouse studies, implying that combining agents did not increase their efficacy.

Field trials

In the field trials, the potato plants were naturally infected by *A. solani* during all years. However, the onset of infection and the disease pressure varied among years as indicated by the infection rates in untreated controls in the large field trials (Fig. 2). 2020 was notable since the infection came late in the season and did not cause as much visible damage compared to the other years. The difference is likely due to climatic differences between the years. The disease pressure was overall higher at Nymö than at Helgegården (Fig. 2).

Small plot field trials

Relative area under disease progress curve (rAUDPC) and the relative area under defoliation curve (rAUC) were used for analyzing the effects of two BCAs, one PRI, and combinations of them on early blight (Table 2, Fig. 3). The rAUDPC and rAUC values were based on scoring data from mid-August to mid-September. Analysis of variance over all three years showed a general significant effect of treatment on rAUDPC ($F_{4, 36} = 6.48$, p-value 0.0005), but there was no significant interaction between treatment and year ($F_{8, 36} = 1.87$, p-value 0.095). However, the F value for the year-effect was large ($F_{2, 36} = 567$, p-value < 0.0001) and shows that the seasonal variations are much larger than the effect of treatments (Fig. 3). All the treatments resulted in significant reductions of rAUDPC according to a post hoc Tukey test (Table 2). For rAUC, the results were similar (Treatment: $F_{4, 36} = 4.46$, p-value 0.005, Year: $F_{2,36} = 869$, p-value < 0.0001, Interaction year treatment $F = 2.27$, p-value 0.044; Table 2). For the treatments that were not included in all years, a separate analysis per year was done that also showed a significant effect of the treatments compared to the controls according to Tukey test (Table 2). In 2018, the effect was only significant for Serenade® and for the combination Serenade® + *P. oligandrum*, and in 2019 only the effect of the combination Serenade® + P. *oligandrum* was significant. If the years 2018 and 2019 are pooled, the results are the same as for all three years (analysis not shown). The low infection pressure in 2020 coincides with

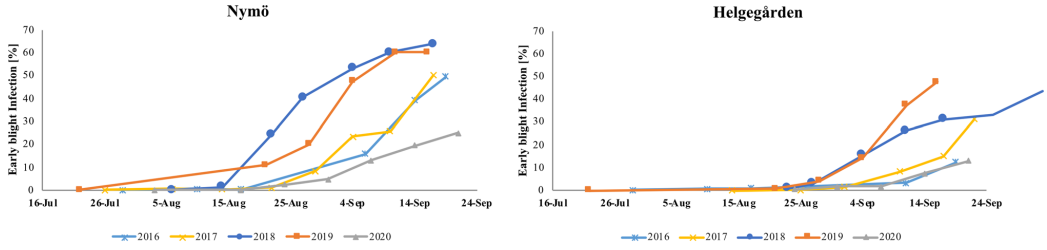

Fig. 2 Infection development disease progress curves for untreated plots during the different seasons and sites of the large trials

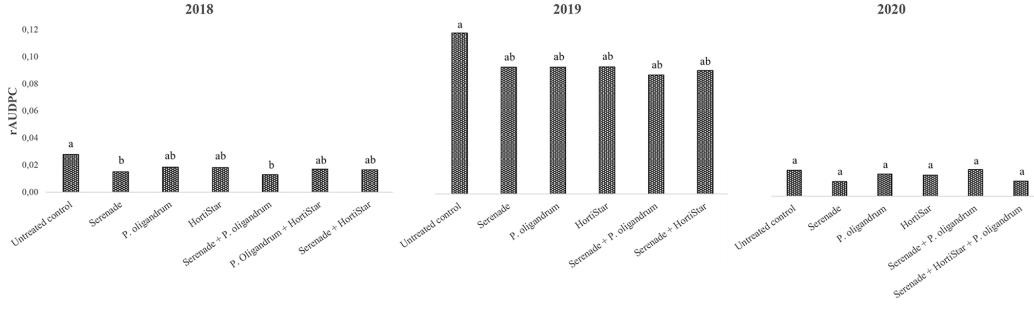

Fig. 3 rAUDPC values for the different years and treatments in the small trials

the lack of significant effect of any of the treatments in that year alone.

The disease reduction was numerically largest with Serenade® or with Serenade® combined with *P. oligandrum* (Table 2, Fig. 3). On average over all the years, the treatments resulted in a disease reduction, measured as rAUDPC, of about 28% for Serenade® and 27% for the combination Serenade® and *P. oligandrum*.

Large plot field trials

In the large field trials, the effects of Serenade®, Actisil® and HortiStar® alone or in combination/alteration with traditional fungicides were evaluated. These treatments were compared with a traditional fungicide application regime. Serenade® and Actisil® were evaluated for two seasons each and HortiStar® for one. The disease scoring and harvest data were recorded for all the large field trials.

Serenade® Serenade® was evaluated in 2019 and 2020. Analysis of variance over both years and sites indicated a general significant effect of treatment on rAUDPC ($F = 99.9$, p-value < 0.0001). The fungicide regime, reduced fungicide regime (evaluated only in 2020) and reduced fungicide regime combined with Serenade® all resulted in significantly lower infection compared to untreated control (Table 3). However, treatment with Serenade® alone did not result in any reduced infection rate in 2019. In 2020, there was a small but significant disease reduction compared to untreated controls at Nymö and when the two trial sites were analyzed together (Table 3).

There was no significant effect of Serenade® on tuber yield, starch content or starch yield in any of the years. However, there was significantly higher yield and starch yield seen as an effect of the fungicide treatments in 2019 at both trial sites and when both trial sites were analyzed together (Table 3a). Further, the starch content was significantly higher in the fungicide treatment at one of the trials site and when both sites were analyzed together.

Actisil® In all the treatments including fungicides, there are significantly lower infection rates compared to untreated controls in 2016 (Table 4a). However, treatments with Actisil® alone did not result in any significant disease reduction. Still, in 2016 one interesting observation was made. Combining half dose fungicides with Actisil® resulted in significantly lower infection than using half dose fungicides alone, and this com-

Table 3 a and b Results from Serenade® treatment in the large trials for 2019 (a) and 2020 (b), 2019: Nymö T1 = 17/6; Helgegården T1 = 19/6, 2020: T0 = 5/6, T1 = 16/6; Helgegården T0 = 10/6, T1 = 17/6, treatment dose in L/ha in parenthesis

Treatment	rAUDPC	rAUC	Yield (ton/ha)	Starchcon-tent%	Starchyield (ton/ha)
2019 (a)					
Helgegården					
Untreated control	0.183 b	0.1953 b	54.7 a	19.3 a	10.6 a
Narita (0.4)T5, T9; Propulse (0.45)T7, T11	0.008 a	0.0770 a	60.0 b	20.0 a	12.0 b
Serenade (2.0)T3, T5, T7, T9, T11	0.185 b	0.2023 b	54.1 a	19.5 a	10.6 a
Serenade (2.0)T3, T5; Narita (0.4)T7, T11; Propulse (0.45)T9	0.016 a	0.0788 a	61.6 b	20.4 a	12.5 b
Nymö					
Untreated control	0.233 c	0.455 c	63.6 a	20.4 a	13.0 a
Narita (0.4)T5, T9; Propulse (0.45)T7, T11	0.070 a	0.1080 a	70.3 b	21.9 b	15.4 c
Serenade (2.0)T3, T5, T7, T9, T11	0.227 c	0.436 c	65.2 ab	20.3 a	13.2 ab
Serenade (2.0)T3, T5; Narita (0.4)T7, T11; Propulse (0.45)T9	0.128 b	0.223 b	66.0 ab	21.2 ab	14.0 b
Mean					
Untreated control	0.207 c	0.325 b	59.2 a	19.9 a	11.8 a
Narita (0.4)T5, T9; Propulse (0.45)T7, T11	0.039 a	0.092 a	65.2 b	20.9 b	13.7 b
Serenade (2.0)T3, T5, T7, T9, T11	0.206 c	0.319 b	59.7 a	19.9 a	11.9 a
Serenade (2.0)T3, T5; Narita (0.4)t7, t11; Propulse (0.45) T9	0.072 b	0.151 a	63.8 b	20.8 b	13.3 b
2020 (b)					
Helgegården					
Untreated control	0.045 b	0.0888 a	56.2 a	23.3 a	13.1 a
Narita (0.4)T4, T8; Propulse (0.45)T6, T10	0.004 a	0.0463 a	54.3 a	23.8 a	12.9 a
Narita (0.4)T5; Propulse (0.45)T8	0.003 a	0.0366 a	57.9 a	23.8 a	13.8 a
Serenade (4.0)T0, T2, T6	0.035 b	0.0997 a	53.6 a	23.8 a	12.8 a
Serenade (4.0)T0, T2, T6;Narita (0.4)t5; Propulse (0.45) T8	0.005 a	0.0301 a	58.8 a	23.5 a	13.8 a
Nymö					
Untreated control	0.075 c	0.4745 c	74.4 a	18.4 a	13.7 a
Narita (0.4)T4, T8; Propulse (0.45)T6, T10	0.011 a	0.2670 a	74.0 a	19.0 a	14.0 a
Narita (0.4)T5; Propulse (0.45)T8	0.012 a	0.2823 a	73.4 a	18.8 a	13.8 a
Serenade (4.0)T0, T2, T6	0.056 b	0.4043 bc	72.7 a	18.4 a	13.4 a
Serenade (4.0)T0, T2, T6; Narita (0.4)t5; Propulse (0.45) T8	0.018 a	0.3168 ab	74.2 a	19.2 a	14.2 a
Mean					
Untreated control	0.060 c	0.2820 a	65.3 a	20.8 a	13.4 a
Narita (0.4)T4, T8; Propulse (0.45)T6, T10	0.008 a	0.1570 a	64.1 a	21.4 a	13.5 a
Narita (0.4)T5; Propulse (0.45)T8	0.007 a	0.1600 a	65.6 a	21.3 a	13.8 a
Serenade (4.0)T0, T2, T6	0.045 b	0.2520 a	63.2 a	21.1 a	13.1 a
Serenade (4.0)T0, T2, T6; Narita (0.4)T5; Propulse (0.45) T8	0.012 a	0.173 a	66.5 a	21.3 a	14.0 a

bination did not have more infection than the treatment with full dose of fungicides (Table 4a). This indicated that there was a combination effect between Actisil® and the fungicides in 2016. The fungicide regime this year was RevusTop® (T1 and T2) followed by Signum® (T3, T5, T7, T9). However, a similar strategy was investigated in 2017 using another fungicide regime: RevusTop® (T4, T8, T12) alternated with Signum® (T6, T10). This year no significant combination effect was found between reduced doses of fungicides and Actisil®.

When the Actisil® trial result was analyzed separately for the years 2016 and 2017, there was no significant effect from any treatments on the yield or starch yield (Table 4).

Table 4 a and b. Results from Actisil® treatment in the large trials 2016 (a) and 2017 (b) the letters show significance ($p < 0.05$) obtained from a Tukey test within the years 2016: Nymö T1 = 15/6; Helgegården = 22/6. 2017: Nymö and Helgegården T1 = 15/6 treatment dose in L/ha in parenthesis

Treatment	rAUDPC	rAUC	Yield (ton/ha)	Starch content%	Starchyield (ton/ha)
Helgegården					
2016 (a)					
Untreated control	0.029 b	0.100 a	49.3 a	24.6 a	12.1 a
RevusTop (0.3)T1, 2; Signum (0.25)T3, 5, 7, 9	0.021 a	0.080 a	50.3 a	24.8 a	12.4 a
Actisil (0.4)T3-T9	0.027 ab	0.081 a	50.9 a	24.8 a	12.6 a
RevusTop (0.15)T1, 2; Signum (0.125)T3, 5, 7, 9	0.025 ab	0.094 a	49.6 a	24.8 a	12.3 a
RevusTop (0.15)T1, 2; Signum (0.125)T3, 5, 7, 9); Actisil (0, 4)T3-T9	0.019 a	0.074 a	50.0 a	24.8 a	12.4 a
Nymö					
Untreated control	0.108 b	0.239 b	80.2 a	21.7 a	17.4 a
RevusTop (0.3)T1, 2; Signum (0.25)T3, 5, 7, 9	0.071 a	0.190 a	81.3 a	22.1 a	18.0 a
Actisil (0.4)T3-T9	0.106 b	0.252 b	78.8 a	22.1 a	17.4 a
RevusTop (0.15)T1, 2; Signum (0.125)T3, 5, 7, 9	0.077 a	0.187 a	81.3 a	22.0 a	17.9 a
RevusTop (0.15)T1, 2;Signum (0.125)T3, 5, 7, 9); actisil (0.4)T3-T9	0.060 a	0.176 a	82.8 a	21.7 a	17.9 a
Mean					
Untreated control	0.068 c	0.169 c	64.7 a	23.2 a	14.8 a
RevusTop (0.3)T1, 2; Signum (0.25)T3, 5, 7, 9	0.046 ab	0.135 ab	65.8 a	23.4 a	15.2 a
Actisil (0.4)T3-T9	0.067 c	0.166 bc	64.9 a	23.4 a	15.0 a
RevusTop (0.15)T1, 2; Signum (0.125)T3, 5, 7, 9	0.051 b	0.141 abc	65.4 a	23.4 a	15.1 a
RevusTop (0.15)T1, 2; Signum (0.125)T3, 5, 7, 9); actisil (0.4)T3-T9	0.040 a	0.125 a	66, 4 a	23, 2 a	15, 1 a
Helgegården					
2017 (b)					
Untreated control	0.025 b	0.102 a	71.6 a	22.1 a	15.8 a
RevusTop (0.3)T4, 8, 12; Signum (0.25)T6, 10	0.004 a	0.088 a	73.5 a	22.7 a	16.7 a
RevusTop (0.15)T4, 8, 12; Signum (0.125)T6, 10	0.011 ab	0.090 a	72.1 a	23.0 a	16.6 a
Actisil (0.4)T4, 6, 8, 10, 12	0.026 b	0.100 a	69.6 a	22.8 a	15.8 a
RevusTop (0.3)T4, 8, 12; Signum (0.25)T6, 10; Actisil (0.4)T4, 6, 8, 10, 12	0.006 a	0.092 a	71.9 a	22.8 a	16.4 a
RevusTop (0.15)T4, 8, 12; Signum (0.125)T6, 10; Actisil (0.4)T4, 6, 8, 10, 12	0.005 a	0.087 a	71.1 a	23.3 a	16.6 a
Nymö					
Untreated control	0.104 c	0.283 ab	78.4 a	21.3 a	16.7 a
RevusTop (0.3)T4, 8, 12; Signum (0.25)T6, 10	0.041 a	0.222 ab	81 a	21.1 a	17.1 a
RevusTop (0.15)T4, 8, 12; Signum (0.125)T6, 10	0.054 ab	0.196 a	81.1 a	20.8 a	16.9 a

Table 4 (continued)

Treatment	rAUDPC	rAUC	Yield (ton/ha)	Starch content%	Starchyield (ton/ha)
Actisil (0.4)T4, 6, 8, 10, 12	0.098 c	0.294 b	77.1 a	21.0 a	16.2 a
RevusTop (0.3)T4, 8, 12; Signum (0.25)T6, 10; Actisil (0.4)T4, 6, 8, 10, 12	0.039 a	0.196 a	80.3 a	21.0 a	16.9 a
RevusTop (0.15)T4, 8, 12; Signum (0.125)T6, 10; Actisil (0.4)T4, 6, 8, 10, 12	0.063 b	0.258 ab	78.8 a	21.1 a	16.7 a
Mean					
Untreated control	0.064 b	0.193 b	75 a	21.7 a	16.3 a
RevusTop (0.3)T4, 8, 12; Signum (0.25)T6, 10	0.023 a	0.155 ab	77.2 a	21.9 a	16.9 a
RevusTop (0.15)T4, 8, 12; Signum (0.125)T6, 10	0.032 a	0.143 a	76.6 a	21.9 a	16.8 a
Actisil (0.4)T4, 6, 8, 10, 12	0.062 b	0,. 197 b	73.4 a	21.9 a	16.0 a
RevusTop (0.3)T4, 8, 12; Signum (0.25)T6, 10; Actisil (0.4)T4, 6, 8, 10, 12	0.022 a	0.144 a	76.1 a	21.9 a	16.6 a
RevusTop (0.15)T4, 8, 12; Signum (0.125)T6, 10; Actisil (0.4)T4, 6, 8, 10, 12	0.034 a	0.173 ab	75 a	22.2 a	16.6 a

Table 5 Results from HortiStar® treatment in the large trials. The letters are showing significant differences ($p < 0.05$) obtained when the two sites were analyzed together (Mean) and separately 2018: Nymö T1 = 15/6; Helgegården T1 = 21/6 treatment dose in L/ha in parenthesis

Treatment	rAUDPC	rAUC	Yield (ton/ha)	Starch content%	Starchyield (ton/ha)
2018					
Helgegården					
Untreated control	0.087 c	0.184 c	60.6 a	19.5 ab	11.8 a
RevusTop (0. 6)T4, 8, 12; Signum (0. 25)T6, 10	0.041 b	0.116 b	65.3 ab	20.4 b	13,. 3 ab
RevusTop (0. 3)T4, 8, 12; Signum (0. 125)T6, 10	0.046 b	0.113 b	62.9 ab	19.2 a	12.1 ab
Narita (0. 4)T3, 7; propulse (0. 45)T5, 9	0.010 a	0.073 a	67.4 b	20.2 ab	13.6 b
RevusTop (0. 3)T4, 8, 12; Hortistar (0, 5)T2, 6, 10	0.051 b	0.125 b	62.6 ab	20.1 ab	12.6 ab
RevusTop (0. 3)T4, 8, 12;Hortistar (0. 5)T2, 4, 6, 8, 10	0.041 b	0.120 b	65.8 ab	20.3 ab	13.4 b
Nymö					
Untreated control	0.301 e	0.433 b	62.2 a	18.6 a	11.6 a
RevusTop (0, 6)T4, 8, 12; Signum (0. 25)T6, 10	0.196 ab	0.385 ab	66.1 a	18.7 a	12.3 a
RevusTop (0. 3)T4, 8, 12; Signum (0. 125)T6, 10	0.240 d	0.428 b	67.2 a	19.1 a	12.4 a
Narita (0. 4)T3, 7; propulse (0. 45)T5, 9	0.181 a	0.328 a	67.5 a	19.1 a	12.9 a
RevusTop (0. 3)T4, 8, 12; Hortistar (0. 5)T2, T6, 10	0.236 cd	0.422 b	65.2 a	18.7 a	12.2 a
RevusTop (0. 3)T4, 8, 12; Hortistar (0. 5)T2, T4, T6, T8, 10	0.212 bc	0.381 ab	64.7 a	18.6 a	12.0 a
Mean					
Untreated control	0.194 d	0.308 c	61.4 a	19.1 ab	11.7 a
RevusTop (0. 6)T4, 8, 12; Signum (0. 25)T6, 10	0.119 b	0.251 b	65.7 b	19.5 ab	12.8 b
RevusTop (0. 3)T4, 8, 12; Signum (0. 125)T6, 10	0.143 c	0.270 bc	65 ab	18.8 a	12 2 ab
Narita (0. 4)T3, 7; propulse (0. 45)T5, 9	0.095 a	0.200 a	67.4 b	19.6 b	13.3 b
RevusTop (0. 3)T4, 8, 12; Hortistar (0. 5)T2, T6, 10	0.143 c	0.274 bc	63.9 ab	19.4 ab	12.4 ab
Revus Top (0. 3)T4, 8, 12; Hortistar (0. 5)T2, T4, T6, T8, 10	0.126 bc	0.250 b	65.3 ab	19.5 ab	12.7 ab

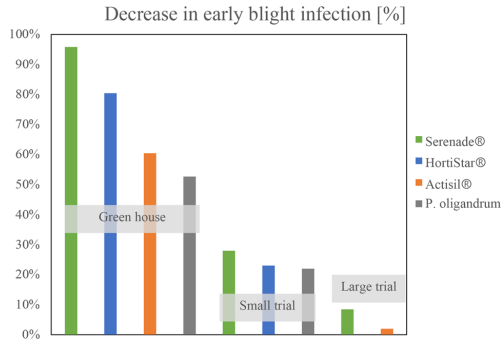

Fig. 4 Comparison between average reduction in early blight infection between the different trial setups for the four different agents. To compare the results from the different trial settings, the percentage of infection reduction from the treatments compared to control is used. For the greenhouse trial, this means reduction in the size of the lesion, and for the field trial the numbers come from the visual hand scoring

HortiStar® HortiStar® was evaluated only in 2018 (Table 5). We investigated the effect of the fungicide regime RevusTop® (T4, T8, T12) alternated with Signum® (T6, T10) and compared with treatments combining half dose fungicides with HortiStar®. A treatment with only HortiStar® was not included. Alternating/combining the fungicide RevusTop® with HortiStar® five times did not result in significantly lower infection rates than the same combination where HortiStar® was applied only three times. The combination treatments with HortiStar® did not have more infection than a similar treatment where RevusTop® was alternated with the fungicide Signum®.

Analyses over both trial sites showed that only fungicide treatments resulted in significantly higher yield and starch yield in average, while the two combinations with HortiStar® or the reduced fungicide regime did not.

Discussion

The EU Directive (2009/128/EC) concerning the sustainable use of pesticides proposes a reduced dependence on synthetic pesticides. Integrated pest management (IPM) should be implemented according to the directive, and BCAs, PRIs, and fertilizers could be part of future IPM strategies. Further, reduced fungicide applications have benefits including sustainability, cost efficiency, and a decreased risk of fungicide resistance development (Odilbekov et al., 2019). In the present study, we evaluated the effect of two forms of *P. oligandrum*, including a lab strain formulation and a commercial BCA product named Polygandron®, the BCAs Serenade® (based on *B. subtilis*), and the silicon fertilizers Actisil® and HortiStar® against early blight in potato. In general, we found good and promising effects of the investigated BCAs and PRIs in greenhouse experiments, small but significant effects in small hand-sprayed field trials but almost no effect in large-scale field trials where the agents were applied with a tractor sprayer (Fig. 4). The synthetic fungicides did, however, efficiently reduce the infection and generally increased the yield.

No effect on the tuber yield was observed in this study, except from the synthetic fungicides. If biological control agents or PRIs/fertilizers are to be used in traditional farming, the effect has to be comparable to that of traditional fungicide, also economically. In organic farming on the other hand, BCAs will only be compared to untreated; however, still they must result in yield improvement. The differences among the years in the field trial is reflecting the fluctuating efficacy of the alternative treatments. A dilemma of using BCAs or PRIs/fertilizers in conventional agriculture is the uncertainty of sufficient disease control that may depend on environmental conditions, disease pressure and microbial interactions.

The efficiency of BCAs for the control of early blight

The oomycete *P. oligandrum* does not only act directly through mycoparasitism, antibiosis, and competition for nutrients, but also interacts with plant roots and stimulates plant defense responses (Bělonožníková et al., 2020) related to the soil microbial community and direct and indirect pathogen inhibition. However, we did not observe any disease reducing effect when *P. oligandrum* was added to the soil in the greenhouse experiments as we did for foliar treatment. The more effective result in foliar treatment could be explained as a direct effect of *P. oligandrum* on the pathogen which is in the same part of the plants. When *P. oligandrum* was used in the soil, perhaps the interaction with roots and stimulation of the plant defense responses was limited due to an unsuitable environment, microbial competition, or a longer time may be required for the interaction to occur in the soil.

Earlier reports indicate that the BCA *B. subtilis* strains have inhibitory effects on *A. solani* in vitro (Zhang et al., 2020); however, little was known of the potential to reduce early blight infections in the field. In a study conducted in Germany, researchers evaluated Serenade® and *Trichoderma* in combination to control early blight in comparison with conventional fungicides. They found an average of 20% inhibitory effect of the biological control treatment, whereas the chemical control agent showed 78% protection (Metz, 2017). In this study, Serenade® also reduced early blight infection to a similar degree (28%) in the small plot trial both alone and combined with *P. oligandrum*. However, in

the large plot trials the effect was much smaller or absent. Metz and Hausladen (2022) also made a large field evaluation 2016–2019 where they yet again experience a drop of efficacy when the trial is scaled up. The BCAs only showed a significant reduction in field in one year out of four.

The highest reduction in lesion size in greenhouse trials was observed in plants treated by Serenade® alone or in combination with other treatments. According to the literature, *B. subtilis* can control a wide variety of pathogens in different plants (Collins and Jacobsen, 2003; Lahlali et al., 2013; Abbasi and Weselowski, 2014; Egel et al., 2019). *B. subtilis* can colonize the leaf surface and compete with *A. solani* for space and nutrients and physically prevent penetration of the pathogen into the leaves (Abbasi and Weselowski, 2014) Secondary metabolites and lipopeptides have also been found to reduce the lesion size of *A. solani* in potato (Abbasi and Weselowski, 2014). The reduction of lesion size observed in the greenhouse plants treated by Serenade® can be the result of these direct mode of actions since the pathogen and Serenade® were in contact on the potato leaves. Induction of plant resistance by Serenade® (Lahlali et al., 2013) may also explain the disease reduction. In the greenhouse experiments, Serenade® alone was as effective as combined treatments including Serenade® and we did not observe any synergistic effects. This result may relate to the fact that when using Serenade® alone, the lesions were so small, they were measured at close to zero, so combined treatments showed no significant difference here.

The efficiency of PRIs for the control of early blight

Both Actisil® and Hortistar® contain silicon that can mechanically strengthen plant cell walls (Ma and Yamaji, 2006). Silicon can also enhance induced systemic resistance in potato plants (Xue et al., 2021). Actisil® was evaluated both in greenhouse and in large trials in 2016 and 2017. In the greenhouse Actisil® significantly decreased the lesion sizes after inoculation with *A. solani*, but in the large field trials there was no effect on the early blight development. Still, Actisil® treatment in combination with fungicides had significant effect on the infection rate in 2016 but this was not the case in 2017. In all greenhouse experiments, HortiStar® caused significant reductions of the lesion sizes and also reduced the disease in the small hand sprayed field trials. HortiStar® was only evaluated in one field season (2018) for the large trials. This season the fungicide Signum® alternated with RevusTop® was used as a reference fungicide regime. Replacing Signum® with HortiStar® gave the same result with respect to disease development rate. However, at that time fungicide resistance against boscalid (a.i. in Signum®) was widely spread and the efficacy of Signum® was strongly reduced (Mostafanezhad et al., 2021). The fungicide reference with Narita® and Propulse® was also included in this trial and would be better for comparison. HortiStar® was not efficient enough to affect yield or infection rate.

Positive effects in the greenhouse do not always translate to efficient disease control in the field

To be able to integrate alternative agents in IPM strategies, there is a need to unravel the reasons behind the discrepancy between the frequently reported successes in greenhouse studies and the poor and variable effects in field trials.

All the field trials were treated with late blight fungicides, which would presumably be toxic to *P. oligandrum* and might be one reason behind the limited effect of *P. oligandrum* in the field trials.

Another possible explanation to the results might be related to the durability of effect. In the greenhouse studies, the agents were applied 24–48 h before the inoculation with *A. solani*. In the field trials, the interval between the treatments was two weeks and it could be that the effect of the treatments diminished some days after treatments. Still, in the small hand-sprayed trials we found a significant effect although much smaller than in the greenhouse with a two-week interval. The durability of the effects of BCAs and PRIs needs to be studied in more detail. The timing of treatments may also be an important factor. In our field trials, we applied the BCAs and PRIs at the same times as chemical treatment would be applied. Maybe the treatments must start earlier if a microorganism should have time to establish on the canopy for example. In 2020, when we observed a weak but significant effect of Serenade® in the large field trial the first application was done much earlier than in 2019 where no effect was observed. In a recently published article, da Silva et al. (2021) showed a significant disease reduction in potato early blight of around 90% after treatment with *Clonostachys* in greenhouse like our results with Serenade®. They further suggest that this BCA could be used in field before planting to reduce the soil inoculum, and not as a direct treatment during the season.

The variation of efficacy of disease suppression between field and greenhouse assays might also be related to differences in the microbiome of the plant. In a greenhouse experiment UV-light, soil, humidity and irrigation will be very different from a field. Studies have shown that the bacterial community in potatoes are recruited from the soil (Buchholz et al., 2019). Microbial agents may be harmed by the continuous UV-light present in the field, and this may also be part of the explanation for the better effect in the greenhouse. A better strategy might be to introduce the biocontrol agent in the soil before planting for possible reduction of soil-borne inoculum of the pathogen. Abiotic factors such as environmental conditions (Rasche et al., 2006) or different

soil types (İnceoğlu et al., 2012) are known to influence the structural and functional diversity of for example 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. Hence it matters greatly, in which environment, and existing interactive microbial community, the biocontrol agent will be amended, and thereby to what extent it will have capabilities of disease control.

Application method

The application methods were different in the greenhouse, the small and the large field trial. In the greenhouse and the small trials, a hand sprayer was used which will have a lower pressure, larger droplets, and a higher coverage of lower foliage than the tractor sprayer used for the large trial. In the small hand-sprayed trials, we also made effort to try to hit all the leaves. The absence of effect of BCAs in the large tractor-sprayed trials could be due to that the agents did not reach the lower leaves resulting in a high initial infection rate. Early blight infection usually starts in the lower aging leaves of the plants. This might explain some of the divergence of the results. If alternatives to fungicides are to be used in conventional farming, the products need to fit the already practiced routines and equipment or that application technologies are developed to better fit BCAs and other alternative agents.

We used doses of the products as recommended by the suppliers. It is possible that higher doses are required to obtain significant effects in the field. In 2020, we used double dose of Serenade® compared to 2019 and the application was done earlier. This might explain why the rAUDPC was significantly reduced in 2020 but not in 2019, but it might also be explained by a different disease pressure.

The BCAs and PRIs we investigated had no or very limited effect on early blight in the field. However, still a small effect could be of importance if there were a combinatory, or even better a synergistic effect, when used in combination or together with reduced amounts of chemical fungicides. In the greenhouse, we observed weak additive effects when two or more agents were combined. In the field trials, no such effect was observed when combining the alternative agents in small trials. In the big field trials, on one occasion Actisil® combined with half dose fungicide resulted in the same level of control as full-dose fungicide. However, it was not repeatable.

Conclusions

In summary, it can be concluded that there is a need for more field-based research on the use of alternative treatments against early blight in potato. The plant biological interactions need to be further evaluated. There seems to be a gap in the understanding of how and when alternative treatments should be applied with tractor sprayers to sustain the effect of the products. It might be of importance to cover all the foliage of the crop, which a flat fan nozzle cannot conduct. Another possible solution might be to formulate the products in a way that gives the BCAs better opportunities to colonize the foliage. Serenade® and Actisil® showed a small potential in reducing the infection of early blight in the field, in some of the years, but no tuber yield increase was observed. If BCAs and PRIs are going to be used against early blight in potato the efficacy of them must be much higher. Maybe that can be improved by optimizing dose rates, application timing and application technology or by development of more efficient agents and formulations. Their use also must be put in perspective involving other IPM measures like more resistant cultivars, optimized nutrition, crop rotations, optimized timing of fungicide treatments by using decision support systems, etc. Breeding for resistance is important and there may be possibilities to also breed for improved response to BCAs and PRIs in the future.

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Declarations

Conflict of interest The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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This thesis aims to broaden our understanding of the mycoparasitic *Pythium spec*ies; through transcriptomics and comparative genomics, the study differentiates these mycoparasites from their phytopathogenic counterparts. The research examines whether complex carbohydrates serve as nutrition for the mycoparasite. The efficacy of *P. oligandrum* for biostimulation and disease control is evaluated. The thesis aims to contribute to sustainable potato cultivation practices by improving our understanding of these mycoparasitic *Pythium* species and their interactions with the rhizosphere microbiome and within the potato cropping system.

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