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Faculty of Agrobiology, Food and Natural Resources

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Protection of Strawberries against *Phytophthora cactorum*

Doctoral Dissertation

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DEDICATION

This dissertation is dedicated to my beloved parents (Ammi & Babajan) for their unconditional love, support, and prayers.

DECLARATION

I, Asad Ali, hereby declare that the dissertation entitled "**Protection of Strawberries against Phytophthora cactorum**" is being submitted in partial fulfilment of the requirement for the award of a doctoral degree at the Czech University of Life Sciences, Prague. All literature sources used in this thesis are properly cited according to the requirements of the Faculty of Agrobiology, Food and Natural Resources, Czech University of Life Sciences, Prague. The matter embodied in this dissertation is my research and has not been submitted elsewhere for any degree or diploma. In addition, I certify that all the experiments, data, information, and analyses included in this thesis are authentic and that they were carried out in compliance with academic standards and ethical guidelines. I take full responsibility for the information and conclusions provided in this document.

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TABLE OF CONTENTS

Abstract

Phytophthora cactorum is considered a devastating hemibiotrophic pathogen causing a major loss to strawberry plants worldwide. Inappropriate and continuous use of fungicides leads to resistance among pathogens. Therefore, it was necessary to evaluate the sensitivity of *P. cactorum* to various fungicides. In the current study, the mycelial growth inhibition potential of active compounds of 8 different systemic fungicides i.e., Azoxystrobin, Cymoxanil, Dimethomorph, Fenamidone, Fluopicolide, Fosetyl-AL, Metalaxyl, and Propamocarb were tested against 141 *P. cactorum* strains, isolated from different localities of the Czech Republic. In- Vitro efficiency of active compounds of aforementioned fungicides, dissolved in dimethyl sulfoxide (DMSO), was analysed by their inhibitory indexes and mycelial growth inhibition, displayed at various concentrations. Azoxystrobin, Cymoxanil, Fosetyl-AL, Fenamidone, and Propamocarb have no or very low effect on mycelial growth inhibition as the recorded Efficient concentration (EC_{50}) values were higher than 1000 µg/ml. In the case of Metalaxyl, 17% of isolates displayed EC_{50}>100 µg/ml and were classified as Metalaxyl resistant, 83% displayed EC_{50} ranges from 0.1-2.00 µg/ml and were categorized as Metalaxyl sensitive. Likewise, isolates tested against Dimethomorph were also divided into 2 heterogenous groups, 95.7% of isolates displayed EC_{50} between 0.1-10 µg/ml and were classified as Dimethomorph sensitive, 4.3% of isolates presented EC_{50}>100 µg/ml and were categorized as Dimethomorph resistant. Fluopicolide was effective in mycelial growth inhibition with an EC_{50} range of 0.7-4 µg/ml, and no resistant isolate was found. The effect of Azoxystrobin, Dimethomorph, Fluopicolide, and Metalaxyl were further evaluated on sporangia formation and zoospore release the potential of 30 *P. cactorum* isolates. The efficacy of commercially available 4 fungicides was also evaluated in the greenhouse experiment and 'Acrobat' with Dimethomorph as an active ingredient ranked high based on the plant health index when compared to the other 3 fungicides.

In the current study biocontrol and biostimulant potential of marine, brown microalgae *Tisochrysis lutea* is also explored. Mycelial growth of Metalaxyl resistant isolates was inhibited by crude bio extract from *T. lutea* with EC_{50} ranges from 0.5-0.9 µg/ml. Additionally, In vivo results revealed the biostimulant and biocontrol potential of crude bio extracts. These results will be helpful for the management and monitoring of the resistance of *P. cactorum* to fungicides and a step towards microalgal-based biological control of this pathogen.

Chapter 1: Introduction

1.1 Introduction

Fragaria × ananassa, strawberry, is an important fruit grown both for edible and commercial purposes. Highly perishable, and rich in organic acids, vitamin C, phosphorus, iron, and other minerals with specific antioxidant compounds, it has a unique shape and a pleasant flavour. It is utilized to produce purees, juice concentrate, jams, juices, and rose red wine. The fruit is produced in 73 countries around the globe and the total area of cultivation is approximately 397603 ha with an annual production of nearly 956,9864 tons (FAO. 2022. FAOSTAT). The European Union is a major producer of strawberries, i.e., 47% of the world output, 165,000 ha under strawberry cultivation. However, the production of strawberries in the Czech Republic decreased from 19,658 tons in 1994 to 1830 tons in 2022 making it the 64th country in the world. In the previous two decades, serious financial losses were incurred due to two reasons. First is the low yield of fruit and second is damage due to diseases. Diseases caused by nematodes, arthropods, bacteria, viruses, and various other biological agents constantly jeopardize the commercial potential of the crop. The phytopathogenic fungi that attack strawberry plants are among the pests that are particularly widespread in terms of the number of genera and species that can cause diseases, and therefore in terms of significant financial losses (Garrido et al. 2011). Strawberry plants are susceptible to more than 50 different fungal genera, such as *Botrytis* spp., *Colletotrichum* spp., and *Verticillium* spp. Besides fungal phytopathogens, *P. cactorum,* an oomycete from the family Peronosporaceae, with cosmopolitan distribution (Pánek et al. 2016) causes crown and leather rot in strawberry plants and fruit respectively which caused major economic loss to the agricultural sector in Europe since 1960 (Porras et al. 2007). Leather rot affects strawberry fruit at any stage while crown rot causes reddish-brown discoloration of the crown which attacks vascular tissue and eventually wilting of the plant occurs (Stensvand et al. 1999).

P. cactorum was discovered by Lebert and Cohn in 1870. This persistent oomycete can infect a very large number of hosts i.e., more than 200 plant species and 100 crops cultivated around the world, especially those that are present in lowland or wetland conditions (Eikemo & Stensvand 2015). Numerous commercially significant crops, including pears, apples, and strawberries are affected by this pathogen, causing crown rots, leather rot, root collar, foliar, and fruit infections (Hantula et al. 2000). Rose (1924) reported the first incidence of leather rot in the USA but gradually cases were reported in Europe and Asia while the first

description of crown rot was reported in Germany in (Deutschmann 1954), in 1988 in Sweden (Olsson 1995), in 1990 in Finland (Parrika 1991) and in 1992 in Norway (Stensvand & Semb 2003). Both these diseases, i.e., Leather rot and crown rot of strawberries are reported to cause serious economic losses globally (Nellist et al. 2019). The infection potential of *P. cactorum* isolated from different hosts to cause crown rot in strawberries was analysed by (Van Der Scheer 1971; Harris & Stickels 1981; Hantula et al. 2000) and they concluded that crown rot can only be caused by isolates, isolated from crown rot infected field or directly from strawberry crowns. While all *P. cactorum* isolates from other hosts can cause leather rot in strawberries but not crown rot.

Eikemo et al. (2004) performed AFLP analysis and showed that crown rot is caused by a genetically different pathotype of *P. cactorum* than its counterpart in leather rot. Origin, natural distribution, and resistance to fungicides in *Phytophthora* spp. are difficult to assess because of the global transportation of plants and seeds (Pánek et al. 2016). De Haan (1896) reported *P. nicotianae* in Indonesia for the first time in 1895 and in the USA, it was described in 1915. Man In 'T Veld et al. (2007) mentioned the presence of this pathogen in Europe since 1927. Goss et al. (2011) described *P. rumarorum* migrations between Europe, USA, and Canada but the unclear origin and spread of this pathogen is still subject to investigation. Man In 'T Veld et al. (2007) mentioned the widespread distribution of *P. cactorum* in Europe while (Jung et al. 2016) based on low genetic variability, close phylogenetic relatedness to other non-native species, and high virulence to host plants considered *P. cactorum* species as Alien to the European continent. De Cock & Lévesque (2004) reported another recently introduced species *P. hedraiandra* similar to *P. cactorum* in Europe but its presence in the USA was also reported (Schwingle et al. 2007). Interspecific hybridization phenomenon which provides more genes for mutations consequently increases the pathogenicity of isolates and helps them to survive in a new environment is reported in *P. cactorum* and other *Phytophthora* spp. by various researchers (Bertier et al. 2013; Burgess 2015; Pánek et al. 2016).

Management of crown rot and leather rot of strawberries caused by this pathogen is dependent on cultural practices and chemical control (Ellis et al. 1998). Former includes crop rotation (Divya & Sudini 2013), soil solarization (Patel et al. 2014), avoiding flooded irrigation, proper soil drainage, use of straw mulches (Ellis et al.1998), and selection of resistant cultivars from reliable supplier accounts a lot in reduction of disease incidence rate (Huang et al. 2004). Baggio et al. (2021) mentioned heat treatment of *P. cactorum* zoospores at 44°C for 5 minutes is effective in inhibiting germination and colony formation. They also reported that thermotherapy of inoculated plants has the potential to reduce *Phytophthora* crown rot in

production fields. Cultural practices are effective to some extent but during wet years and epidemics, alternate strategies like chemical control should be used to avoid crop loss. Mefenoxam, Fosetyl-AL (Rebollar-Alviter et al. 2007b), Azoxystrobin, Pyraclostrobin (Rebollar-Alviter & Ellis 2005), and Dimethomorph (Duncan 2002) are the most effective fungicides to control leather rot and crown rot in strawberries. Extensive use of fungicides leads to resistance among *P. cactorum* isolates. Jeffers et al. (2004) found *P. cactorum* isolates resistant to Mefenoxam in the USA for the first time Brown et al. (2004). reported resistance to Fosetyl-AL in other oomycetes. Recently Marin & Peres (2021) reported that fungicides Cymoxanil, Fluopicolide, Mandipropamid, and Oxathiapiproline completely inhibited mycelial growth of *P. cactorum* isolated from Florida at a concentration of 1 μg/ml. Resistance to fungicides varies geographically and depends on the frequency of applications of fungicides, therefore, In the current study, the effect of eight fungicides, with different modes of action, on the various life stages of *P. cactorum* isolates, isolated from the Czech Republic is evaluated. The Efficacy of commercially available fungicides was checked in the greenhouse experiment. Additionally, to keep in mind resistance to fungicides and global environmental concerns biological control and biostimulant potential of marine brown microalga was also evaluated against *P. cactorum.*

Chapter 2: Hypothesis, Aim, and Objectives

2.1 Hypothesis

- *P. cactorum* isolates continuously exposed to fungicides become resistant.
- There should be variation in the pathogenicity of *P. cactorum* isolates, isolated from different hosts and different sites.
- If the efficacy of a particular fungicide is decreasing gradually, then sensitive and resistant isolates must be present at the same time in the population.
- If both sensitive and resistant populations are present at the same time, then there must be a chance of increasing resistant isolates in the population.

2.2 Aim and Objectives

Protection of strawberry plants against *P. cactorum.*

1. Culturing and preservation of *P. cactorum* isolates.
2. Evaluation of fungicide's potential to inhibit *P. cactorum.*
3. Find out resistance and sensitivity patterns among isolates.
4. Analyse commercially available fungicides to inhibit *P. cactorum* infections in strawberry plants in the Greenhouse.
5. Effect of various fungicides on different life stages of *P. cactorum.*
6. Genetic differences among resistant and sensitive isolates.
7. Biological control of *P. cactorum.*

Chapter 3: Review of Literature

3.1 Strawberry – The Host

Strawberry is a member of the family Rosaceae just like other agriculturally important plants from the same family such as apple (*Malus domestica*), almond (*Prunus dulcis*), peach (*Prunus persica*), plum (*Prunus domestica*), raspberry (*Rubus idaeus*), sweet cherry (*Prunus avium*) and roses (*Rosa* spp.) (Longhi et al. 2014). The strawberry genus, *Fragaria*, comprises more than 20 species which are distributed worldwide mainly in the northern hemisphere (Liston et al. 2014). Variation in the ploidy level of *Fragaria* is also observed which ranges from diploids to decaploids. Woodland strawberry (*Fragaria vesca*) is the most widely distributed species which has a diploid set of chromosomes (2n=2x=14), while domesticated garden strawberry (*Fragaria ananassa*) has octoploid (2n=8x=56) i.e., set of eight chromosomes.

When two octoploid Fragaria species i.e., *Fragaria virginiana* from North America and *Fragaria chiloensis* brought from Chile were inadvertently mixed in the 1700s in France, which resulted in the production of the first garden strawberry (Cai et al. 2017). Strawberry breeding operations began in England in the 1800s and then spread to France and the USA. As a consequence, superior hybrids with the firmness, flavour, and colour of *F. virginiana* and the enormous fruit size of *F. chiloensis* were produced. The superior attributes of the *F. ananassa* variants led to their ultimate dominance over the other *Fragaria* species, making them the most commonly cultivated strawberry in fields and gardens. There are hundreds of *F. ananassa* cultivars available now for various purposes and climates, and there are dozens of breeding operations going on worldwide resulting in the production of about 40 to 50 varieties each year (Faedi & Baruzzi 2016). Strawberry (*Fragaria ananassa* Dutch.) is an important horticultural crop with an annual yield of over 8 million tons (FAO. 2022. FAOSTAT). Strawberry is cultivated in every country from temperate to subtropical regions with a worldwide cultivated area of approximately 400 000 hectares. Highly perishable, rich in organic acids, vitamin C, phosphorus, iron, and other minerals, and with specific antioxidant compounds, it has a unique shape and a pleasant flavour. It is utilized for the production of purees, juice concentrate, juice, jams, preserves, and rose-red wine. According to statistical data provided by the Food and Agriculture Organization of the United Nations in 2022 biggest producer of strawberries is China followed by the USA and Turkey. In Europe,

Spain is the largest producer. More details about the top 20 strawberry producer countries, top 20 strawberry importers, and top 20 strawberry exporters are given in Supplementary Table 1, Supplementary Table 2, and Supplementary Table 3 respectively (Supplementary data). According to official data provided by the Czech Ministry of Agriculture to FAO, production of strawberries in the Czech Republic decreased from 12547 tons in 2000 to 1830 tons in 2022 (Figure 1). According to the Atlas Big 2022, Australian database, the Czech Republic is ranked 64th in strawberry production with a total strawberry cultivation area of 440 hectares with 4814 kg/hectare production. A Continuous increase in the quantity of imported strawberries has been observed since 2000. Year-wise detail of the production and imported quantity of strawberries is given in Figure 1 (Data provided by the Czech Ministry of Agriculture).
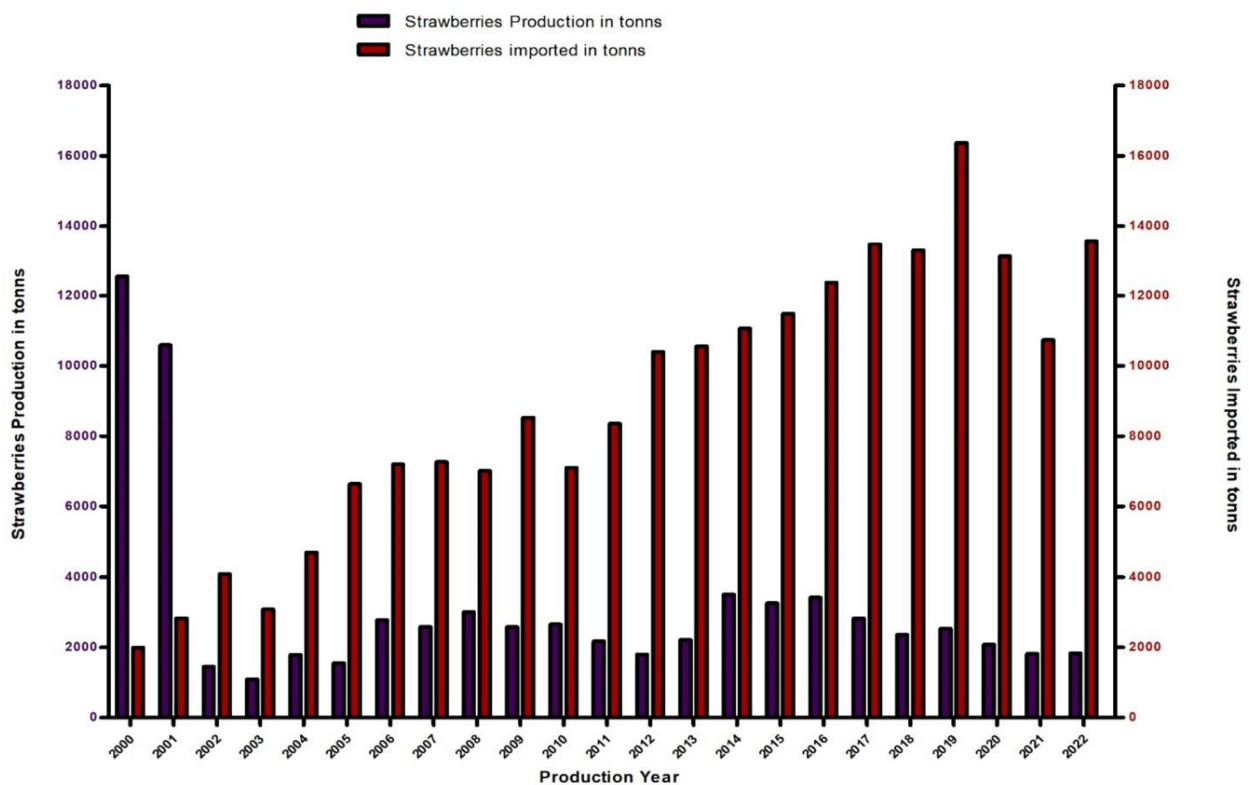
Figure 1: Production and import of Strawberries in the Czech Republic, Data provided by the Czech Ministry of Agriculture to FAO

3.2 *Phytophthora cactorum*: The Pathogen Causing Diseases

In 1870, Lebert and Cohn first discovered *P. cactorum* on cactus. This persistent oomycete can infect a very large number of hosts i.e., more than 200 plant species and 100 crops

cultivated around the world, especially those that are present in lowland or wetland conditions (Eikemo & Stensvand 2015). It has the potential to decrease the production of many economically important crops such as apples, pears, and strawberries, and it causes several infections such as crown rots, leather rot, root collar, foliar, and fruit infections (Hantula et al. 2000). Among *Phytophthora* species, *P. cactorum* causes two different diseases in strawberries i.e., *Phytophthora* crown rot and *Phytophthora* leather rot (Garrido et al. 2011).

3.2.1 *Phytophthora* Crown Rot

The first incidence of crown rot was reported in Germany in 1954 (Deutschmann 1954). Mostly found in poorly drained and irrigated soil, disease incidence rises when the weather is hot and rainy for prolonged periods. On some soils, the disease can stay for several years after being introduced. Crown rot is quite common in frigo plants or plants that have been kept in cold storage. The earliest signs of disease are plant growth retardation or withering of young leaves, which can be found at any time throughout the season. Plants that have been infected may stay stunted, or their leaves may become blue, and the entire plant may quickly wither until it collapses completely (Duncan 2002). A longitudinal cut of the crown displays water soaked and light brown tissue when it is first infected, but as the infection advances, significant necrosis emerges that is evenly brown and not limited to the vascular tissue. The crown apex, base, and centre become reddish brown. The colour of the roots attached to the infected crown also changes into black. Fungal oospores can easily be isolated from infected roots (Peres & Baggio 2019).

3.2.2 *Phytophthora* Leather Rot

The first disease incidence of leather rot was reported in the United States in 1924 (Rose 1924) but gradually cases were reported in Europe and Asia. Heavy rains are linked to major outbreaks of leather rot, but the disease can also spread during the winter season with numerous fog and dew. The most distinguishing feature of this infection is the fruit's foul odour and flavour. Leather rot can cause severe economic loss in strawberry production, with reported damage as high as 50% in severe cases (Eikemo & Stensvand 2015). *P. cactorum* can infect plants at any stage of plant development and cause disease. Symptoms of dark brown or green with brown edges appear in infected immature or green fruits. Infection can develop on ripe, mature fruit without changing colour or bleached, and vary in tint from pale lavender to purple. The entire fruit becomes dark as the infection progresses, and its rough

texture gives it a leathery appearance (Louws & Ridge 2014). When there is a lot of moisture in the air, fine white fungal growth emerges on the fruit's surface. During the final stage of infection, green and mature fruits dry up completely and are eventually mummified. *P. cactorum* lives as oospores on mummified fruit from season to season. The oospores can survive longer in the soil and can be activated when favourable conditions arise (Paulus 1990). The effect of crown rot and leather rot caused by *P. cactorum* can be visualized in Figure 2.
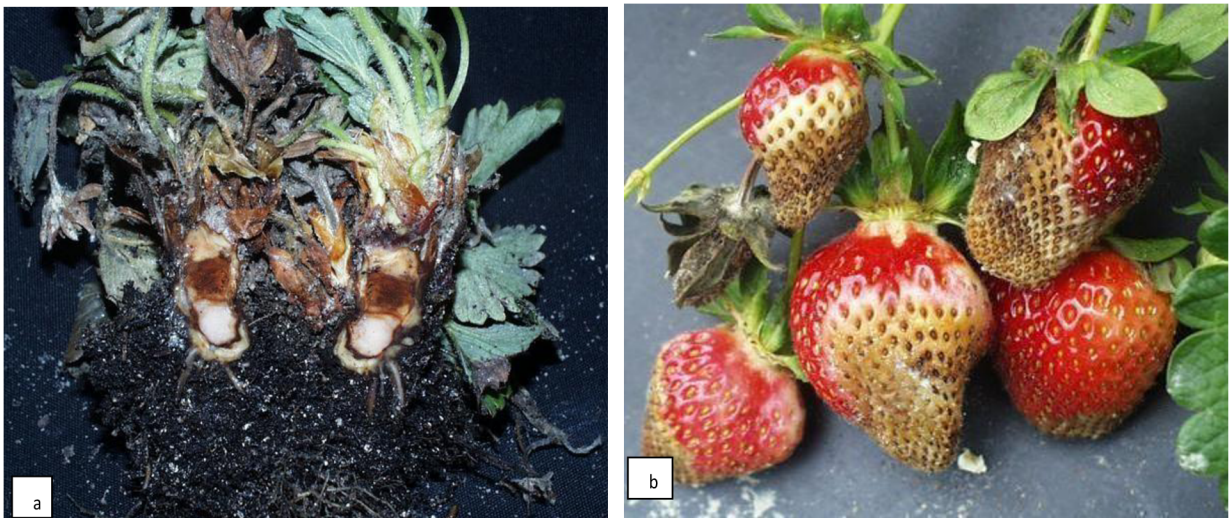
Figure 2: a) Crown rot of strawberry plant, b) Leather rot of strawberry (Adapted: Louws & Ridge 2014).

3.3 Taxonomy of *P. cactorum*

Oomycetes were originally classified in the kingdom Mycota because their lifestyle and filamentous growth are very similar to fungi. However, there are fundamental differences between these two groups. For instance, the cell wall of oomycetes is composed of cellulose and glucan whereas chitin is the main constituent of the fungal cell wall (Thines 2014). Within the kingdom Chromista, oomycetes belong to the lineage of biflagellate "heterokont" also known as "stramenopiles"(Adl et al. 2005). They form the Chromalveolate "super-kingdom" with its alveolate cousins, which comprise the apicocomplexa, dinoflagellates, and ciliates, which were first classified by (Beakes et al. 2012). Rhizaria (amoeboid Foramifera and Radiolaria) may be included in the "super kingdom," constituting the "RAS supergroup" also referred to as "SAR" (Grattepanche et al. 2018).

Peronosporales, Pythiales, Albuginales, and Saprolegniales are the four main orders in the class of oomycetes. The genus *Phytophthora* belongs to the order of Peronosporales along with downy mildews. Various important plant pathogens are included in this genus. Most of them are parasites, for example, Downy mildew and Albuginales are biotrophic parasites (obtain nutrients from living plant cells) whereas Pythium species are necrotic parasites (obtain food by killing plant cells). To date, more than 150 *Phytophthora* species have been described, and their numbers are increasing (McGowan & Fitzpatrick 2020). It is speculated that the total number of *Phytophthora* species may be close to 600. These species can cause huge damage to agriculture and natural ecosystems (Kamoun et al. 2015). *Phytophthora* species is divided into ten major clades based on multiple phylogenetic markers and *P. cactorum*, together with *P. hedraiandra, P. idaei, and P. pseudotsugae,* was classified as a member of clade I, specifically subclade Ia (Blair et al. 2008; Martin et al. 2014). Table 1 shows the classification of *P. cactorum* up to the species level.

Table 1: Taxonomy of *P. cactorum* (H Ho 2018)

Clade	SAR
Phylum	Oomycota
Order	Peronosporales
family	Peronosporaceae
Genus	*Phytophthora*
Species	*P. cactorum*

3.4 Morphology and Life Cycle of *P. cactorum*

P. cactorum is a soilborne pathogen found throughout the world. However, it is most prevalent in temperate areas with a wide host range, infecting over 200 plant species (Hantula et al. 2000). *Phytophthora* species are hemibiotrophic, meaning they go through two stages of the food cycle: biotrophic feeding on living host cells and necrotrophic feeding on dead plants. *P. cactorum* mycelia's vegetative hyphae are aseptate (coenocytic) and, similar to other *Phytophthora* species, may form various propagules such as zoospores, sporangia, chlamydospores, and oospores, which aid in their distribution and germination (Sophien 2003). Sporangia are also known as zoosporangia and are asexual structures filled with tiny numerous zoospores. The sporangia are prematurely deciduous, papillary, and have an elliptical or ovoid shape and short pedicels. Low temperature and high moisture content

promote the differentiation of sporangia's multinucleate cytoplasmic content, which then divides into mononucleate biflagellate zoospores through cytoplasmic cleavage (Bush et al. 2006). Zoospores can swim in water films with their flagella and may be attracted by chemical secretions from the roots. Disease caused by *Phytophthora* usually worsens in plants when there is inadequate drainage or waterlogging since the zoospores require water to survive. Other types of spores that are produced by certain *P. cactorum* and some other *Phytophthora* species are chlamydospores and oospores. Chlamydospores are asexual propagules that may persist for lengthy periods in soil and plant tissues. Sexual spores are oospores, they can also survive in harsh conditions in soil or an infected plant for a longer period until environmental circumstances are favourable for germination (Martin et al. 2012). In the sexual reproduction cycle, a male antheridium fertilizes a female oogonium, resulting in the formation of a thick-walled oospore. In contrast to amphigynous antheridia that encircle the oogonial stalk, antheridia in *P. cactorum* are paragynous, meaning they are connected to the side of the oogonium. In contrast to other heterothallic *Phytophthora* species (e.g. *P. infestans*) reproduction is homothallic, meaning it can occur without the interaction of two separate thalli or mating types (H Ho 2018).
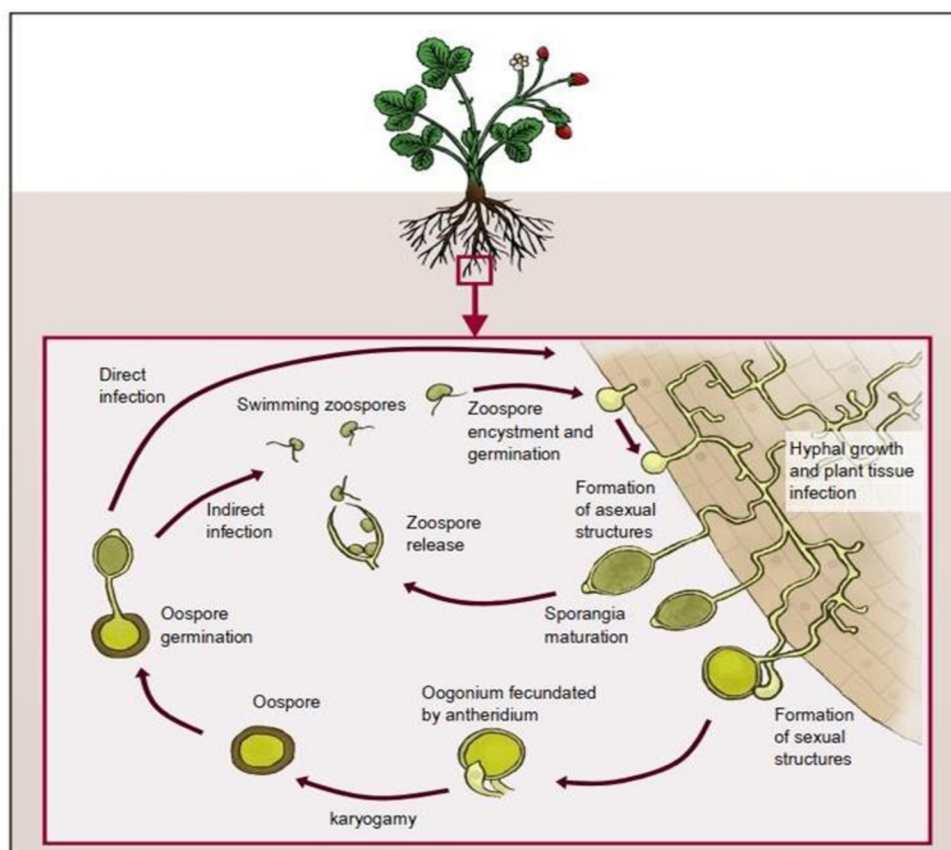
Figure 3: Life Cycle of *P. cactorum* in strawberry. Adapted from (Alcals 2008).

3.5 Infection Cycle of *Phytophthora* Species

The infection process of *Phytophthora* species begins when there is enough water present either in the soil or on the leaf surface, it reaches to host plant by swimming utilizing their biflagellate zoospores and where they attach to the plant host using special adhesive proteins (Ah-Fong et al. 2017). Zoospores use chemotactic and electrotactic approaches to identify the specific host as these zoospores detect some specific substances released by the host like amino acids, sugars, and ions. For example, *P. sojae* detects its host by identifying isoflavone compounds (Hardham & Blackman 2010). Several other factors also help zoospores to identify their host's secretions like guanidine-binding (G) protein subunits, alpha (Gα), beta (Gβ), and gamma (Gγ) (van den Hoogen et al. 2018).

Following effective zoospore movement, *Phytophthora*'s ability to adhere and establish as biotrophs within host tissues is an important aspect of its successful infection. Before encystment, these pathogens inflect their ventral grooves in the direction of the host, releasing thrombospondin protein towards the host plant surface (Robold & Hardham 2005). Using these proteins, pathogen cells are firmly attached to the external site of the potential host, leading to cell entry, and further promoting the development of disease structure, which is a necessary condition for penetration. To protect germinating cysts from desiccation, oomycete releases different proteins, the majority of which are cellulose-binding elicitor lectin (CBEL), mucin and jacalin-like proteins, and acidic cell wall proteins (Gaulin et al. 2002; Larousse et al. 2014).

After the pathogen successfully attaches to the host, penetration into the plant apoplast is essential for effective infection (Doehlemann & Hemetsberger 2013). Following the pathogen invasion of the host cell, the plant cells defend themselves by releasing catalytic proteases that block the pathogen from invading the cell further (van der Hoorn & Kamoun 2008). To overcome the attack of the host, the pathogen also releases enzyme inhibitors. Cell wall-degrading enzymes (CWDEs), elicitins, and enzyme inhibitors are among the apoplastic effectors secreted by *Phytophthora* species (Krishnan et al. 2019). To reduce the host cell wall barrier, CWDEs target hemicellulose, 1,3-glucan, cellulose, glycoprotein, and pectin which helps the pathogen to enter and invade the host successfully (Judelson & Ah-Fong 2019). Glyceraldehyde hydrolases, pectin lyase, carbohydrate esterase, glycosyltransferases, and carbohydrate-binding molecules are some of the most described CWDEs in *Phytophthora* species (Hardham & Blackman 2018). Because CWDEs target the host cell wall, the plant's first line of defence, known as pattern-triggered immunity (PTI), recognizes them as microbe-

associated molecular patterns (MAMPs), which are recognized by the host plant recognition receptors (PRRs). The pathogen in response secretes apoplastic effectors, primarily protease inhibitors, to thwart host defences. Such as, to block *Phytophthora* Inhibited Protease 1 (PIP1) defence responses in tomato plants, *P. infestans* secretes cystatin-like cysteine protease inhibitors EPIC1-EPIC4 and EPIC2B (Tian et al. 2007). *Phytophthora* species produce another structurally conserved protein called elicitins while still in the apoplastic region. During pathogen-host interaction, elicitin overexpressed gets recognized by host PRRs and produces a hypersensitive reaction (HR) that leads to the death of host cells (Adachi et al. 2015).

CRNs and RXLRs are cytoplasmic effectors that fight on the same battlefield but use different strategies to affect host immunity. They are transported inside the host cell and guided to various subcellular compartments (Whisson et al. 2007). PTI is promoted by CRNs and suppressed by RXLRs. CRNs are involved in the necrotrophic stage of oomycete hemibiotrophy, whereas RXLRs are involved in the biotrophic step. The pathogen enters the cell-killing stage after completing the biotrophic phase, where it produces CRNs before exiting the dead host to infect fresh plants via sporulation (Stam et al. 2013). However, because some *Phytophthora* species can remain on perennial hosts for a long period, much like a chronic disease, it's important to note that not every *Phytophthora* species destroy their hosts when they sporulate (Wang et al. 2020).
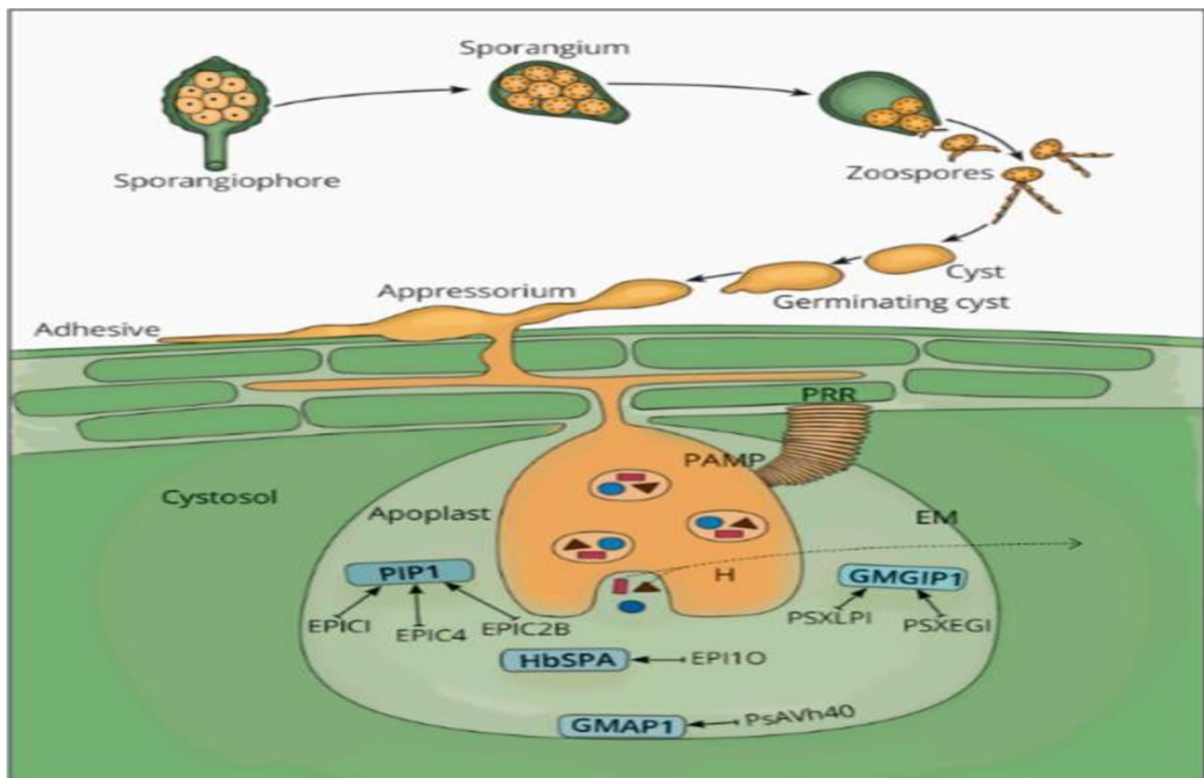
Figure 4: Infection process model of *Phytophthora* species, showing release, attachment, and penetration of zoospores in host cells, Adapted from (Chepsergon et al. 2020).

3.6 Control Strategies

3.6.1 Early Disease Diagnosis

Early disease diagnosis and pathogen identification are the key to protecting crops from diseases. After observing the aforementioned symptoms of crown rot or leather rot in the field, basic microbiological techniques are used to identify the pathogen. Culturing the pathogen on a PARPH medium and observing the mycelial growth pattern is the initial step followed by colony morphology, sporangia structure, oospore presence, and zoospore production have to be observed. Molecular methods are the most reliable approach to identifying a *Phytophthora* isolate. It is possible to use PCR to amplify, purify, and sequence the ITS 1 and 2 regions of rDNA and the cox 1 and 2 regions of mitochondrial DNA. Following that, sequences can be compared to those of confirmed *Phytophthora* species listed

in databases such as GenBank and Phytophthora ID (Jeffers & Aldwinckle 1987).

3.6.2 Cultural Practices

Control of leather and crown rots is based on the combination of cultural practices (Rebollar-Alviter et al. 2007a). Cultural practices include crop rotation (Divya & Sudini 2013), soil solarization (Patel et al. 2014), avoiding flooded irrigation, proper soil drainage, use of straw mulches (Ellis et al. 1998) and selection of resistant cultivars from reliable supplier accounts significantly in reduction of disease incidence rate (Huang et al. 2004). Use of healthy plants, although symptoms may not be apparent at the time of field setting. If tips were infected, *Phytophthora* can show up in the plug plants and these plants should be culled or treated before field setting. Baggio et al. (2021) mentioned heat treatment of *P. cactorum* zoospores at 44°C for 5 minutes is effective in inhibiting germination and colony formation. They also reported that thermotherapy of inoculated plants has the potential to reduce *Phytophthora* crown rot in production fields. Selection of a proper healthy site with adequate soil drainage and avoiding fields with a history of disease can reduce disease incidence rate. The Use of raised beds for plants can reduce fruit damage (Claire et al. 2018).

3.6.3 Chemical Control

Mefenoxam, a phenylamide fungicide, is widely used due to its great efficacy, systemic activity, and oomycete specificity. This phenyl amide fungicide affects ribosomal RNA synthesis and consequently reduces pathogen growth and inhibits zoospore germination (Fisher & Hayes 1982). As Mefenoxam is site site-specific fungicide it is classified as at high risk of resistance. Fosetyl-AL and several other phosphite fungicides are registered against leather rot in many countries and these fungicides provide efficient results in disease reduction (Rebollar-Alviter & Ellis 2005). Moreover, they also reported that strobilurin fungicides provide the best control of leather rot similar to Mefenoxam. Another registered fungicide is Fosetyl-AL. Fosetyl-Al can be applied to foliage with ease and effectiveness due to its comparatively low toxicity and basipetal movement in plants. There is also no solid evidence of resistance development in *Phytophthora*, as described by (Duncan 2002). Since the 1950s, soil fumigation with methyl bromide has been the recommended technique to treat soilborne diseases; however, due to the possibility of ozone depletion, its usage was prohibited after 2005. Dichloropropene, Chloropicrin, Metolachlor, Trifloxysulfuron, Metam potassium, Iodomethane, Dimethyl disulfide, 1,3-Dichloropropene, Fluensulfone, Fluopyram, or Fosthiazate are some of the substitutes for methyl bromide that are presently being

researched. Another prospective substitute for Methyl bromide is Ethanedinitrile (EDN), a broad spectrum fumigant with nematicidal, fungicidal, insecticidal, and herbicidal properties (Douda et al. 2021). In the Czech Republic registered fungicides against oomycetes in strawberries are Aliette 80 WG, Delan 700 WDG, Flowbrix, Previcur Energy, and VitiSan with active compound Fosetyl-AL, Dithianon, Cupric oxychloride, Fosetyl+Propamocarb, and Potassium bicarbonate respectively. According to the Czech Ministry of Agriculture, the chronological validity of the current licenses of the aforementioned fungicides are March 2026, August 2025, December 2025, March 2026, and October 2037. These timelines emphasize how crucial it is for farming operations to switch to alternative products. Certain fungicides may be subject to limitations or prohibitions as laws change to prioritize the environment and public health because of worries about their safety or potential effects on the environment. In response, alternative products and methods that provide efficient disease management while limiting negative consequences are becoming more and more popular among farmers and other agricultural stakeholders. Biopesticides, plant extracts, cultural methods, and integrated pest management (IPM) techniques are a few examples of these substitutes that offer environmentally responsible and long-lasting options for crop protection. Adopting substitute products guarantees regulatory compliance while enhancing agricultural system resilience, decreasing reliance on pesticides, and promoting long-term sustainability for farmers and the environment.

3.6.3.1 Fungicide Resistance

Chemical control of strawberry diseases has become a challenge due to the selection of fungicide resistance for different fungicide classes (Forcelini et al. 2016). Marin & Peres (2021) recently reported increasing Mefenoxam resistance in *P. cactorum* isolates. They reported that in Florida, USA from 1997 to 2015, *P. cactorum* isolates were sensitive to Metalaxyl with $EC_{50}<1.0$ µg/ml but a gradual annual increase in resistance i.e., 9, 10, 21, and 23% was observed from 2015 to 2019 with $EC_{50}>100$ µg/ml. In the same study, they didn't find any resistance among *P. nicotianae* isolates. They also checked the mycelial growth inhibition potential of Azoxystrobin against *P. cactorum* isolates, collected from 1997 to 2020 from strawberry fields in Florida and they divided isolates into two groups i.e. Azoxystrobin sensitive isolates with EC_{50} value less than 0.1 µg/ml and Azoxystrobin resistant isolates with EC_{50} greater than 50 µg/ml. All the resistant isolates were those, collected after 2010 which proves a gradual increase in resistance. Another study conducted by Jeffers et al. (2004b) shows that all 6 isolates from strawberry fields where Metalaxyl is continuously used are

highly resistant to Metalaxyl compared to *P. cactorum* isolates from three other locations. Sensitivity or resistance to Metalaxyl or any other phenylamide fungicide varies on intra as well as inter-specific levels, even isolates originating from one population (Taylor et al. 2002). First time resistance to Cymoxanil was reported in Italy in 1997, followed by Germany in 2000 and France in 2002. As the mode of action of this fungicide is protective and curative, the most important factor for this resistance is probably the extensive use of this chemical as a curative spray (Gullino et al. 1997) and probably the low frequency of application of Cymoxanil in the USA is the main reason of low resistance in *P. cactorum* isolates. Continuous use of fungicides leads to resistance among isolates, therefore alternate strategy strategies which can reduce the burden of fungicide resistance on pathogens population and also eco-friendly and less toxic like biocontrol are considered as better options to cope with phytopathogens (Syed Ab Rahman et al. 2018).

3.7 Biological Control

3.7.1. BCAs

Continuous use of chemical fungicides affects the environment, contaminates the water table, and poses serious health problems in animals and humans. To avoid these serious repercussions biological control is an effective, user-friendly, and green strategy to control plant diseases. The term 'Biological Control' was first proposed by Smith in 1919. Biological control is defined as the use of indigenous microorganisms or their products to inhibit the activities and population of disease-causing plant pathogens (Nega 2014). It may also be defined as the use of microbial antagonists to control the diseases causing pathogens in a cropping system. The Microorganism that inhibits or represses the growth of pests or pathogens is termed as the biological control agent (BCA) (Roh et al. 2007).

BCAs commonly used for phytopathogens are viruses, bacteria, and fungal strains. They are usually isolated from different soil zones such as phyllosphere, endosphere, or rhizosphere. The Most commonly used fungal BCAs against various pathogens of the genus *Phytophthora* are the *Trichoderma* and *Aureobasidium pullulans* (Zohaib Anjum 2019; Iqbal et al. 2021). Different species of *Bacillus* such as *B. amylolyquefaciens*, *B. subtilis, B. megaterium, B. licheniformis, B. cereus, B. velezensis* (Oh et al. 2011; Moradi et al. 2018) and *Serratia ficaria, Enterobacter cloacae* (Okamoto et al. 2000), *Pseudomonas fluorescens* and *P. syringae* (Anandhakumar & Zeller 2008) are most common bacterial BCAs used against *Phytophthora* species. Arbuscular mycorrhizal fungi such as *Claroideoglomus* and *Glomus*

have been successfully tested with *P. cactorum* (Hautsalo et al. 2016).

3.7.2 Mode of Action of BCAs

BCAs prevent pathogens from infecting the host plant by using several direct and indirect mechanisms. Direct mechanisms include the production of antimicrobial metabolites such as *Trichoderma virens*, *B. amyloliquefaciens*, *B. subtilis, Pseudomonas putida, B. megaterium,* etc. *B. subtilis* can produce more than two dozen different antimicrobial compounds (Stein 2005). Secretion of extracellular hydrolytic enzymes such as chitinase, cellulases, endoglucanase, and amylase which hydrolyse chitin, cellulose, hemicellulose, proteins, and DNA is another mechanism used by BCAs. These enzymes help the BCAs to control plant diseases by hydrolysing their polymeric compound. Studies have shown that the *B. subtilis* J9 strain releases extracellular protease and chitinase against *B. cinerea* to protect strawberry plants from grey mold (Badiaa et al. 2012). Quorum quenching, also known as quorum sensing inhibition, is a recently described mechanism to control Phytopathogens. In this mechanism, bacterial virulence is weakened by the inactivation of quorum-sensing molecules. Quorum sensing is the crosstalk mechanism of microorganisms which is helpful in the adaptation of specific strategies. Pathogenic bacteria also use this mechanism to regulate the expression of virulence genes and quorum sensing is performed by numerous small molecules known as quorum sensing molecules which are very diverse in structure and morphological appearance. N-acyl-L-homoserine lactones (AHLs) are the most common signalling molecules among pathogenic bacteria (Sieper et al. 2014). Some *Bacillus* species can produce AHL lactonase which catalyses the hydrolysis of the AHL ring like *B. thuringiensis* suppresses plant pathogen *Erwinia carotovora* which is dependent on quorum sensing molecules for proliferation (Dong et al. 2004). Competition for nutrients such as oxygen, carbon, and other nutrients and space is another strategy used by BCAs. Iron present in ferric form is extremely low in the rhizosphere up to 10-18 M in concentration. Microorganisms needed iron for their growth, for this purpose they released iron binding ligands known as siderophores. Different siderophores or iron chelating compounds are produced by bacteria such as Azotobacter and Pseudomonas. These bacteria quench iron with the help of siderophores thus making it unavailable for pathogen and inhibiting their growth. Recent studies have shown that the rhizosphere microbial community structure is greatly influenced by iron (Kramer et al. 2020). In indirect mechanisms, BCAs act on plants. They trigger the plant immune system which helps plants to develop systemic acquired resistance (SAR) or induced systemic resistance (ISR). For example, the *B. mycoides* strain induces ISR in sugar

beet (Bargabus et al. 2004). Plant hormones production such as indole acetic acid (IAA), auxin, and gibberellic acid is another indirect mechanism employed by BCAs (Duca et al. 2014).
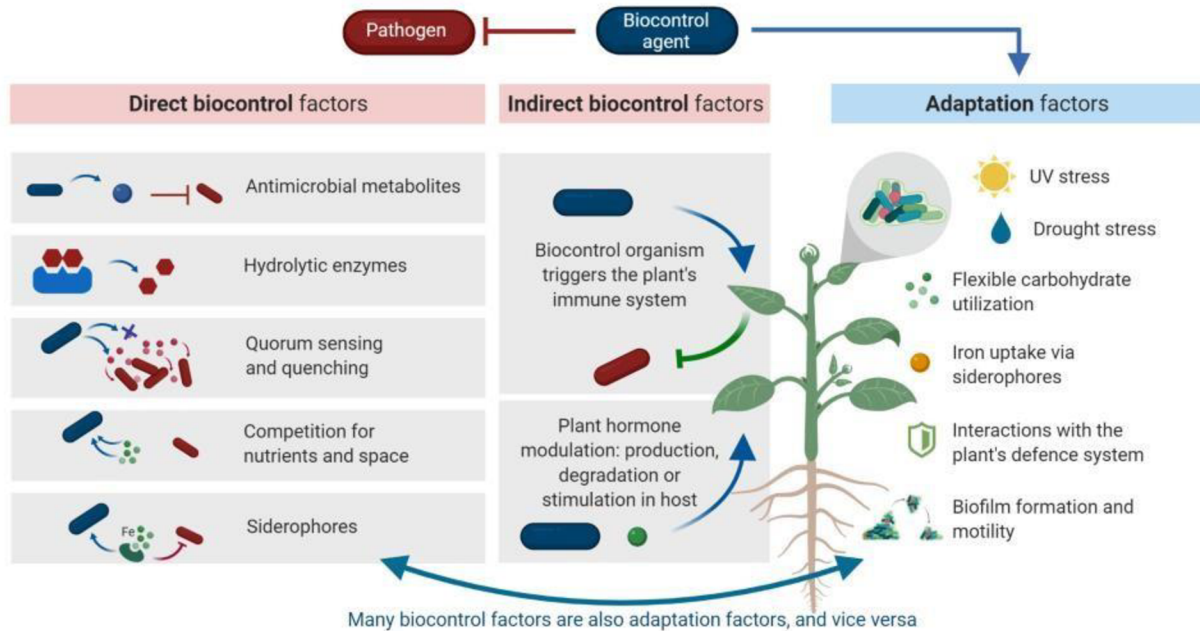
Figure 5: Direct and indirect mechanisms of biocontrol agent Adapted from (Legein et al. 2020).

3.7.3 Microalgal Based Biocontrol of Phytopathogens

The sustainability of agriculture is threatened by chemical biocides, which has led to a quest for sustainable alternatives for disease control. Using biological alternatives, like fungus and bacteria, is advantageous for the environment and efficient. Because of their increased disease resistance, less need for toxic pesticides, higher nutritional value, and increased agricultural yields, cyanobacteria, mainly green microalgae, have been recognized as possible biocontrol agents. On the other hand, there is no published research on the application of brown microalgae in plant disease biocontrol (Poveda & Díez-Méndez 2023).

3.8 Disease Suppression and Pathogen Control

Biocidal metabolites, such as hydrolytic enzymes and antibacterial chemicals, are produced by cyanobacteria and can inhibit or kill pathogenic bacteria, fungi, or nematodes. By causing structural and functional alterations, these substances disrupt the cytoplasmic membrane, deactivate enzymes, and prevent the production of new proteins. Algal extracts have antibacterial and bioactive components that can help control pathogens and prevent soil-borne

illnesses. Applying cyanobacterial/microalgae cultures that are growing in the form of fresh or dried biomass can accomplish this (Poveda & Díez-Méndez 2023). Numerous substances originating from cyanobacteria have been linked to biocontrol, pesticidal, and insecticidal effects, enhancing plant resistance and stimulating the production of defense enzymes in plants. Anabaena and Oscillatoria cyanobacteria have demonstrated antibiotic efficacy against a range of pathogens, such as *Bacillus subtilis*, *Staphylococcus aureus*, and *Micrococcus flavus*. Additionally, these strains have antifungal efficacy against *Rhizopus stolonifera, Botrytis cinerea,* and *Alteraria* alternative. Anabaena species are cyanobacterial strains with dual functions in biocontrol and biostimulant qualities. For example, *Anabaena variabilis* formulations were shown to be herbicide-tolerant, to boost crop production and soil fertility in rice fields, to decrease the severity of illness, and to increase plant development in tomato seedlings. Cyanotoxins, which are produced by cyanobacteria, can be harmful to both people and animals. Cyanotoxins may build up in plants, even in food crops grown for human consumption. It is essential to critically evaluate the impacts of cyanobacteria employed as biocontrol on various animal systems. Further research is required to fully understand the indirect effects of antibiotics on macro and microfauna and flora, even though studies have shown that they are effective against some types of bacteria and cyanobacteria (Rojas et al. 2020; Carpine & Sieber 2021). It has been discovered that cyanobacteria, in particular *Microcoleus vaginatus*, reduce the numbers of plant pests like nematodes by producing peptide poisons and nematicidal chemicals. Research has demonstrated that nematode populations and root galling can be decreased by inoculating tomato seedling roots with *M. vaginatus* culture filtrates. Meloidogyne incognita is likewise susceptible to the nematicidal action of Oscillatoria chlorina powder. Plant growth can be improved by cyanobacteria injection before seedling inoculation (Singh et al. 2011). Additionally, research has demonstrated that *Aulosira fertilissima* culture filtrates suppress the root-knot nematode *Meloidogyne triticoryzae*. Compared to artificial N fertilizer (urea), cyanobacterial biofertilizer has been shown to reduce mosquito populations in rice fields. Grain output was raised using blue-green algae fertilizer, while mosquito populations remained the same. Against a variety of plant diseases and pests, Cyanobacterium Nostoc strain ATCC 53789 has shown antifungal, insecticidal, nematicidal, cytotoxic, and herbicidal activities. As a result, it's important to identify plant diseases and pests in various crops and design effective tactics for microalgal and microorganism biocontrol. The use of cyanobacterial treatments can successfully integrate with standard agronomic procedures while preventing toxicity to non-target species. To minimize toxicity to non-target organisms and increase crop output, it is

essential to identify and develop effective microalgal/microorganism biocontrol techniques (Asimakis et al. 2022).

Chapter 4: Material and Methods

4.1 Sampling and Receiving of Isolates

P. cactorum strains, were received from Crop research institute, Ruzyne, Czech Republic. These strains were isolated from woods and different commercial strawberry fields in the Czech Republic by leaf baiting technique. In this method, 10 g of soil sample was poured into a sterile transparent plastic box and then 100 ml of distilled water was added. After gentle stirring 3 strawberry leaves were dipped into it and the box was covered with a lid on a bench top at room temperature. After every 24 hours lesions on the lower side of the leaves were observed. Proper lesions appeared after 72 hours. Leaves were then washed with distilled water and lesions were cut and placed on PARPH (Pimaricin, 10 mg; Ampicillin, 100 mg; Rifampicin, 10 mg; Pentachloronitrobenzene, 50 mg; Hymexazol, 50 mg; Difco corn meal agar, 15 g; and distilled water, 1 liter), a *Phytophthora* selective medium as described by (Oudemans 1999). Petri dishes were incubated at 21°C for 72 to 96 h. After incubation time, the growth of the pathogen when observed around the lesions, and isolates were re-cultured to obtain pure pathogenic strains. The isolates were purified and recultured on V8 agar medium and stored at 4°C. Refreshing of cultures was done after three months with the same medium. Antibiotics used in the PARPH medium were obtained from Sigma Aldrich, Germany.

4.2 *P. cactorum* Isolates Used

The following 141 *P. cactorum* isolates mentioned in

Table 2 were received from the Crop Research Institute, Ruzyně, Czech Republic. The majority of the isolates were isolated from strawberry fields while isolates from serial numbers 40 to 49 were isolated from wood of trees.

Table 2: *P. cactorum* isolates used in this study, isolates with mark (●) are used in sporangia formation and zoospore release assay

S.no	Isolate ID	S.no	Isolate ID	S.no	Isolate ID	S.no	Isolate ID	S.no	Isolate ID	S.no	Isolate ID
1	415F	30	272/09	59	17-15-8●	88	17-30-3	117	17-09-14a●
2	416	31	277/09	60	17-7-27a	89	17-12-12	118	17-09-12●
3	417	32	282/09	61	17-30-9	90	18-10-17a	119	18-07-14●
4	421	33	426/10	62	17-12-18b	91	17-23-3a	120	18-02-3
5	440	34	434/11	63	17_8_17b●	92	17-24-20	121	17-45-1a
6	451	35	503/11	64	18-10-18c	93	17-24-26	122	17-37-11
7	1383	36	549/11	65	17-53-3	94	17-15-10	123	17-12-3●
8	17_11_17	37	634/13	66	18-37-7c	95	17-23-19	124	17-11-16
9	17_11_19	38	66/07	67	18-07-2-s12	96	17-12-31	125	18-10-16
10	17_11_3	39	CBS	68	17-57-F1	97	17-24-12	126	17-12-8
11	17_12_1b	40	CBS111725●	69	17-30-6	98	17-3-23a	127	17-12-18
12	17_12_5a	41	ICMP11853●	70	18-10-12	99	18-10-11	128	17-12-18a●
13	17_12_6a	42	M5620●	71	17-30-12b	100	17-37-13	129	17-12-20
14	17_12_6b	43	M5624●	72	18-10-4a	101	18-07-2-s1●	130	17-12-16●
15	17_12_7	44	M5652●	73	17-24-8a	102	18-12-1b	131	17-12-17a
16	17_3_12	45	M5654●	74	17-24-4	103	18-07-2-S5●	132	17-30-8
17	17_3_24	46	PD20017401	75	18-33-3	104	17-37-13	133	17-12-28
18	17_4_10	47	Ph4●	76	17-12-17b	105	18-10-11	134	17-12-30
19	17_4_1a	48	Ph8●	77	17-24-4b	106	17-3-23a●	135	17-12-27a
20	17_4_2	49	PS-719●	78	17_03_5●	107	17-24-12	136	17_12_27●
21	17_4_3	50	17-24-4a	79	18-07-12a	108	17-3-10	137	17-15-10b●
22	17_4_5	51	18-07-2S12●	80	17-12-9	109	17-37-7a	138	17-23-8
23	17_4_7	52	17-34-7	81	17-30-18	110	17-15-1●	139	17-23-9
24	17_4_7b	53	17-37-15	82	18-02-1b●	111	17-37-7c	140	17-24-3a
25	17_4_9	54	17-26-14●	83	17-30-13	112	17-23-1d	141	17-12-4
26	17_7_25	55	17-24-5c	84	18-07-6	113	17-7-12a●	-	-
27	17_8_10	56	17-44-12	85	17-12-24	114	17-12-23		
28	17_8_6	57	17-45-1b	86	17-12-6c	115	17-4-8●		
29	17_9_14b●	58	17-60-26	87	17-12-5c	116	17-3-23		

4.3 Fungicides Selection

For evaluation, active compounds of 8 fungicides against *P. cactorum,* with a different mode of action, were selected which are Azoxystrobin, Cymoxanil, Dimethomorph, Fluopicolide, Fosetyl-Al, Fenamidone, Metalaxyl, and Propamocarb. All these active compounds were

obtained from Sigma-Aldrich (Germany). Active compounds of each fungicide were used in 5 different concentrations with negative control i.e., 0, 0.001, 0.01, 0.1, 1, 10 µg/ml. To preserve fungicide potential, all active compounds were dissolved in Dimethyl sulfoxide (DMSO) and stored at 4°C in the dark. Details of active compounds used in this study are given below in Table 3.

Table 3: Active Compounds Used in This Study

Chemical	Commercial name	FRAC group	Mode of action
Azoxystrobin	Heritage, Amistar	11	QoI
Cymoxanil	Curzate	27	Inhibits synthesis of nucleic acids
Dimethomorph	Acrobat	40	Inhibition of cellulose synthesis
Fenamidone	Fenstop	11	QoI
Fluopicolide	Infinito	43	Delocalization of spectrin like protiens
Fosetyl-AL	Aliette	P07	Inhibit spore germination
Metalaxyl	Ridomil	4	Inhibits protein synthesis
Propamocarb	Banol	28	Inhibit membrane biosynthesis

4.4 Fungicides Evaluation

For the poisoned plate assay V8 juice Agar amended with aforementioned active compounds

of fungicides is used. Each active compound is assessed in five different concentrations along with a control for every isolated *P. cactorum* strain. Fungicide concentrations were prepared in DMSO and further diluted in respective amounts of DMSO, already sterilized by UV for 20 minutes. Then this solution was added to autoclaved V8 juice agar when the temperature was around 55° to 60°C (before solidification) followed by proper mixing and then poured 15 ml of agar juice in each Petri dish.18 Petri dishes were prepared for evaluation of a single fungicide. This assay was performed in triplicate to acquire the best possible results. The Composition of V8 juice agar is given in Table 4. The In-Vitro efficacy of the aforementioned 8 active compounds was evaluated against all *P. cactorum* strains isolated from different regions of the Czech Republic by poisoned plate assay. Active compounds with concentrations 0, 0.001, 0.01, 0.1, 1, 10 μg/ml were added to V8 agar medium. A 6 mm borer was used to prepare mycelial discs from the margin of 8 days old *P. cactorum* culture and incorporated in the centre of poisoned plates. After incubation at 25°C for 8 days in the dark, control Petri dishes are observed with 80% growth. Mycelial growth was measured for all treatments both vertically and horizontally by using a digital Vernier caliper and growth in mm at different concentrations was obtained for the finding of mycelial growth inhibition % and calculation of EC_{50} values. Mycelial growth inhibition was calculated for all fungicides by the following formula.

$$\text{Mycelial growth inhibition \%} = \frac{growth\ in\ control - growth\ in\ treatd\ petri\ dish}{growth\ in\ control} * 100$$

Table 4: V8 Juice Agar Composition

S.NO	Composition	Quantity /liter
1	V8 juice	200 ml
2	$CaCO_3$	4 g
3	Distilled water	800 ml
4	Agar	16 g

4.5 Sporangia Formation Assay

Dimethomorph, Fluopicolide, Azoxystrobin, and Metalaxyl were selected for sporangia formation assay based on effective mycelial growth inhibition. A Sporangia formation assay was performed as reported by (Ma et al. 2018), with slight modifications. Three agar plugs of 6 mm from the margins of the actively growing *P. cactorum* colony were transferred to 90 mm Petri dishes and were added with 20 ml of sterile distilled water already amended with the aforementioned concentrations of respective active compounds. After incubation at 25°C for 48 h under 10 h photoperiod, the mycelial plugs were transferred to Eppendorf tubes and crushed followed by the addition of 1 ml liquid from a respective Petri dish. Eppendorf tubes containing crushed mycelial plug and 250 µl liquid were vortexed for 2 minutes at 500 rpm and then the number of sporangia was counted by using a haemocytometer. This assay was performed in triplicates.

4.6 Zoospore Discharge Assay

Azoxystrobin, Dimethomorph, Fluopicolide, and Metalaxyl were used for the zoospore discharge assay. This assay was performed according to the protocol developed by (Ma et al. 2018) with slight modifications. Sporangia were produced by transferring 6 mm mycelial plugs of growing *P. cactorum* colony to Petri dishes containing 20 ml sterile distilled water. After incubation at 25°C for 48 hours the distilled water in Petri dishes was decanted and 20 ml sterile distilled water amended with active compounds of desired concentrations was added to each Petri dish and followed by refrigerating at 4°C for 2 hours. After treatment at 4°C Petri dishes were incubated at 25°C for 2 days under 10 hours photoperiod for release of zoospores. The Number of empty sporangia was counted by using a haemocytometer and EC_{50} values were determined. This assay was performed in triplicates.

4.7 Comparison of the Genetic Makeup of RPA190 Gene in Metalaxyl Resistant and Sensitive Isolates

4.7.1 Screening of Metalaxyl Resistant and Sensitive Isolates for Molecular Studies

Based on EC_{50} and mycelial growth inhibition percentage 4 highly resistant and 4 sensitive isolates were selected for further molecular studies. In this study, we compare the genetic makeup of the RPA 190 gene of Metalaxyl resistant and sensitive isolates. Single nucleotide polymorphism (SNPs) in the RPA 190 gene is responsible for Metalaxyl resistance in *Phytophthora infestans*. At the same time, according to our knowledge, there is no study on

the relation of RPA190 and Metalaxyl resistance in *P. cactorum* is reported.

4.7.2 Culturing for DNA Isolation

For isolation of DNA, Resistant and sensitive isolates were first recultured on V8 juice agar and incubated at room temperature in the absence of light. After 5 days when 80% growth was observed, 1 mycelial disc of 6mm, with borer, was made at the margin of the colony and shifted to sterilized 100ml V8 juice broth medium and put onto a shaking incubator at room temperature for one week for maximum mycelial development. After one week when enough mycelia were developed, all media was filtered through sterilized mesh cloth. Mycelia of each strain was collected and preserved at -80°C.

4.7.3 DNA Isolation

Isolation of DNA was performed by GenElut Plant Genomic DNA Miniprep Kit provided by Sigma Aldrich Germany. Preserved mycelial samples of each strain were grinded into fine powder in liquid nitrogen using a mortar and pestle. 100 mg of the grinded powder was transferred to a microcentrifuge tube. 350 µl of Lysis Solution (Part A) and 50 µl of Lysis Solution (Part B) was added to the tube and mixed properly followed by incubation at 65° C for 10 minutes. For precipitation of debris 130 µl of precipitation solution was added to the mixture and mixed by inverting and placed on ice for 5 minutes followed by centrifugation (12,000–16,000 x g) for 5 minutes. The supernatant was pipetted onto the GenElut filtration column. Then filtration columns were centrifuged at maximum speed for 1 minute. This removes any cellular debris further. The filtration column was discarded, and collection tubes were retained. Then 700 µl of the binding solution was added to the liquid in the collection tube and subsequently added to the binding column (with a red O-ring) followed by centrifugation at 12,000 x g for 30 seconds to 1 minute. After centrifugation flow through liquid was discarded. Then binding column was washed 2 times with 500 µl of wash solution already amended with ethanol followed by centrifugation for 3 minutes at maximum speed for drying. In the last step of elution binding columns were transferred to fresh 2 ml collection tubes and 100 µl of prewarmed (65°C) elution solution was added and centrifuged at maximum speed for 1 minute. The elution step was performed twice for maximum recovery of genomic DNA. As eluate contains pure genomic DNA it was stored 2–8°C for short term storage and then used for further analysis.

4.7.4 Primer Designing for RPA190 Gene Responsible for Metalaxyl Resistance and Preparation of PCR Product for Sequencing

According to (Chen et al. 2018) multiple mutations such as V1476G, P980S, and F382Y in RPA190 contribute to Metalaxyl resistance, and resistance to Metalaxyl can emerge in at least two independent pathways in *P. infestans*. Because of limited data on Metalaxyl resistance in *P. cactorum.* Primers were designed according to the aforementioned detected mutations in *P. infestans*. The primers were designed using Primer-BLAST and Primer 3 software. Gradient PCR for finding the optimized annealing temperature of primers was performed and the annealing temperature was set for each primer based on the vividity of gel electrophoresis images. Polymerase chain reaction (PCR) was performed using a C1000 thermocycler (Bio-Rad, Hercules, CA, USA), using 30 ng of total gDNA per reaction. The thermocycler was programmed at an initial denaturation step at 95 °C for 5 minutes, followed by 40 cycles of 5 s at 95°C, 10 s at 56 and 61 °C (based on the annealing temperature of the primer pair), and 2 minutes at 72°C along with a final extension step for 10 minutes at 72 °C. The PCR amplified products were separated in the 1.5% agarose gel and subsequently purified using a MinElute Gel Extraction Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. The concentration of gel-purified product was checked by nanodrop and confirmed by gel electrophoresis and then samples were sent for custom DNA Sequencing (Eurofins Genomics Germany, Ebersberg, Germany). Details of designed primers are given in Table 5.

Table 5: Details of designed primers to detect mutations in RPA190

Primer name	Sequence (5' to 3')	Annealing temperature (°C)	Amplicon Length (bp)	Mutation points covered (Numbers according to *Phytophthora infestans*)
Forward AAMFP1:	TGCAGTTGATGTGGCAGAAC	61	466	F382
Reverse AAMRP1:	TTACCCATCATGTGCTTGCG			
Forward AAMFP2:	CTGACGCTGACTACAATGCC	56	431	P980
Reverse AAMRP2:	GTGCTTGATCAGACAACGCT			
Forward AAMFP3:	CCACAGCAGCGAAAATGGTA	56	422	V1476
Reverse AAMRP3:	CGTTGCTCTTCTTGTTGCCA			

4.8 RNA Extraction

Four Metalaxyl resistant and four Metalaxyl sensitive isolates were grown on V8 juice agar

for one week. Mycelial plugs of 6mm were cut from the margins of freshly grown isolates and added to clear v8 juice broth. Cultures were grown for one week at room temperature in a shaking incubator at 150 RPM. After one week when enough mycelia was developed, media from all 8 flasks was filtered through sterilized mesh cloth. Mycelia of each strain was collected and preserved at -80°C until further use. A hybrid RTM kit with a Cat. No.305-101 (GeneAll Biotechnology Co., Ltd., Seoul, Korea) was used for total RNA isolation. About 100 mg of mycelia from the aforementioned isolates were homogenized in 1 ml of RiboEx and then frozen rapidly with liquid nitrogen. To completely dissociate nucleoprotein complexes, homogenized samples were incubated at room temperature for 5 minutes followed by the addition of 200 ul of chloroform. After the addition of chloroform samples were shaken vigorously and placed at room temperature for 2 minutes followed by centrifugation at 12000 RCF for 15 minutes AT 4°C. This step fractionates the mixture into three phases; a lower layer with biomass, an interphase, and an upper colourless aqueous layer which is about 50% of the volume of a RiboEx used at the start for homogenization. The same amount of RBI buffer was added to the sample and mixed by inverting followed by filtering with the minispin column at 13000 RCF for 30 seconds. After the retentate was attached to the mini spin column, 500 ul of SW1 buffer was added and centrifuged at 13000 RCF for 30 seconds. This step took away all phenolic compounds and other traces of solvents used in the extraction process. Finally, 50 ul of nuclease free water was added to the centre of the minispin column and centrifuged at 13000 RCF for 1 minute at room temperature to elute RNA. Integrity and concentration of extracted RNA were analysed with nanodrop followed by gel electrophoresis. Extracted RNA was treated with DNases (kit obtained from Thermofisher Scientific Germany). Purified RNA was stored at -80°C for further analysis.

4.9 Gene Expression Analysis

Initially, 1000 ng *P. cactorum* RNA was used for cDNA synthesis using a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems™, Waltham, MA, USA). RPA190 expression analysis was done in CFX Connect Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA, USA) using cDNA (~13 ng), as a template (respectively). A combination of ubiquitin-conjugating enzyme (Ubc), β-tubulin (Tub-b), and 40S ribosomal protein S3A (WS21) were used as reference/housekeeping genes (Chen et al. 2011). Gene-specific primers for quantitative real-time PCR experiments are listed in Table 6. The results were calculated using the 2−ΔΔCt method and a comparison between the S and R strains was performed using a two-sample t-test function from the RT-qPCR experiment was repeated

with five biological replications.

Table 6: Detail of gene specific primers for qPCR

Primer name	Sequence (5' to 3')	Annealing temperature (°C)	Amplicon Length (bp)
Forward RT-PCR FP1:	TGTACGGCTCAGCAAACAAC	62.6	130
Reverse RT-PCR RP1:	CCGTACACTTCGTAGCATGC		
Forward RT-PCR FP2:	GTGCGAGCATGTACGGTATG	62	145
Reverse RT-PCR RP2:	AGCGTCAGATCCTCCATAGC		

4.10 Greenhouse Experiment

The Efficacy of 4 Commercially available fungicides mentioned in Table 7 with positive and negative control against *P. cactorum* was analysed in the greenhouse with an average temperature of 25°C at day and 12°C at night. The Karmen variety was selected for this experiment because, in our previous study, we screened out Karmen as the most resistant cultivar against *P. cactorum*. The five most fungicide resistant *P. cactorum* isolates were cultured in clear V8 broth medium (centrifuged v8 juice) and incubated at 25°C in a shaking incubator for 10 days followed by filtering and grinding of solid mycelial residue. The Soil was inoculated with 10 ml grinded mycelial paste followed by the addition of 5 ml fungicides to each pot. After 24 hours young strawberry plants were shifted to inoculated pots. The Efficiency of fungicides was analysed by monitoring 10 various parameters i.e., number and weight of leaves, number, and weight of fruits, number and weight of blooms, diameter and weight of root neck, length, and weight of roots. Each treatment was repeated 10 times to acquire the best possible results.

Table 7: Fungicides Used in Green House Experiment

Variant	Active compounds
Acrobat	Dimethomorph
Ridomil+Ortiva	Metalaxyl+Azoxystrobin
Acrobat+ Retengo	Dimethomorph+Pyraclostrobin
K+ *P. cactorum*	Plants infected with *P. cactorum*
Control	Plants grown in potting soil

4.10.1 Comparison of Statistical Data from Greenhouse Experiment with Newly Developed Plant Health Index Score

All parameters in the greenhouse were measured and the Plant health index score (PHI) was calculated from data from the greenhouse experiment by the following formula.

$$plant\ health\ index\ \% = \frac{\Sigma\ mean\ of\ all\ parameters\ of\ treated\ plant}{\Sigma\ mean\ of\ all\ parameters\ of\ control} * 100$$

The performance of treatment was calculated by subtracting the PHI of the treated plant from the PHI of control. Performance greater than control is positive and demonstrated as assisted growth due to treatment while performance less than control was named as negative. Negative performance is the loss of plant health due to treatment.

$$performance\ of\ treatment = PHI\ of\ treated\ plant - PHI\ of\ control\ plant$$

4.10.2 Visual Symptomatic Scaling

The root neck of *P. cactorum* infected strawberry plants appears dark brown and hence upon the intensity of dark visual appearance we scale the degree of infection into 3 classes. A healthy root neck with no symptoms of crown rot looks white and is ranked as alpha. Root necks with slight symptoms were classified in bravo class while root necks that are

completely symptomatic and appear brown were classified as Charlie. Each treatment was performed 10 times and a total of 50 root neck pictures were observed for 5 treatments comprised of 3 fungicide treatments and 1 positive and 1 negative control.

4.11 Biological Control of *P. cactorum* Using Microalgae: *Tisochrysis lutea*

T. lutea, initially isolated from the tropical coastline region of the Pacific, (Bendif et al. 2013) was obtained from the culture repository of the Bioprocess Engineering department, Wageningen University, The Netherlands. The growth medium was prepared by the addition of $NaNo_3$ (3.04 g/l) and HEPES (4.76 g/l) to natural seawater from the North Sea (The Netherlands). pH of the medium was adjusted to 8.0 with NaOH. After autoclaving, the medium was amended with 2 ml/L of sterilised Nutribloom plus, commercially available culture medium stock obtained from NECTON, S.A. (Olhão, Portugal). The strain was revived in a 100 ml Erlenmeyer flask containing 20 ml medium at 25°C, light intensity 140 μmol, 2% CO_2, 85 RPM with 18:6 day: night photoperiod in infors shaking incubator. After 10 days, the culture was refreshed by adding 10 ml of the culture into a 100 ml Erlenmeyer flask containing 90 ml of medium and placed in an incubator with the aforementioned conditions. The microalgal culture was refreshed monthly.

4.11.1 Growth of *T. lutea* in Photobioreactors

To obtain biomass in bulk, *T. lutea* was cultivated in a Horizontal tubular (HTI) photobioreactor with a capacity of 300 (l) liters. HTI requires about 20 l of inoculum. To upscale inoculum preparation microalgal culture was first cultivated in a 500 ml Erlenmeyer flask according to the aforementioned protocol. After 10 days when dense growth was observed, this amount was used as inoculum to start Labfors 5 photobioreactor (INFORS HT) with a reactor volume of 1800 ml. After 2 weeks biomass was harvested and used as inoculum to start a 20 l flatpanel photobioreactor. After 15 days flatpanel reactor was harvested and used as inoculum to start the HTI photobioreactor. Two batches of *T. lutea* biomass were harvested from HTI at the interval of 20 days. The growing culture of *T. lutea* in respective photobioreactors can be observed in Figure 6.
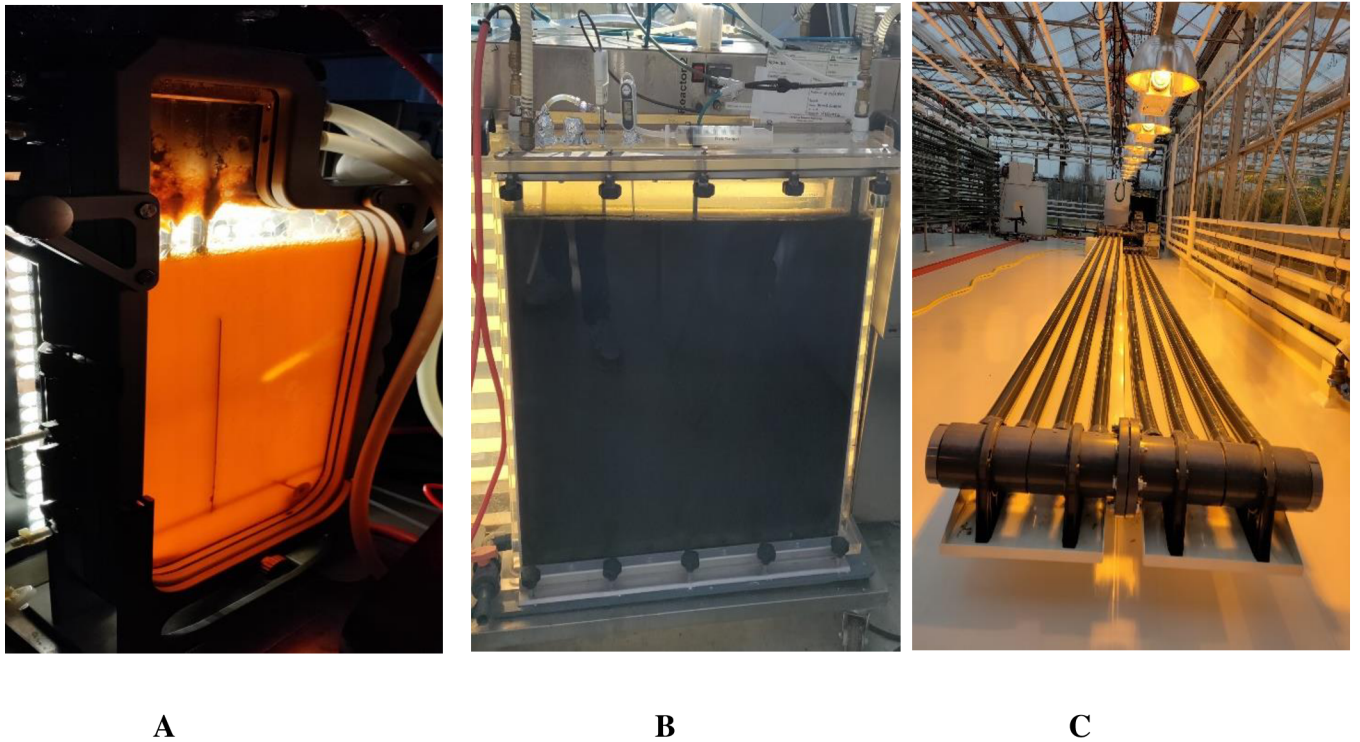
Figure 6: Growing culture of *T. lutea* in A) Infors photobioreactor, B) Flatpanel photobioreactor, C) Horizontal tubular photobioreactor

4.11.2 Harvesting and Storage of Biomass

Daily monitoring of optical density (OD), pH, temperature, and light are the key parameters to obtain dense and optimal biomass. After 7 days of inoculation, the HTI reactor was harvested. 80% i.e. 240 l of the biomass was collected in a separate tank and centrifuged. For this purpose Evodos centrifuge (Figure 7) with a product feed of 500 l/h, 3800 RPM was used. The remaining 60 l of culture was used to run the second batch of the HTI reactor and was harvested the same way after 10 days. Concentrated biomass obtained after centrifugation was washed 2 times with 0.5 M ammonium formate buffer to remove extra salts and collected in sterile bags followed by vacuum sealing and stored at -20°C until further use.

<div align="center">A   B</div>

Figure 7: A) Evodos centrifuge **B)** Collection of biomass after centrifugation from Evodos centrifuge

4.11.3 Cell Wall Disruption and Freeze Drying

The concentrated biomass of *T. lutea* was thawed at room temperature, at this stage cells are intact. To recover intracellular compounds intact cells need to be disrupted. Disruption of cells was performed by Beadmill as shown in Figure 8. Concentrated biomass was diluted with distilled water to achieve the final concentration of 100 g/l. Cells were disrupted using a beadmill with 6500 RPM for 6 minutes. Disruption of cells was confirmed by microscopy and Beckman coulter counter, Multisizer 3. All of the disrupted biomass was freeze dried and as a result dried disrupted powder of *T. lutea* called Crude Bio extract (CBE) was collected as shown in figure 8 and saved at -20°C for further tests.

<center>A</center>
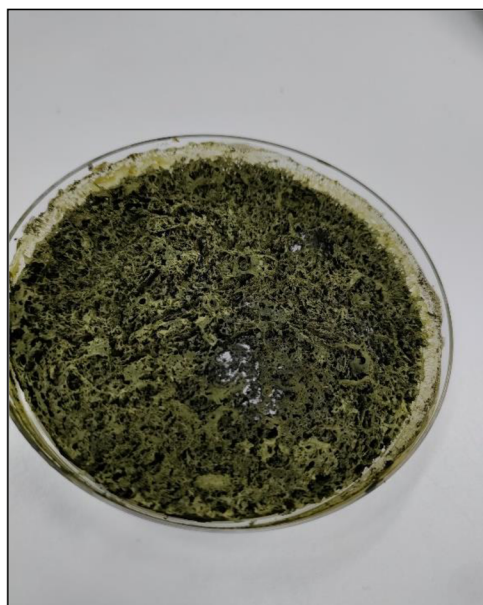
<center>B</center>

Figure 8: A) Bead mill for disruption of *T. lutea* cells B) Dried disrupted biomass after freeze-drying

4.11.4 Screening for Antifungal Potential

Several microalgae have been reported to exhibit antifungal potential, but we believe that brown algae especially *T. lutea* was the first study we conducted. CBE was screened for its antifungal potential against *P. cactorum* at different concentrations. Initially, 25 mg/ml of CBE was checked against mycelial inhibition. Later various concentrations i.e. 1,2,3,4,5,10,20,30, and 40 mg/ml were checked against 8 *P. cactorum* isolates i.e. R1, R2, R3, R4, S1, S2, S3, S4.

4.11.5 Biphasic Extraction of Bioactive Compounds

The extraction of bioactive compounds from the biomass depends on the compatibility of solvents and the suitable extraction technique. Four different solvents based on the polarity index were selected for this purpose. Solvents used were n-Hexane, chloroform, methanol, and milli Q water. 1 g of dried disrupted biomass was dissolved in 150 ml of aforementioned solvents followed by stirring for 48 hours. Solvents were removed by using a rotary evaporator and extracts recovered were stored at 4°C for further analysis. All of the organic

solvents used were of analytical grade obtained from Sigma Aldrich Germany.

4.11.6 FTIR, SEM, and Partial Characterization of Biomass

Fourier-Transform Infra-Red spectrometer was used to check the various functional groups present in extracted bioactive compounds. FTIR model used was Bruker Tensor 27 equipped with liquid nitrogen cooled MCT- detector, (mercury cadmium telluride), for sensitive measurements. The concentration of total organic carbon (TOC), total nitrogen (TN), total carbon (TC), and total inorganic carbon (TIC) present in biomass was calculated by using the SHIMADZU Total Organic Carbon Analyzer available at the Department of Bioprocess Engineering (BPE), Wageningen University, The Netherlands. To check the interaction of *P. cactorum* and crude bioextract, Scanning electron microscopy (SEM) was performed at Wageningen Electron Microscopy Centre, The Netherlands. *P. cactorum* isolate, (R1) treated with a CBE concentration of 5 mg/ml was selected for SEM. For control, R1 grown on V8 agar medium was used.

4.11.7 UHPLC-HRMS-MS DDA (Data Dependent Analysis)

An Agilent 1290 UHPLC system connected to a Thermo Fisher Q-Exactive Focus Quadrupole-Orbitrap Mass Spectrometer with an electrospray ionization (HESI) interface was used to acquire the data. 48 samples (5 µl) were injected and separated chromatographically on a reversed-phase C18 HSS T3 Acquity column (Waters, Milford, MA, USA), with a 2.1 × 100 mm size and 1.8 µm particle size. Buffer A (water containing 0.1% formic acid) and Buffer B (acetonitrile containing 0.1% formic acid) were the mobile phases utilized in the chromatographic separation process. Eluent A (water) and Eluent B (acetonitrile), both acidified with 0.1% formic acid, were used as mobile phases to apply a gradient profile. The following procedure was used to create a multi-step elution dual-mode gradient: at 0.0 min (5% B; 0.40 ml/min), the gradient started as follows: The original circumstances were restored to 5% B in 1 minute; the entire run time was 21 minutes, including 5 minutes for re-equilibration. The conditions were as follows: 11 min 35% B, 12.5 min 70% B, 13.5 min 99% B, held for 1.5 min till 15.0 min. The column was kept at 40°C and was fed at a rate of 0.4 ml/min. The interface's parameters were set as follows for the HESI (Heated Electrospray Ionization) source to function well in both negative and positive ion modes: Capillary temperature 320°C, S-lens RF level 47.0, aux gas heater temperature 0°C, spray voltage 3.2

kV, sheath gas flow rate 30 ml/min, aux gas flow rate 15 ml/min, and sweep gas flow rate 33 ml/min. The mass spectra were acquired in DDA (Data Dependent Analysis) by full scan MS1 and MS2 in positive and negative ionization mode on a Q-Exactive high resolution Orbitrap-type MS (ThermoFisher, Bremen, Germany) in a scan range from 100 to 1000 m/z; the full MS resolution was 70,000 FWHM, AGC target 3e6, maximum IT 200ms and with a profile spectrum data type. The dd/MS2 analysis was performed with a resolution of 17,500 FWHM with three different collision energies: 15,30,45 eV, AGC target 2e5, maximum IT 50ms, loop count of 3, minimum AGC target of 8.00e3, intensity threshold 1.6e5, apex trigger 2 to 15 s, dynamic exclusion of 4.0 s, charge exclusion 2-8, >8 and a profile spectrum data file (Stincone et al. 2023).

4.11.8 Data Pre-Processing and Feature Annotation Workflow

This workflow describes a basic pipeline for untargeted LC-MS (or LC-MS/MS) data preprocessing. Its primary objective is to convert the extremely complicated raw LC-MS data into a list of features and related signal intensities that were found in all of the samples that were studied. Upon exporting these feature lists, additional downstream analysis (such as identification, search against spectral libraries, statistical analysis, etc.) can be performed. A schematic representation of the workflow is shown below:
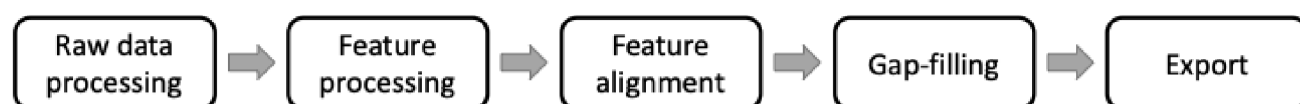
To get a list of all the identified features (each represented by an RT and m/z value) from the raw LC-MS data, "feature processing" is performed after mass detection and data import. The creation of extracted ion chromatograms (EICs) for every identified mass was the initial stage in the "Feature processing" procedure. After being "detected" in each file, the features were compiled into "feature lists" and underwent additional processing and alignment to establish connections between the corresponding characteristics in all samples. One of the most important processes in the preprocessing of data is the feature resolving step, which allows the separation of co-eluting and overlapping chromatographic peaks. Lastly, feature alignment made it possible for matching features in every sample to align. The data was exported and then put into SIRUS software for identification after being pre-processed using MZmine 3

Open source. Depending on the sample type, data were submitted individually.

4.11.9 *T. lutea* as Biostimulant and Biocontrol Agent

The Greenhouse experiment was designed to check the antifungal and biostimulant potential of crude extract from *T. lutea*. Frozen Strawberry plants of ever bearing cultivar, Ostara, with plant health passport were obtained from a commercial supplier i.e. de-Kemp BV, The Netherlands. The experiment was divided into 2 parts i.e. Biostimulant Part and Biocontrol Part. Plants were grown in potting soil. The pots used were 17 cm in diameter with a capacity of 700 g of soil. The experiment was started in mid-June and finished in the last week of September. The total span of the experiment was 100 days. Plants were watered daily. Concentrations of crude bioextract used were 2, 4,6, 8, and 10 g/l with a control added with distilled water. To prepare crude bioextract suspension, dried disrupted biomass of *T. lutea* was dissolved in distilled water according to desired concentrations.

Each plant received 10 ml of CBE with the aforementioned concentration at the interval of 10 days. A total of 9 applications of CBE were applied in the span of the experiment (100 days). Each plant receives 90 ml of CBE in 100 days. Commercially available fertilizer specifically designed for strawberries i.e. Kristalon was also applied according to the recommended dose, to one of the treatments to check the comparison with CBE-treated plants. In the biocontrol part strawberry plants were inoculated with *P. cactorum* to induce infection. The same concentrations of CBE were applied as mentioned before. The reason was to check whether the infection caused by *P. cactorum* can be suppressed by the addition of CBE or not. Furthermore, plants treated with *P. cactorum* only were considered as positive control. Commercially available fungicide to control *P. cactorum* i.e. Aliette with Fosetyl-AL as an active ingredient was also used in one treatment to compare the efficacy of CBE, positive control, and also with Aliette. Plant growth parameters like total length and weight of the whole plant, length and weight of shoot plus root, number of leaves, number and weight of fruits, diameter, and weight of root neck were recorded. Each treatment was performed in seven replicates. The greenhouse experiment was performed at the Nergena greenhouse facility at Wageningen University, The Netherlands.

4.12 Softwares Used

4.12.1 GraphPad Prism

GraphPad Prism software (9.0.0) for Windows OS (GraphPad Software, San Diego, CA, USA), was used to calculate EC_{50} values for each fungicide used in the mycelial growth inhibition assay, the sporangia formation and zoospore release assay according to a protocol described by (Li et al. 2014). The data of the growth values for all fungicide concentrations tested against each isolate were converted into logarithmic form, and all measured data was then normalized to convert the heterogeneous variance of the data to homogenous. This kind of normalization was carried out following the identification of the plateaus in the data concerning the inhibition response; that is, the highest level of inhibition in the experiment, brought about by the highest concentration of fungicide, was identified as a 100% response of isolates, and the absence of inhibition at zero concentration was regarded as a 0% response of isolates. The EC_{50} values were determined by applying a nonlinear regression curve fit model following the standardization of the data.

4.12.2 BioEdit

BioEdit was used for sequence alignment and comparison of sequences. Sequences were imported to the software by clicking ''import' under the file menu after opening the software. As we can compare multiple sequences, so all the sequences were imported. Once the sequences were imported, a chromatogram for each sequence popped up which showed initial peaks of the sequence, which were not homogenized because of the presence of the sequence of primer. The unhomogenized sequence at the start and end of the sequence was deleted. Reverse complement sequences were created after importing sequences from reverse primers. Then pairwise alignment was performed. At the end consensus sequences were created by clicking ''Alignment'' and then ''create consensus sequence''. After that new consensus sequences were generated, and all 24 consensus sequences of 3 primers (for 4 resistant and 4 sensitive isolates) were checked by the colour differentiation of nucleotides.

Chapter 5: Results

5.1 In-Vitro Mycelial Growth Inhibition Assay

3 out of 8 fungicides i.e., Metalaxyl, Dimethomorph, and Fluopicolide displayed inhibitory effects against *P. cactorum* isolates and EC_{50} values were calculated. Other active compounds i.e., Azoxystrobin, Cymoxanil, Fenamidone, Fosetyl-AL, and Propamocarb were not effective in in-vitro inhibition of mycelia at the highest concentration of 10 µg/ml. Compared to Metalaxyl and Fluopicolide, Dimethomorph has strong in-vitro mycelial inhibition potential. Against Dimethomorph and Metalaxyl, isolates were divided into 2 heterogenous groups i.e., sensitive, and resistant. Details of the efficacy of active compounds are given below.

5.1.1 Metalaxyl

Variation in EC_{50} and growth inhibition was observed against Metalaxyl because of which isolates were divided into two heterogeneous groups i.e., Metalaxyl Sensitive and Metalaxyl resistant. For Metalaxyl sensitive isolates EC_{50} ranges from 0.1 to 10. Isolates with EC_{50} values higher than 10 µg/ml were recognized as resistant.
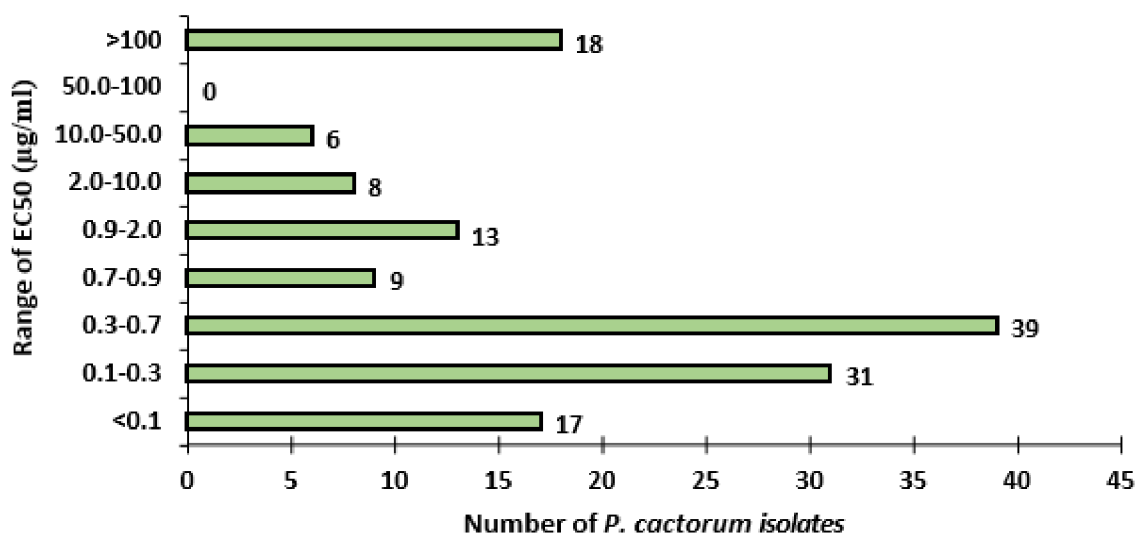

Figure 9: Range of EC_{50} value, recorded against Metalaxyl.

5.1.1.1 Percentage of Metalaxyl Resistant and Sensitive Isolates among Population

P. cactorum population, based on resistance level, was divided into two main groups as shown in Figure 10.

1. Metalaxyl resistant: Out of 141 *P. cactorum* isolates, 24 isolates showed EC_{50} values higher than 10 and were considered resistant to Metalaxyl. In the overall population, 17% of isolates were resistant.
2. Metalaxyl Sensitive: 117 isolates out of 141 have EC_{50} values between 0.1 and 10 and were placed in the sensitive group and 82.9 % of the population was sensitive to Metalaxyl.
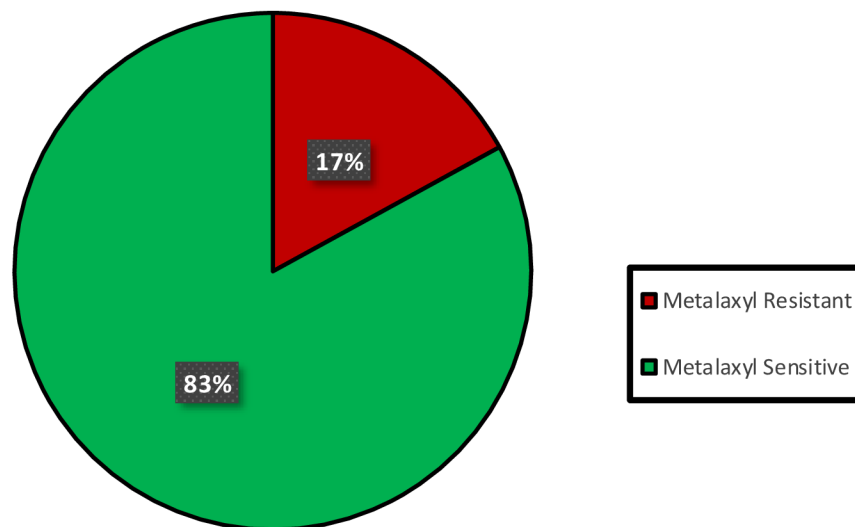

Figure 10: Metalaxyl resistant and sensitive isolates: percentage presence in the population.

5.2 Dimethomorph

Compared to Metalaxyl, Dimethomorph was more effective in inhibiting mycelial growth. EC_{50} values and resistant percentages can be observed in Figure 11 and Figure 12 respectively. Likewise, Metalaxyl, based on resistance level *P. cactorum* population was also divided into two groups.

I. Dimethomorph Sensitive: EC_{50} values for Dimethomorph sensitive isolates range from 0.1 to 10 and 95.7% of *P. cactorum* isolates were sensitive to Dimethomorph.

II. Dimethomorph Resistant: EC_{50} values for this group range from 222.5 to 634778 and 4.3 % isolates from the population were resistant to Dimethomorph.
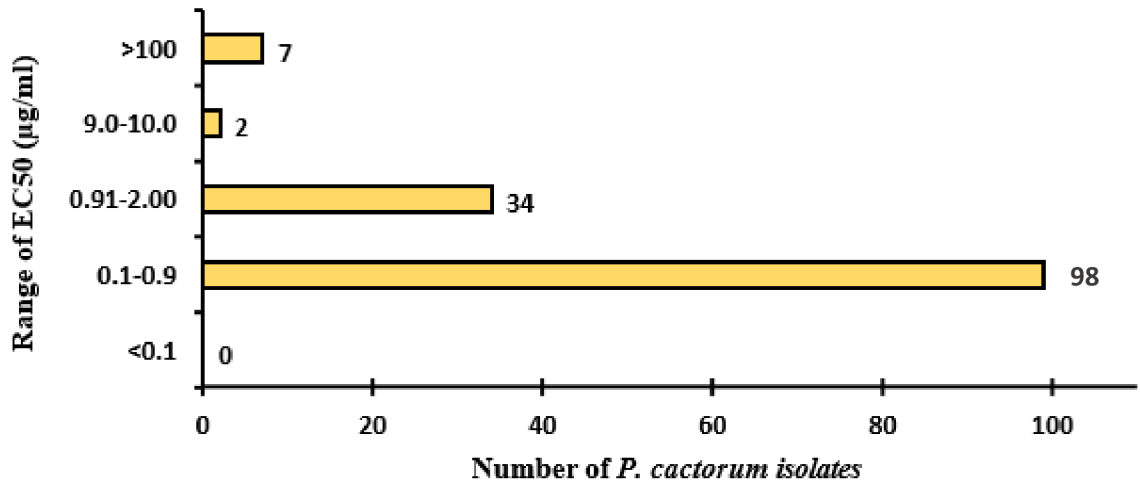
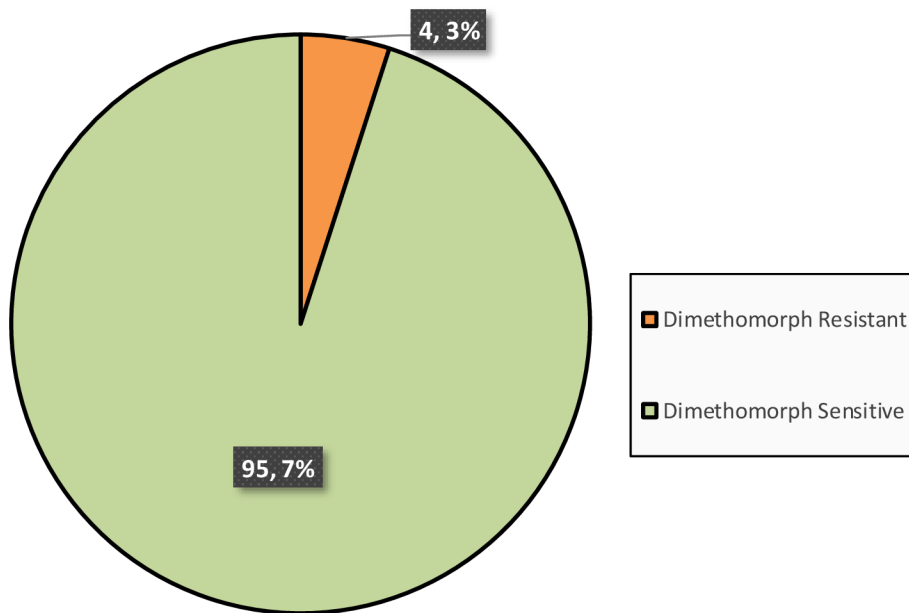**Figure 11:** Range of EC_{50} value, recorded against Dimethomorph.

Figure 12: Dimethomorph resistant and sensitive isolates: percentage presence in the population.

5.3 Fluopicolide:

The lowest EC_{50} values were recorded against Fluopicolide, and mycelial growth was inhibited in all isolates. EC_{50} ranges from 0.7 to 4. No resistant isolates were found but this active compound has fungi static activity i.e., steady growth at the highest concentration of 20 µg/ml was observed even after seven days.

5.4 Azoxystrobin, Cymoxanil, Propamcorab, Fenamidone and Fosetyl-AL

Mycelial growth of *P. cactorum* was not inhibited by Azoxystrobin, Cymoxanil, Propamocarb, and Fosetyl-AL. EC_{50} values recorded against these active compounds were greater than 5000 µg/ml. EC_{50} recorded for Fenamidone ranges from 76.1 to 369.6 µg/ml but its effect was fungistatic as the growth of pathogens was observed after one week even at the concentration of 100 µg/ml.

5.5 Sporangia Formation Assay

Metalaxyl, Dimethomorph, Fluopicolide, and Azoxystrobin were selected for sporangia formation assay against 30 isolates. Dimethomorph was efficient in inhibiting sporangia formation with EC_{50} ranges from 0.07692 to 1.78 and a mean value of 0.343012. In the case of Metalaxyl and Azoxystrobin based on EC_{50} values, isolates were divided into 2 heterogenous groups i.e. (SPF) sporangia formation ineffective and SPF effective. Sporangia formation of 26 isolates out of 30 was arrested by Metalaxyl and EC_{50} value ranges from 0.05605 to 2.894 with a mean value of 0.683951. SPF ineffective group comprised of 4 isolates with EC_{50} ranges from 279217 to 66858235. Azoxystrobin arrested sporangia formation of 29 isolates with EC_{50} ranges from 0.03881 to 9.429 while sporangia formation of 1 isolate was not affected by Azoxystrobin and recorded EC_{50} value was 65628. The EC_{50} value recorded for Fluopicolide ranges from 0.1066 to 0.6331 with a mean value of 0.268287.

5.6 Zoospore Release Assay

A zoospore release assay was also performed for 30 isolates. Among Azoxystrobin, Dimethomorph, Fluopicolide, and Metalaxyl, Fluopicolide was observed as efficient in inhibiting the release of zoospores with EC_{50} ranges between 0.1479 and 4.807 and the mean value of 0.731767. For Azoxystrobin, Dimethomorph, and Metalaxyl range of EC_{50} was 0.1984 to 140.6, 5969 to 8241254881, and 3839 to 39810861 respectively. Azoxystrobin was efficient in controlling zoospore release but in 1 isolate the recorded EC_{50} was 140.6 while 29 isolates had an EC_{50} value less than 10. Metalaxyl and Dimethomorph do not affect the zoospore release of *P. cactorum* isolates even in the presence of the highest concentrations i.e., 100 µg/ml.

5.7 Comparison of the Genetic Makeup of RPA190 Gene in Metalaxyl Resistant and Sensitive Isolates

5.7.1 Screening of Metalaxyl resistant and Sensitive Isolates for Molecular Studies

For comparison of the genetic makeup of the RPA190 gene of Metalaxyl resistant and sensitive isolates 4 highly resistant and 4 sensitive isolates based on EC_{50} were screened out. Metalaxyl concentrations were increased, and growth inhibition percentages were recorded. The highest concentration of Metalaxyl used was 100 µg/ml. Growth of sensitive isolates was inhibited at a concentration of 1 µg/ml, but no growth inhibition of resistant isolates was observed even at 100 µg/ml and hence they were selected for this study. Details of resistant and sensitive isolates are given below in Table 8.

Table 8: EC_{50} recorded for Metalaxyl resistant and sensitive isolates.

Isolates	No of isolates	EC_{50}
Resistant 1	78	1342520801
Resistant 2	115	8310521589
Resistant 3	103	8815969175
Resistant 4	51	4755197
Sensitive 1	59	0.1592
Sensitive 2	136	0.1849
Sensitive 3	113	0.1964
Sensitive 4	123	0.2294

5.7.2 Detection of Mutations and Comparison of RPA190 Gene of Resistant and Sensitive Isolates

Chen et al. (2018) reported single nucleotide polymorphisms (SNPs) in RPA190 as

responsible factors for Metalaxyl resistance in *P. infestans*. According to our knowledge so far there is no report which explains the relationship of the RPA190 gene to Metalaxyl resistance in *P. cactorum*. So, this study was performed for the first time to find out the differences between the RPA190 gene of Metalaxyl resistant and sensitive isolates. Three primers were designed (mentioned in table 4) from the RPA190 gene of *P. infestans* and checked the differences between resistant and sensitive isolates. We didn't find any difference among the RPA190 gene of resistant and sensitive isolates with mutations previously reported in *P. infestans* as depicted in Table 9, hence we concluded that there might be some other gene responsible for Metalaxyl resistance in *P. cactorum*. Compared translated sequences are provided in supplementary figures 1-4.

Table 9: Comparison of P. cactorum Resistanat (R) and Sensitive (S) Isolates with previously identified SNPs in P. infestans

Species	Profile	Mutation	Amino acid sequence
P. infestans	R		360　　　　370　　　　380　　　　390　　　　400
P. cactorum	S1		P V F M G D K Q F E H A Q N S H L S K I M T Y S E S I V Q S D Y Y K R Q A A T T S D
P. cactorum	R1		P A F S G D K Q F E H A Q N S H L S K I M T L S E S I V Q G D Y Y K R Q A A T T S D
P. cactorum	S2		P V F M G D K Q F E H A Q N S H L S K I M T L S E S I V Q G D Y Y K R Q A A T T S D
P. cactorum	R2	F382Y	P V F M G D K Q F E H A Q N S H L S K I M T L S E S I V Q G D Y Y K R Q A A T T S D
P. cactorum	S3		P V F M G D K Q F E H A Q N S H L S K I M T L S E S I V Q G D Y Y K R Q A A T T S D
P. cactorum	R3		P V F M G D K Q F E H A Q N S H L S K I M T L S E S I V Q G D Y Y K R Q A A T T S D
P. cactorum	S4		P V F M G D K Q F E H A Q N S H L S K I M T L S E S I V Q G D Y Y K R Q A A T T S D
P. cactorum	R4		P V F M G D K Q F E H A Q N S H L S K I M T L S E S I V Q G D Y Y K R Q A A T T S D
P. infestans	R		410　　　　420　　　　430　　　　440　　　　450
P. cactorum	S1		Q V N L S R K L A L W T E L Q N A V N L L V D S S K A K P G T D V A Q G I K Q V I E I
P. cactorum	R1		Q V N L S R K L A L W T E L Q N A V N L L V D S S K A K P G T D V A Q G I K Q V I E I
P. cactorum	S2		Q V N L S R K L A L W T E L Q N A V N L L V D S S K A K P G T D V A Q G I K Q V I E I
P. cactorum	R2	T443A	Q V N L S R K L A L W T E L Q N A V N L L V D S S K A K P G T D V A Q G I K Q V I E I
P. cactorum	S3		Q V N L S R K L A L W T E L Q N A V N L L V D S S K A K P G T D V A Q G I K Q V I E I
P. cactorum	R3		Q V N L S R K L A L W T E L Q N A V N L L V D S S K A K P G T D V A Q G I K Q V I E I
P. cactorum	S4		Q V N L S R K L A L W T E L Q N A V N L L V D S S K A K P G T D V A Q G I K Q V I E I
P. cactorum	R4		Q V N L S R K L A L W T E L Q N A V N L L V D S S K A K P G T D V A Q G I K Q V I E I
P. infestans	R		960　　　　970　　　　980　　　　990　　　　1000
P. cactorum	S1		Q I S C G L G Q Q A L E G R R V P I L D S G R S L P S F E P F D P A P R A G G Y V T D
P. cactorum	R1		Q I S C G L G Q Q A L E G R R V P I L D S G R S L P S F E P F D P A P R A G G Y V T D
P. cactorum	S2		Q I S C G L G Q Q A L E G R R V P I L D S G R S L P S F E P F D P A P R A G G Y V T D
P. cactorum	R2	P980S	Q I S C G L G Q Q A L E G R R V P I L D S G R S L P S F E P F D P A P R A G G Y V T D
P. cactorum	S3		Q I S C G L G Q Q A L E G R R V P I L D S G R S L P S F E P F D P A P R A G G Y V T D
P. cactorum	R3		Q I S C G L G Q Q A L E G R R V P I L D S G R S L P S F E P F D P A P R A G G Y V T D
P. cactorum	S4		Q I S C G L G Q Q A L E G R R V P I L D S G R S L P S F E P F D P A P R A G G Y V T D
P. cactorum	R4		Q I S C G L G Q Q A L E G R R V P I L D S G R S L P S F E P F D P A P R A G G Y V T D
P. infestans	R		1450　　　1460　　　1470　　　1480　　　1490
P. cactorum	S1		D Q V F N S V G R G F V G K L L T L I S R E M K K S G V T V S A A A E K N N F K A P S
P. cactorum	R1		D Q V F N S F G R G F V G K L L T L I S R E M K K S G V T V S A A A E K N N F K A P S
P. cactorum	S2		D Q V F N S F G R G F V G K L L T L I S R E M K K S G V T V S A A A E K N N F K A P S
P. cactorum	R2	V1476G	D Q V F N S F G R G F V G K L L T L I S R E M K K S G V T V S A A A E K N N F K A P S
P. cactorum	S3		D Q V F N S F G R G F V G K L L T L I S R E M K K S G V T V S A A A E K N N F K A P S
P. cactorum	R3		D Q V F N S F G R G F V G K L L T L I S R E M K K S G V T V S A A A E K N N F K A P S
P. cactorum	S4		D Q V F N S F G R G F V G K L L T L I S R E M K K S G V T V S A A A E K N N F K A P S
P. cactorum	R4		D Q V F N S F G R G F V G K L L T L I S R E M K K S G V T V S A A A E K N N F K A P S

5.7.3 Gene Expression Analysis

The expression values between the control (DMSO) and the fungicide-treated isolates were compared to analyse the possible involvement of gene overexpression in fungicide resistance. Significant differences in the expression patterns were detected for R1, R3 and R4 isolates. However, no differences between the treatment and control were detected for R2 and S isolates. Interestingly, in the cases of R1 and R3, the expression values for control are higher than the treated ones. This might indicate that the pathogen might have developed alternative mechanisms of resistance that are not reflected in gene expression levels. However, in the case of R4, almost 10X high expression levels were detected for the treatment, as compared to the control. This indicated that the expression of the RPA190 in this isolate was induced by the application of fungicide. In conclusion, gene overexpression can be assumed to be associated with fungicide resistance in the case of R4. The detailed results can be seen in Table 10.

Table 10: Significance level of RPA190 gene expression, Metalaxyl resistant and Sensitive Isolates

Isolate	Avg ($2^{-\Delta\Delta Ct}$)	St dev	P-value
S1 DMSO	0.04	0.04	0.95
S1 MET	0.03	0.06	
R1 _DMSO	0.66	0.22	0.00*
R1_MET	0.03	0.02	
R2 _DMSO	0.02	0.02	0.80
R2_MET	0.02	0.03	
R3 _DMSO	0.05	0.01	0.05*
R3_MET	0.02	0.02	
R4 _DMSO	0.06	0.02	0.01*
R4_MET	0.60	0.37	

*Indicates significance at a 5% significance level.

5.8 Greenhouse Experiment

Commercially available fungicides were used to evaluate their potential in the greenhouse. 10 different parameters were checked, and the efficacy of fungicides was compared by

comparing plant health index. Each treatment was repeated 10 times to acquire the best possible results. Plant health index (PHI) and performance of treatment were calculated. Acrobat fungicide displayed the highest PHI and performance of treatment values (Table 11) followed by Ridomil+Ortiva. Based on the greenhouse experiment we can suggest that Acrobat fungicide with Dimethomorph as the active compound is efficient in inhibiting *P. cactorum* infections compared to other fungicides used.

Table 11: Data of Green House Experiment with Standard deviation (±)

Parameters	Acrobat	Ridomil+Ortiva	Acrobat+ Retengo	K+Phythopthora	Control
Number of leaves	5.3 (±1.06)	5.9(±2.02)	4.9(±1.73)	5.2(±1.48)	5.1(±0.88)
Weight of leaves	4.289(±1.80)	6.07(±2.58)	3.3(±2.61)	1.889(±0.91)	4.749(±1.27)
Number of fruits	0.3(±0.48)	0(±0)	0.2(±0.63)	0.6(±1.26)	0.2(±0.42)
Weight of fruits	0.229(±0.39)	0(±0)	0.119(±0.38)	0.149(±0.31)	0.149(±0.32)
Number of blooms	0(±0.00)	0(±0)	0(±0)	0.4(±0.97)	0(±0)
Weight of blooms	0(±0.00)	0(±0)	0(±0)	0.039(±0.10)	0(±0)
Diameter of root neck	6.983(±1.13)	7.398(±1.06)	6.412(±1.09)	6.802(±2.14)	7.122(±0.62)
Length of roots	10.4(±4.12)	7.8(±3.77)	6.7(±3.87)	5.85(±2.19)	7.1(±3.34)
Weight of roots	5.592(±5.59)	4.652(±1.81)	4.807(±1.62)	4.002(±1.59)	5.725(±1.25)
Weight of root neck	0.618(±0.61)	0.727(±0.17)	0.485(±0.16)	0.588(±0.24)	0.693(±0.19)
The sum of the mean of all parameters	33.711	32.547	26.923	25.519	30.838
PHI	109.32	105.54	87.30	82.75	100.00
Performance of treatment	9.32	5.54	-12.70	-17.25	0.00

5.9 Visual Symptomatic Scaling

Contrary to physical appearance and PHI scoring, the combination of Acrobat+Retengo fungicide with active compound Dimethomorph+Pyraclostrobin respectively showed maximum protection of roots from root rot by *P. cactorum* and all plants remained healthy with no symptom of disease. Ridomil+Ortiva stands as the second most effective disease control fungicide. Cross sections of root necks infected by *P. cactorum* can be compared with healthy root necks in Figure 13 while results of symptomatic scaling are available in Table 12.

Table 12: Scaling Based on Visual Symptoms of Crown Rot

Treatment	Alpha	Bravo	Charlie
Acrobat	5	5	0
Ridomil+Ortiva	8	2	0
Acrobat+Retengo	10	0	0
***Phytophthora* treated**	0	0	10
Control	10	0	0
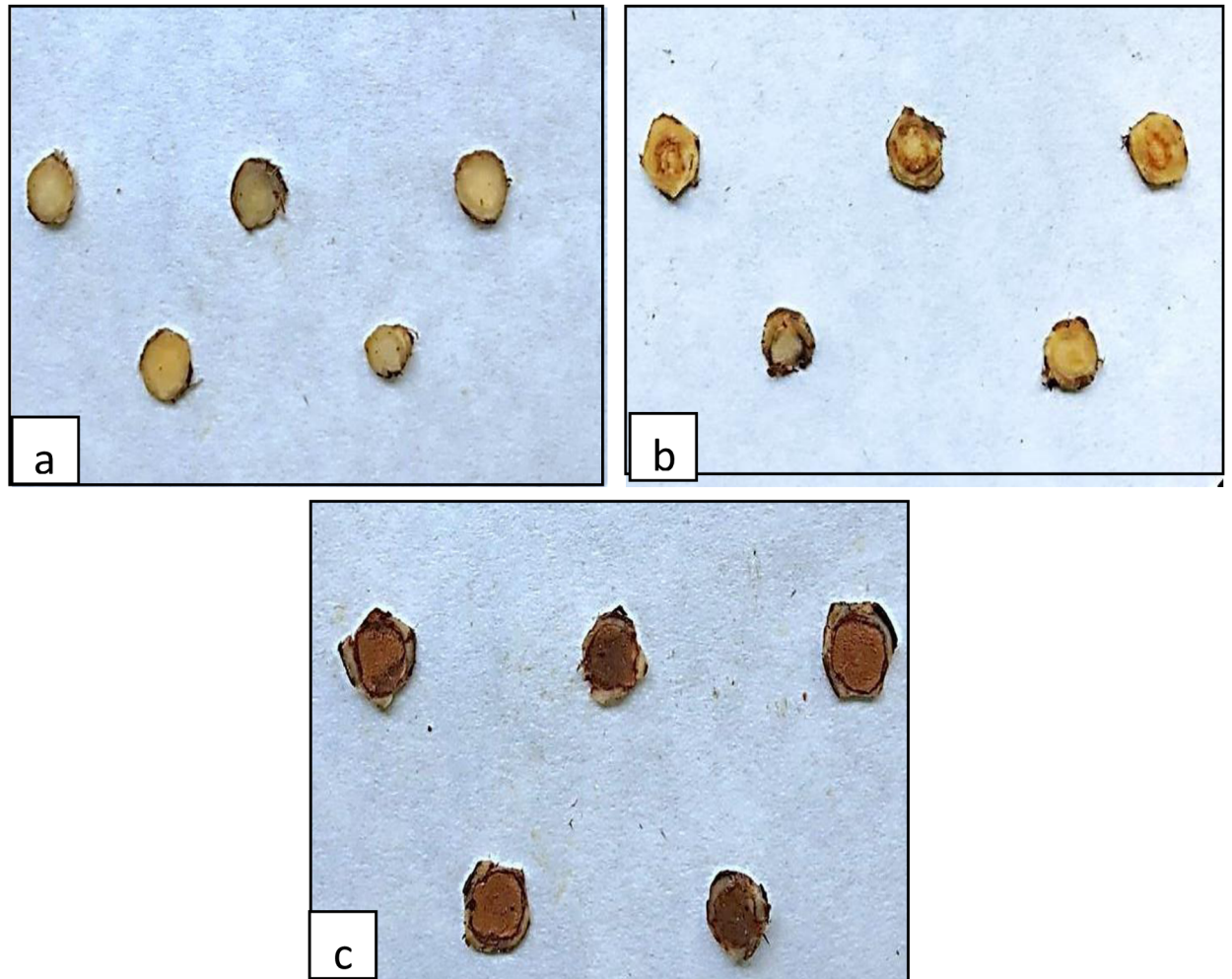
Figure 13: Cross section of strawberry root neck, a) Alpha class: with no symptoms of crown rot b) Bravo class: mild symptoms of crown rot c) Charlie class: severe symptoms of crown rot

5.10 Biological Control of *P. cactorum* Using Microalgae (*T. lutea*)

5.10.1 Growth of *T. lutea* in Photobioreactors

From our previous experience, we realized that *T. lutea* can grow normally at 25°C and hence all the photobioreactors were adjusted at room temperature. For our greenhouse and antifungal bioassay, we need about 500 g of dried disrupted biomass which corresponds to 2.5 kg of wet biomass because of the water presence inside cells i.e. about 80%. To upscale inoculum preparation *T. lutea* was first cultivated in an infors photo bioreactor with a capacity

of 1800 ml. Maximum optical density (OD) was observed on day 7th and then gradually decreased which can be observed in Figure 15. Infors reactor was harvested on day 11 which was used as inoculum to start the flatpanel photobioreactor with a capacity of 20 l. Starting OD of the flatpanel reactor was 0.04 and the final was 2.832 after 9 days as depicted in Figure 16, as the culture in the flatpanel reactor was dense enough, it was harvested and used as inoculum to start the HTI photobioreactor with a capacity of 300 l and a yield of about 1.6 kg in a single batch. The first batch of *T. lutea* was harvested after 7 days with recorded OD of 2.24 as shown in Figure 17 followed by centrifugation. Concentrated biomass obtained was washed 2 times with 0.5 M ammonium formate buffer and stored at -20°C. The main pigments present in this microalgae are chlorophyll, carotenoids, and a particularly enormous quantity of fucoxanthin which imparts a golden brown colour to the culture. Microscopic images of *T. lutea* obtained during experiments are given in Figure 14.
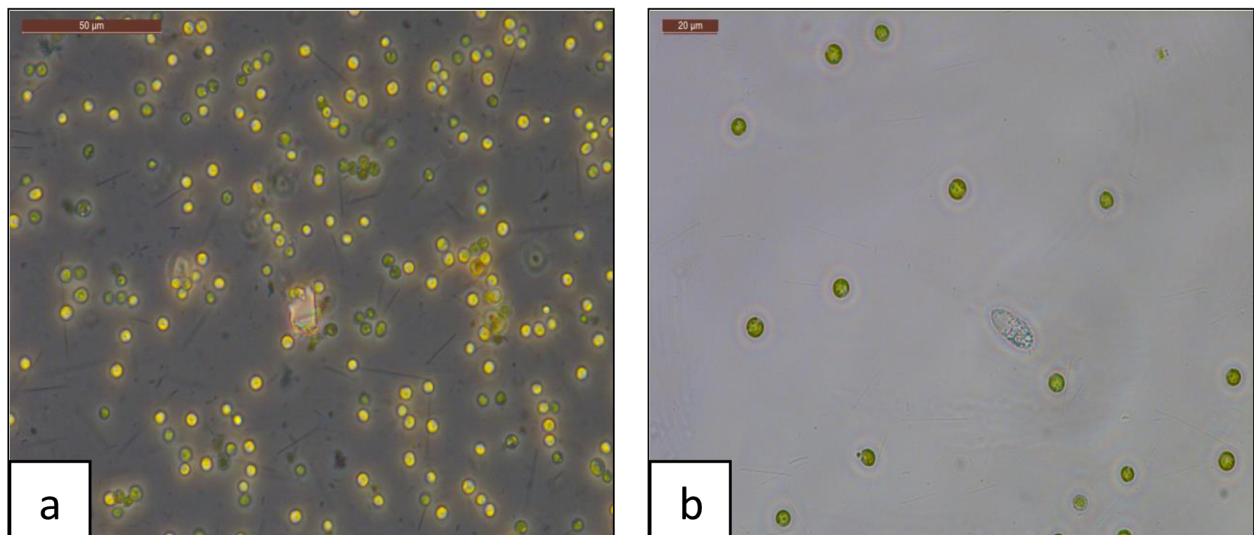

Figure 14: *T. lutea* microscopic images a) 10 X magnification b) 40 X magnification
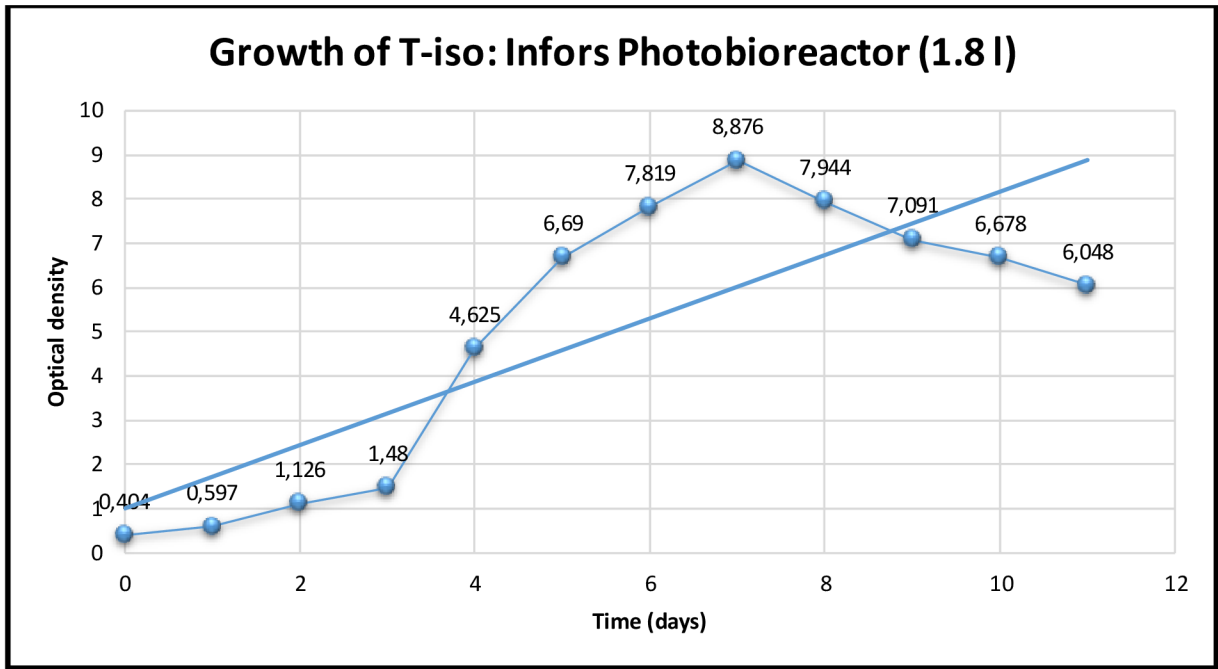
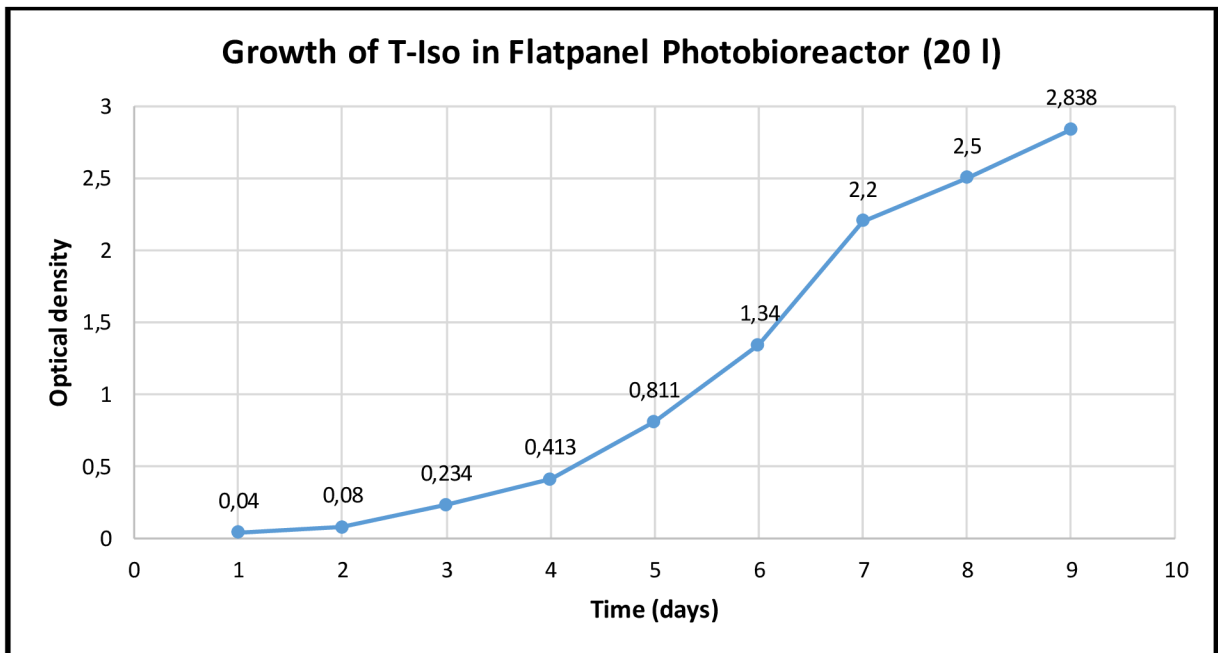**Figure 15:** Growth curve of *T. lutea*, infors photobioreactor (1.8 l)

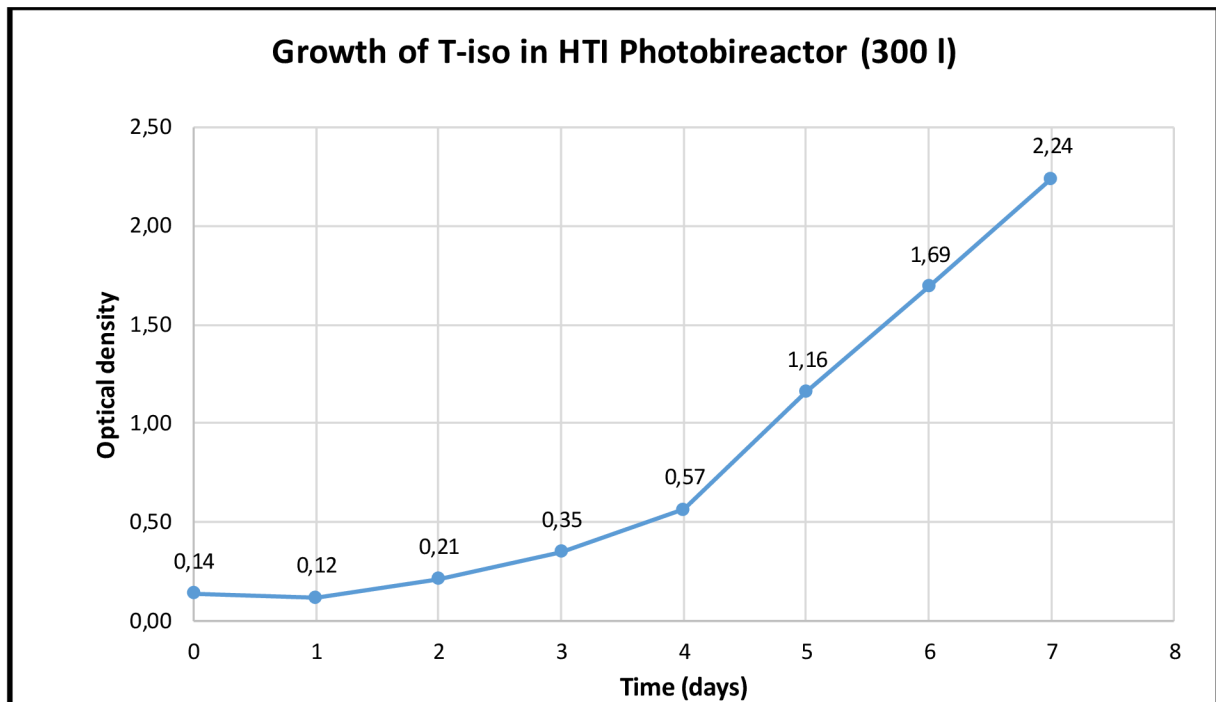**Figure 16:** Growth curve of *T. lutea*, flatpanel photobioreactor (20 l)

Figure 17: Growth curve of *T. lutea*, HTI photobioreactor (300 l)

5.10.2 *T. lutea* Cell Size Measurements

Stored concentrated biomass was thawed at room temperature and diluted to a concentration of 100 g/l. The diameter of the cells was checked using the Beckman coulter counter, Multisizer 3. Intact, healthy *T. lutea* cells range from 4-7 µm in diameter which corresponds to our findings in Figure 18 (a). Cells were then disrupted with Beadmill, and post disruption coulter counter analysis depicts the change in diameter of the cells, as at this stage more than 90% of the cells were disrupted (Figure 18 b). After confirmation of cell disruption, disrupted biomass was freeze dried and stored at -20°C.
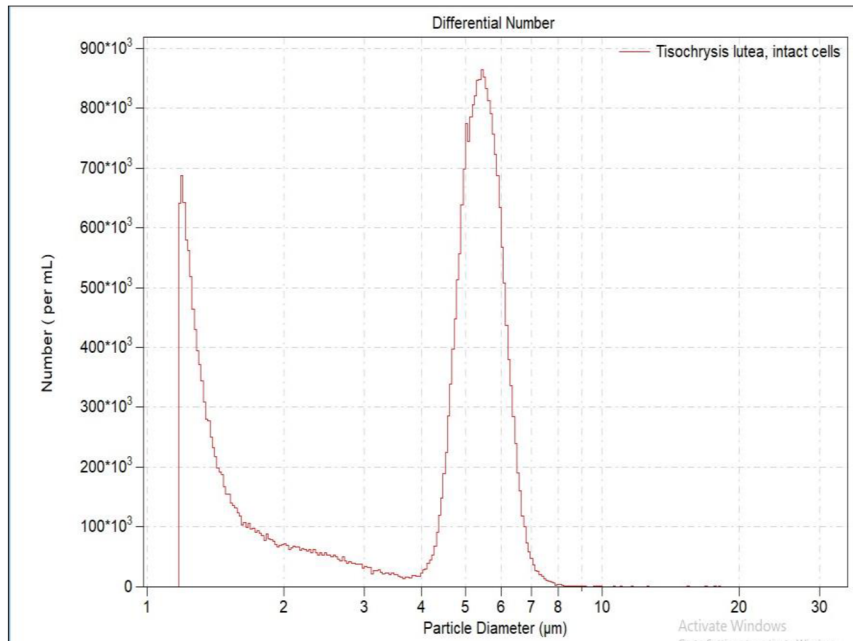
A
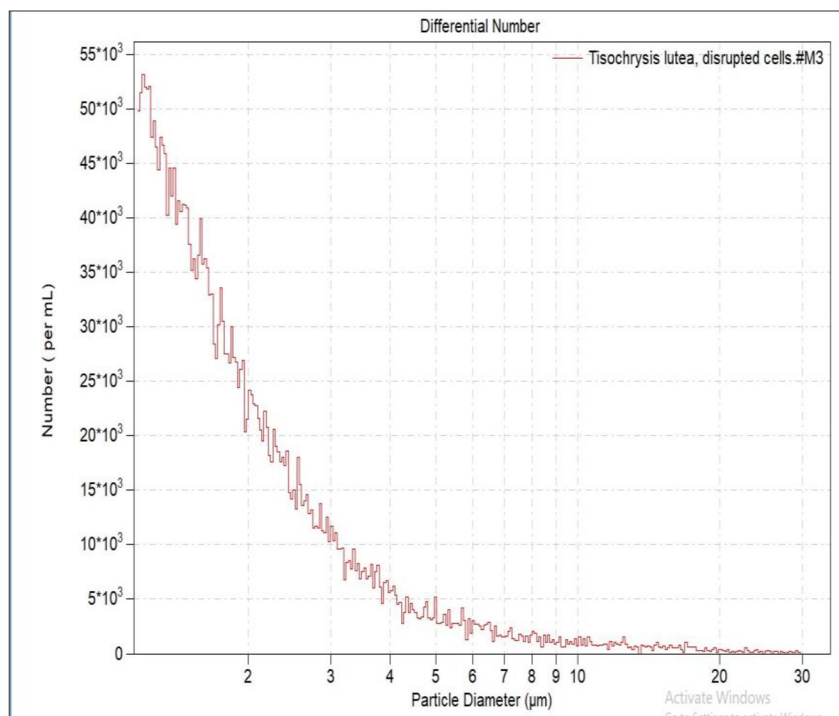
B

Figure 18: Cell size measurement with multisizer, a) Intact *T. lutea* cells b) Disrupted cells

5.11 Antifungal Bioassay

The concentrations used to find out EC_{50} values were 1, 2, 3, 4, 5, 10, 20 and 30 mg/ml. Eight *P. cactorum* isolates were selected out of 141 isolates for this assay, 4 isolates were highly resistant to Metalaxyl while the remaining 4 were highly sensitive. Mycelial growth of all

these isolates was inhibited after the addition of CBE. Recorded EC_{50} values and mycelial growth inhibition percentages are given in Table 13.

Table 13: Mycelial growth (MG %), mycelial growth inhibition % (MGI), and EC_{50} values recorded for *P. cactorum* isolates after treatment with CBE

Isolates		Concentration of crude bio extract (CBE) in mg/ml								EC_{50}
		0	1	2	3	4	5	10	20	
R1	MG %	100.00	26.72	23.28	19.40	17.28	17.24	14.66	14.22	**0.5918**
	MGI %	0.00	73.28	76.72	80.60	82.72	82.76	85.34	85.78	
R2	MG %	100.00	33.61	31.93	29.41	16.81	13.87	12.61	12.35	**0.8059**
	MGI %	0.00	66.39	68.07	70.59	83.19	86.13	87.39	87.65	
R3	MG %	100.00	32.20	32.67	29.66	19.07	15.51	15.13	12.84	**0.8334**
	MGI %	0.00	67.80	67.33	70.34	80.93	84.49	84.87	87.16	
R4	MG %	100.00	27.59	22.41	18.10	17.24	16.68	15.78	15.39	**0.5855**
	MGI %	0.00	72.41	77.59	81.90	82.76	83.32	84.22	84.61	
S1	MG %	100.00	36.55	31.51	27.73	23.95	16.39	13.87	12.61	**0.9003**
	MGI %	0.00	63.45	68.49	72.27	76.05	83.61	86.13	87.39	
S2	MG %	100.00	27.39	24.90	19.92	18.67	18.30	17.55	17.30	**0.6441**
	MGI %	0.00	72.61	75.10	80.08	81.33	81.70	82.45	82.70	
S3	MG %	100.00	32.49	30.38	23.63	23.04	22.78	19.41	18.35	**0.8657**
	MGI %	0.00	67.51	69.62	76.37	76.96	77.22	80.59	81.65	
S4	MG %	100.00	28.98	28.47	28.21	24.26	23.11	21.96	21.57	**0.8686**
	MGI %	0.00	71.02	71.53	71.79	75.74	76.89	78.04	78.43	

5.12 Bi-phasic Extraction

1 gram of CBE was dissolved in 150 ml of solvent and the recovered extracts were i.e., n-hexane 375.5 mg, methanol 313 mg, chloroform 543.2 mg, and Milli Q water 230 mg. Milli Q extracts were recovered using a freeze drier. Antifungal activity of the extracts was performed against the S4 isolate. It was found that antifungal potential is because of some water-soluble compounds (Figure 20). Recovered extracts are given in Figure 19.
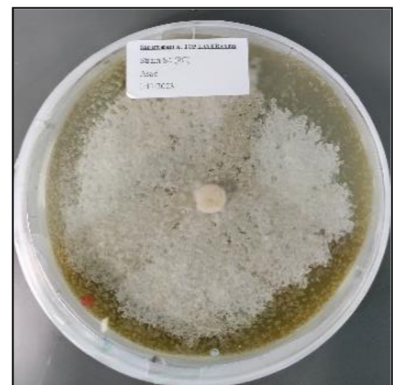
Figure 19: n-haxane, methanolic, chloroform, and water-soluble extracts recovered from CBE with Rotavap
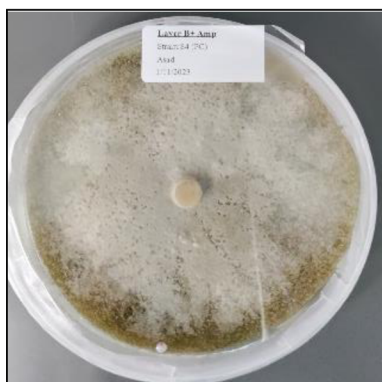

A

B

C

D

E

Figure 20: *P. cactorum* tested on 3 phases of n-hexane, recovered during extraction of metabolites
a) V8 agar: Control,   b) on n-hexane,   c) top layer   d) middle layer   e) Water soluble extracts

5.13 Scanning Electron Microscopy

Scanning electron micrographs when compared, showed (figure 24) complete inhibition of sporangia of R1 isolate when treated with CBE at 5 mg/ml. Mycelia appears to be flattened and drained while that of control looks healthy and turgid. Numerous sporangia can be observed in the control R1 isolate. The average mycelial diameter of the control was about 3.3 µm while in CBE treated R1 isolate diameter of mycelia was around 4.2 µm. This increase could be because of drying as visible followed by flattening and flaccidity of mycelia. The average size of sporangia recorded was about 17.2 µm. The micrographs below are at 350 X magnification.
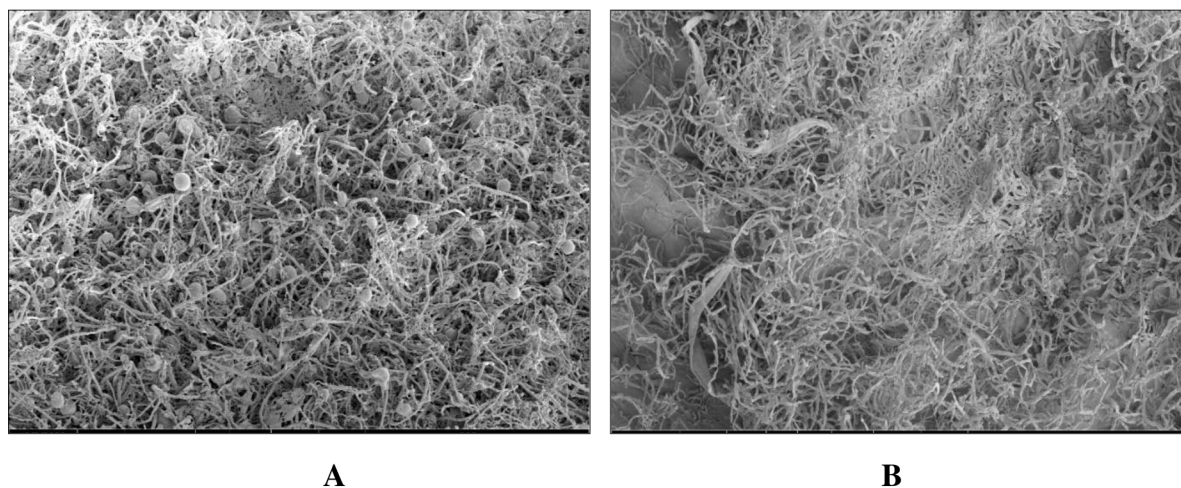

A   **B**

Figure 21: Scanning Electron Micrographs of (A) R1 control (B) R1 treated with CBE, 5 mg/ml

5.14 Fourier Transform Infrared Spectroscopy (FTIR)

The chemical bonds or functional groups present in the dried crude bioextract were predicted by FTIR. The bonds were determined by interpreting the infrared absorption spectra. Figure 22 showed the FTIR spectrum of the dried crude bioextract dissolved in chloroform, while Figure 23 showed the interpretation of the chemical bonds in the methanolic extracts, Figure 24 showed results of n-hexane, and Figure 25 depicted water-soluble compounds in crude bio extract.

Strong bonds in the functional group region found in chloroform extracts range from 3011 cm−1709 cm− 1 which showed the presence of alkanes, amine salts, aldehyde group, aliphatic ketones, and carboxylic acid while in fingerprint region sulfoxide, sulfonic acid, amine groups

are detected. Similar results were also observed in methanolic extracts i.e., alcohol, alkane, and nitro compounds were detected in the functional group region while in the fingerprint region besides sulfonic acid and sulfoxide, secondary alcohol structures were also predicted. In n-hexane extracts list of peaks were a bit higher than in semipolar solvent i.e., chloroform. Conjugated aldehyde, alkenes, nitro compounds, and methylene groups were detected in the functional group region while in the fingerprint region, additional compounds detected were aromatic amines, vinyl ether, ester, monosubstituted benzene derivatives, and secondary alcohol peaks. Details of detected peaks and corresponding functional groups are given in Table 14.
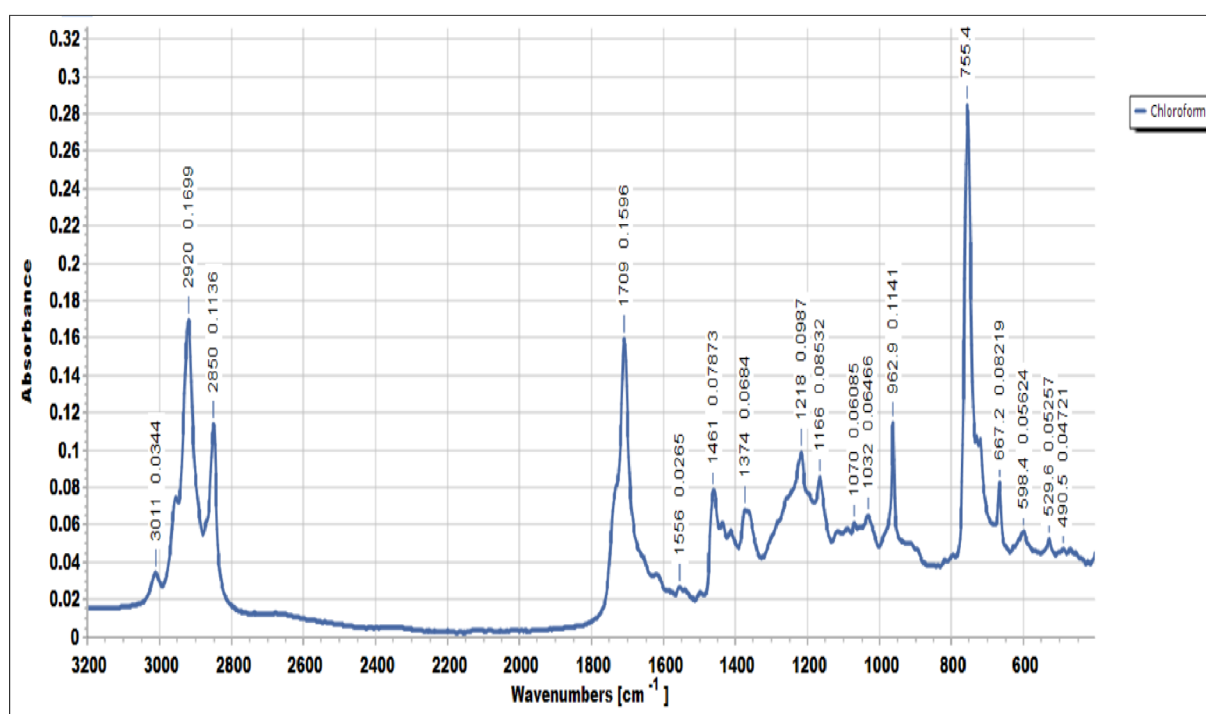

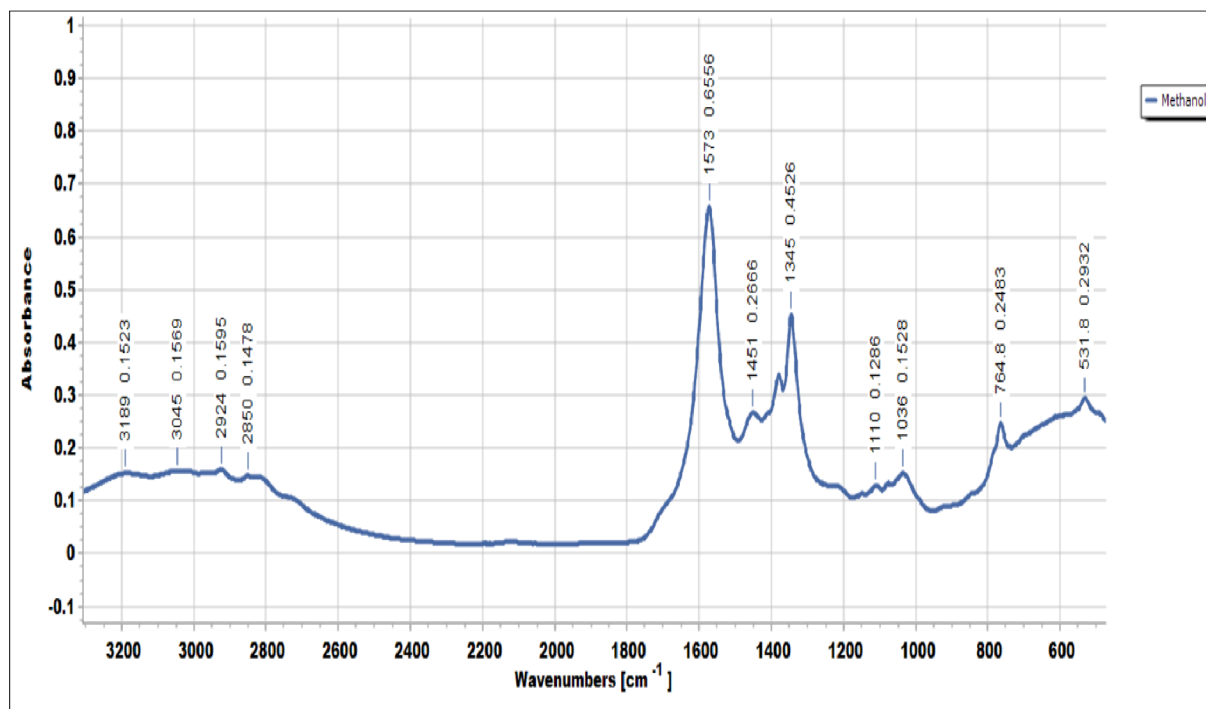**Figure 22:** FTIR spectrograph of metabolites extracted in chloroform

Figure 23: FTIR spectrograph of metabolites extracted in Methanol
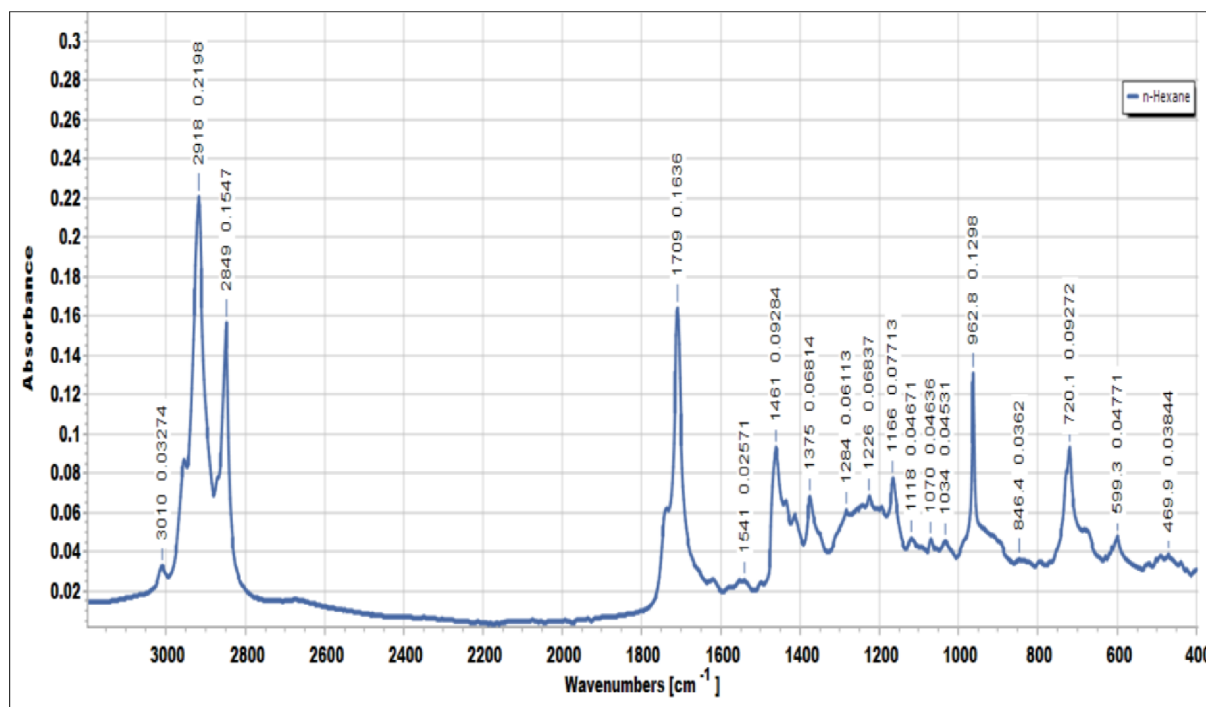

Figure 24: FTIR spectrograph of metabolites extracted in n-hexane
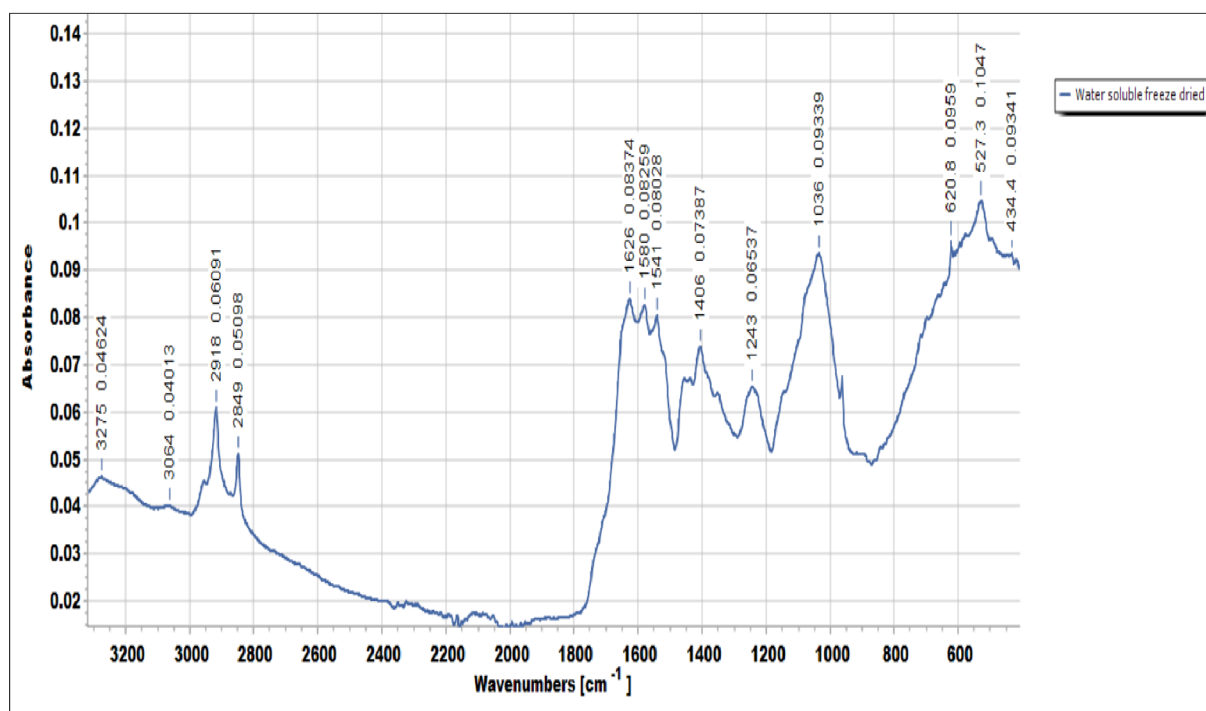
Figure 25: FTIR spectrograph of crude bio extract, extracted in Milli Q

Table 14: FTIR analysis, detected peaks with respective functional groups in CBE

	FTIR Peaks (cm¹)	Functional Group	
Chloroform	3011	C-H	alkane
	2920	N-H	amine salt
	2850	C-H	aldehyde
	1709	C=O	aliphatic ketone/ carboxylic acid
	1556	N-O	nitro compound
	1461	C-H	alkane
	1374	C-H	alkane
	1218	C-N	amine
	1166	S=O	sulfonic acid
	1070	S=O	sulfoxide
	1032	S=O	sulfoxide
	963	C=C	alkene
	755	C-H	1,2 disubstituted
	667	C=C	alkene

	3189	O-H	alcohol
Methanol	3045	C-H	alkane
	2924	C-H	alkane
	2850	C-H	alkane
	1573	N-O	nitro compound
	1451	C-H	alkane (methyl group)
	1345	S=O	sulfonic acid
	1110	C-O	secondary alcohol
	1036	S=O	sulfoxide
	764	C-H	1,3-disubstituted
	3010	C-H	alkene
	2918	C-H	alkane
	2894	C-H	alkane
	1709	C=O	conjugated aldehyde
	1541	N-O	nitro compound
	1461	C-H	alkane (methylene group)
	1375	C-H	alkane (methyl group)
n-Hexane	1284	C-N	aromatic amine
	1226	C-O	vinyl ether
	1166	C-O	ester
	1118	C-O	secondary alcohol
	1070	S=O	sulfoxide
	1034	S=O	sulfoxide
	962	C=C	alkene
	846	C=C	alkene
	720	C-H	monosubstituted benzene derivative
	3275	O-H	carboxylic acid
	3064	C-H	alkene
	2849	C-H	alkane
	1626	C=C	alkene
	1580	N-H	amine
Water soluble	1541	N-O	nitro compound
	1406	S=O	sulfonyl chloride
	1243	C-O	alkyl aryl ether
	1036	S=O	sulfoxide

5.15 UHPLC-HRMS-MS DDA (Data Dependent Analysis)

Water extraction of crude extract highlighted a superior number of molecular features (ESI+ and ESI-). The number of Water-soluble compounds are more than methanol soluble ones. In total 2640 peaks for water soluble compounds were detected while methanol soluble were around 2115. About 150 compounds were identified from crude bio extract through UHPL analysis and the majority of them appear to be carbohydrates, fatty acids, amino acid derivates, and peptides, as these are the major constituents of cells. Traces of terpenoids and alkaloids were also detected. Spectrographs from the analysis showing observed peaks with (ESI +) (ESI -) are given below in Figure 26, Figure 27, Figure 28, Figure 29 with description mentioned in captions.
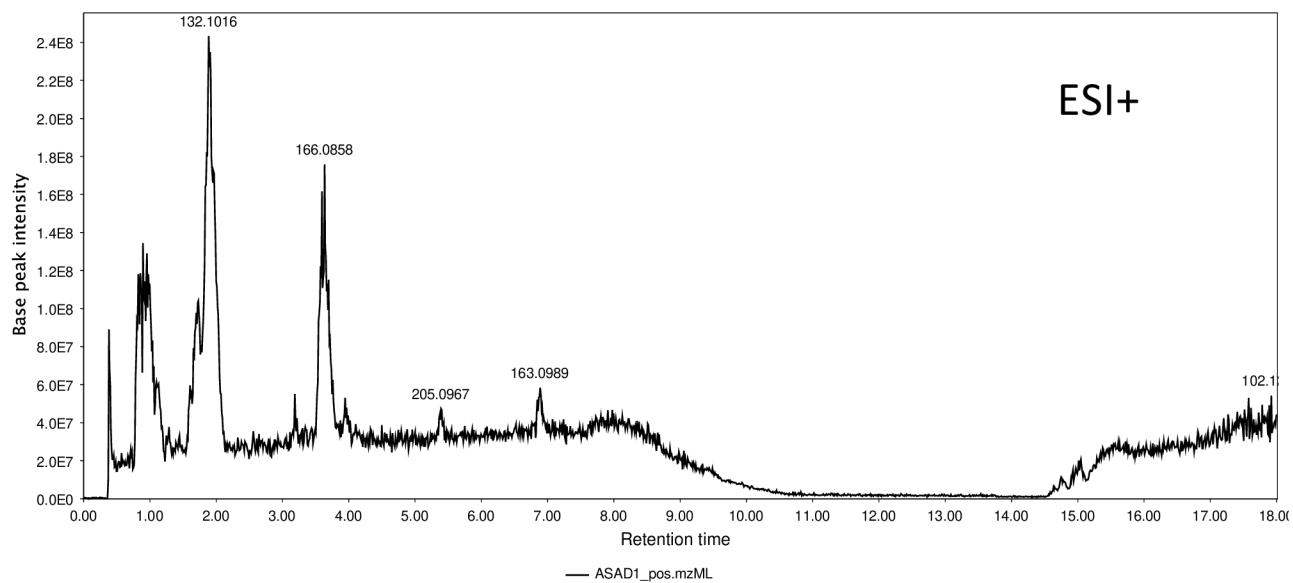
Figure 26: UHPLC spectrograph of crude bio extract sample extracted in MeOH/ H2O (50:50), ESI+
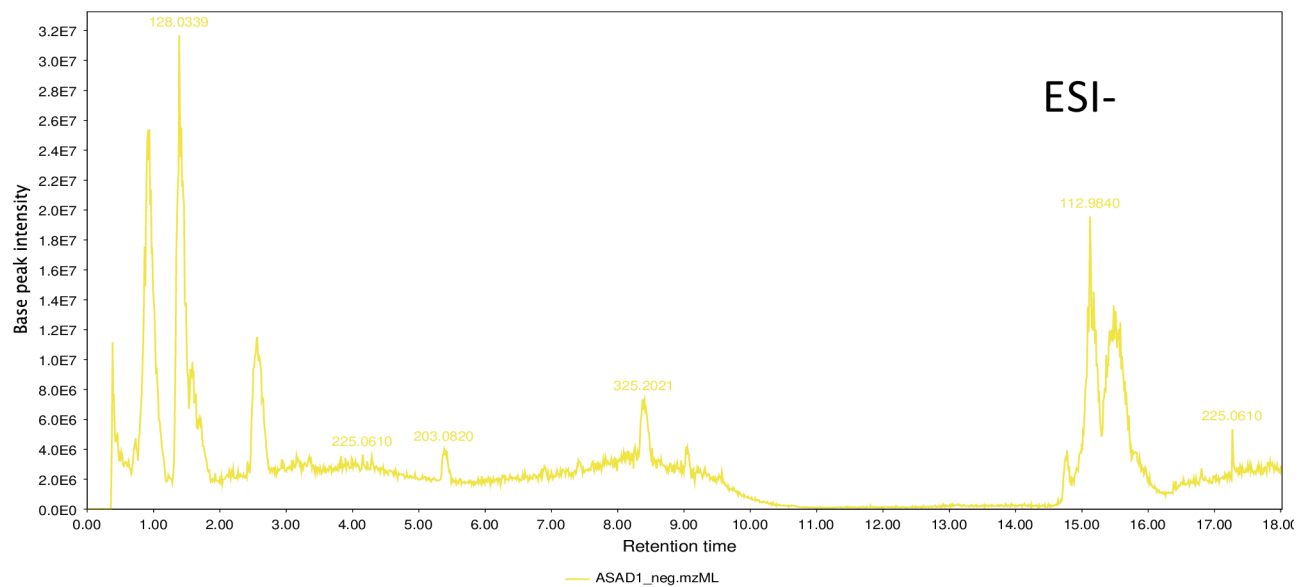

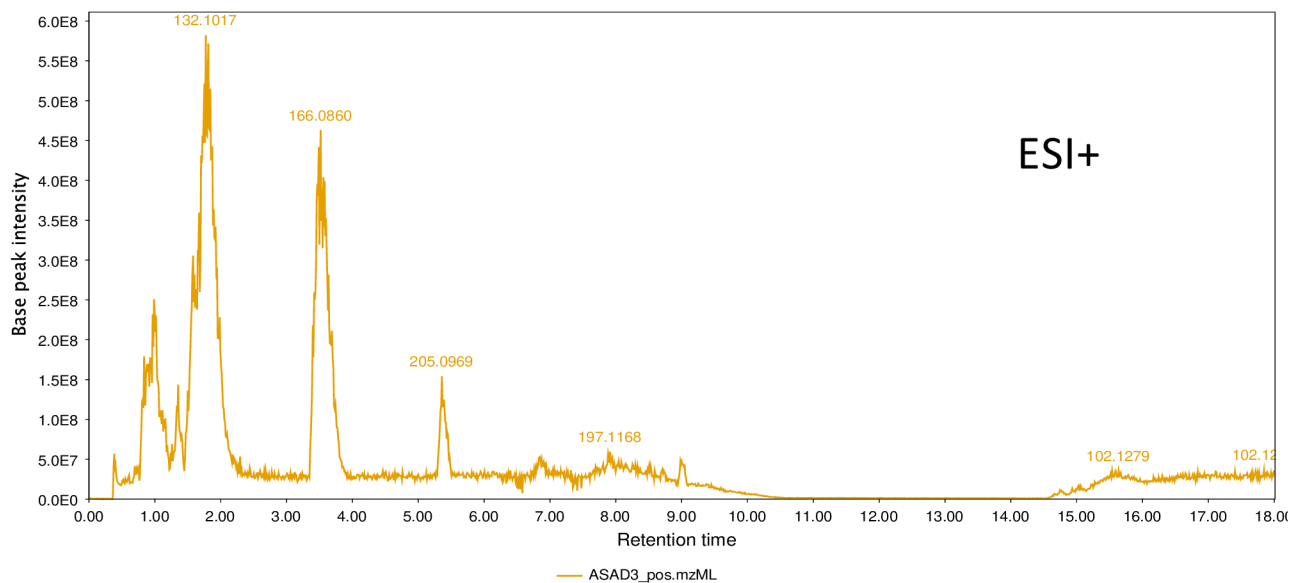**Figure 27:** UHPLC spectrograph of crude bio extract sample extracted in MeOH/ H2O (50:50), ESI-

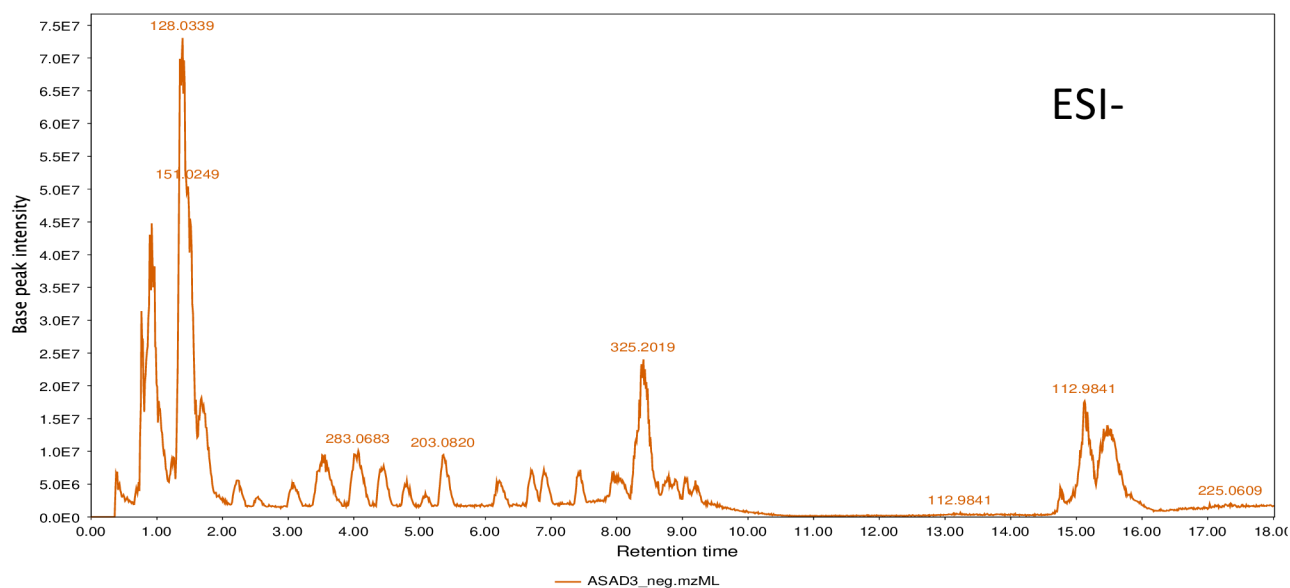**Figure 28:** UHPLC spectrograph of crude bio extract sample extracted in H2O, ESI+

Figure 29: UHPLC spectrograph of crude bio extract sample extracted in H2O, ESI-

5.16 *T. lutea* as Biostimulant and Biocontrol Agent: Greenhouse Experiment

Different growth parameters and strawberry production were recorded in the greenhouse experiment. Table 15 shows treatments executed in the experiment. Growth parameters recorded were the total length of plants, length of shoots, the total weight of the plant, the total weight of shoots, the weight of roots, the weight and diameter of root neck, and strawberry production in grams. Plant health index percentage was calculated for all

61

treatments, followed by the transformation of percentage data into Arcsine values. It was concluded that in the biostimulant part, treatment E i.e., 8 g/l has a maximum PHI value compared to the control but no significant difference was observed according to the Kruskal-Wallis test i.e. p>0.99. At concentrations higher than 8 g/l slight reduction in health index was observed. In the biocontrol experiment 10 g/l has a maximum PHI value of 8 g/l while in overall comparison, commercially available fungicide against *P. cactorum* i.e., Fosetyl-AL (treatment M) has the highest plant health index value. Kruskal-Wallis test revealed a significant difference in growth parameters between the control and treated plants, ** indicates high significance between control and T.K (p < 0.0008), *** indicates significance difference between control and T.L (p = 0.0086), *** indicates high significance between control and TM (p <0.0001) as shown in Figure 33. Production of strawberries was not significantly affected by the addition of CBE in both experiments. However, in the biocontrol experiment when plants were inoculated with *P. cactorum* (treatment G) production of strawberries was significantly lower than the plants treated with CBE and FoAL. It was also concluded that increasing concentrations of CBE can positively affect the plant defense system. Data description of total harvested strawberries in grams, and plant health index calculated for both experiments is given in Figure 30, Figure 31,

Figure *32*

Figure 33.

Table 15: Different treatments and concentrations of CBE used in the greenhouse experiment

		Biostimulant Part
Treatment A	T.A	**(Control) Potting soil + strawberry plant**
Treatment B	T.B	Potting soil + strawberry plant + CBE concentration 2 g/l
Treatment C	T.C	Potting soil+ strawberry plant + CBE concentration 4 g/l
Treatment D	T.D	Potting soil+ strawberry plant + CBE concentration 6 g/l
Treatment E	T.E	Potting soil+ strawberry plant + CBE concentration 8 g/l
Treatment F	T.F	Potting soil+ strawberry plant + CBE concentration 10 g/l
		Biocontrol Part
Treatment G	T.G	Potting soil+ strawberry plant + *P. cactorum*
Treatment H	T.H	Potting soil+ strawberry plant +*P. cactorum* +CBE concentration 2 g/l

| Treatment I	T.I	Potting soil+ strawberry plant +*P. cactorum* +CBE concentration 4 g/l
Treatment J	T.J	Potting soil+ strawberry plant +*P. cactorum* +CBE concentration 6 g/l
Treatment K	T.K	Potting soil+ strawberry plant +*P. cactorum* +CBE concentration 8 g/l
Treatment L	T.L	Potting soil+ strawberry plant +*P. cactorum* +CBE concentration 10 g/l
Treatment M	T.M	Potting soil+ strawberry plant + Fosetyl-AL+ *P. cactorum*
Treatment N	T.N	Potting soil+ strawberry plant + Fosetyl-AL
Treatment O	T.O	Potting soil+ strawberry plant + Kristalon
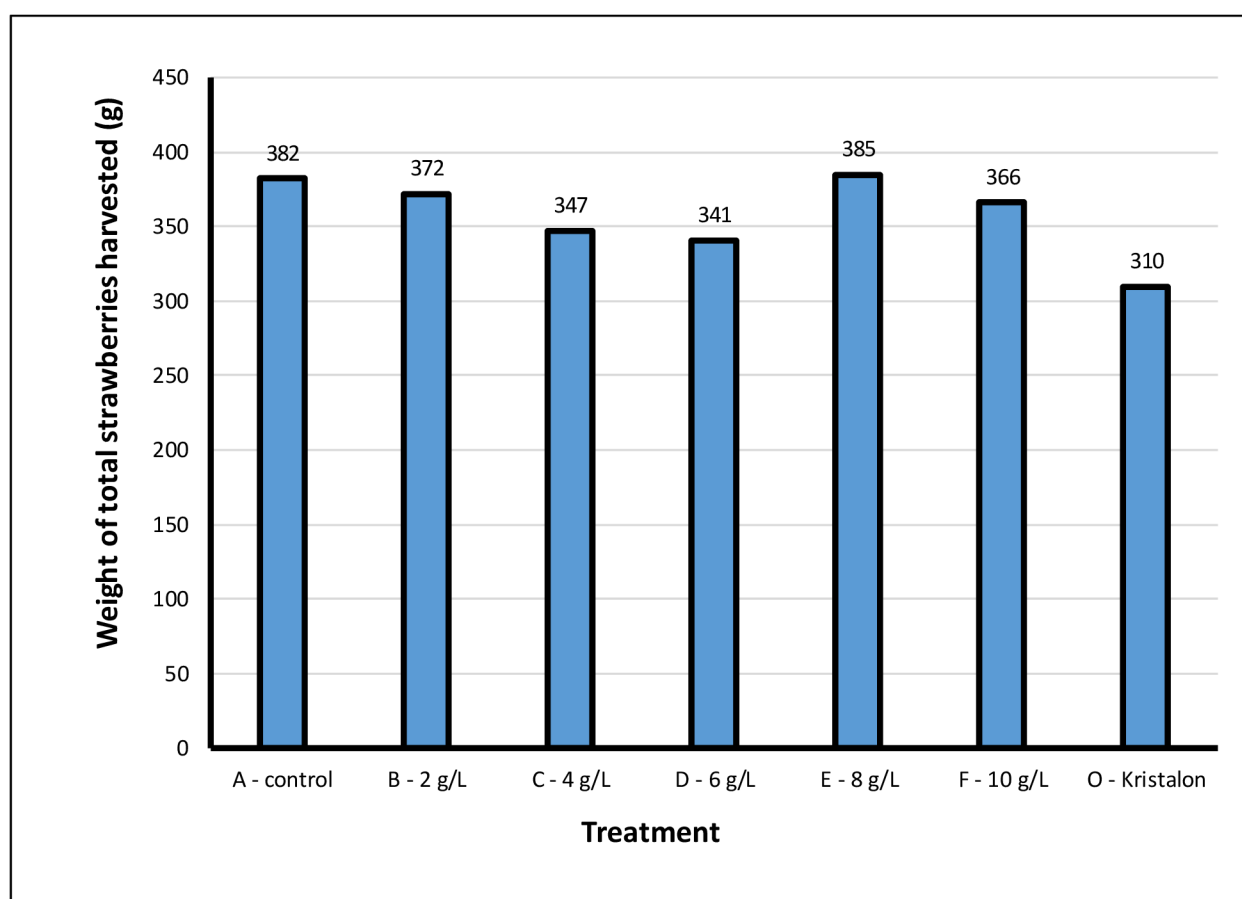
Figure 30: Biostimulant part: Total production of strawberries (g) with different concentrations of CBE
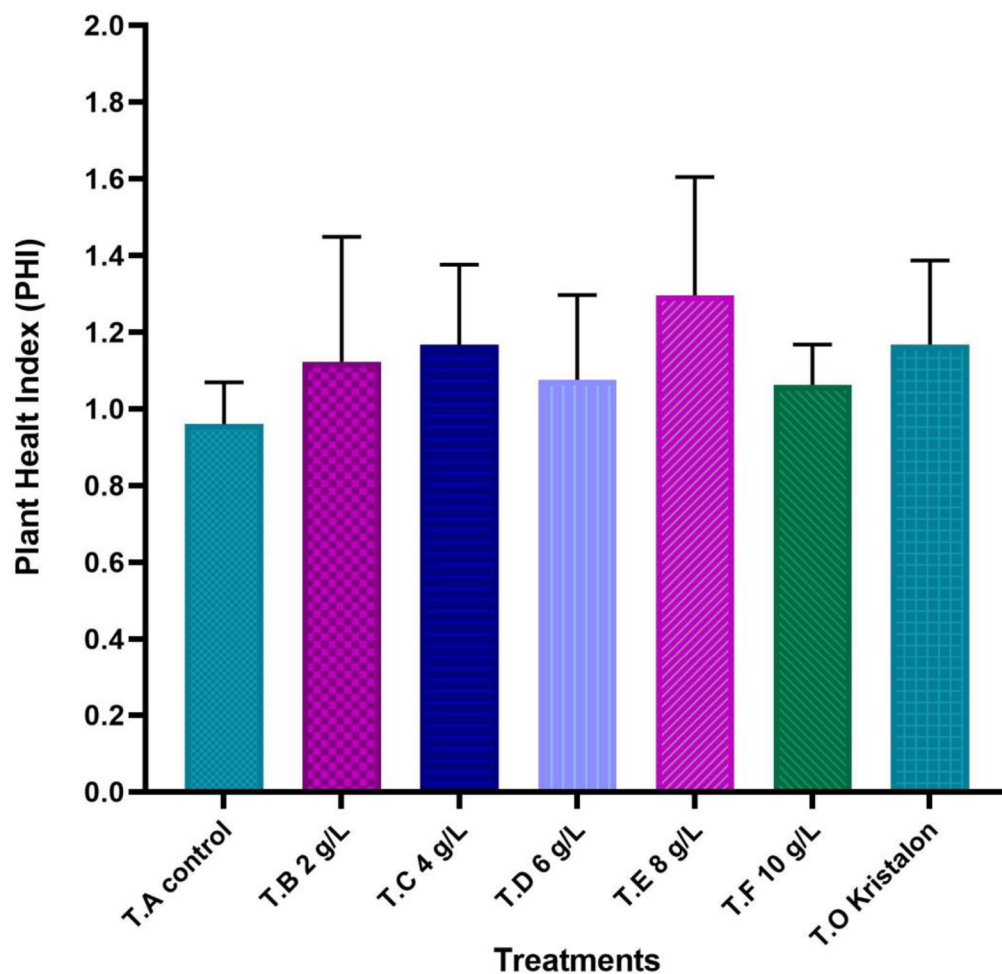
Figure 31: Plant health index (%) of biostimulant experiment with treated concentrations of CBE, T.A represent control without any treatment. Data was normalized (arcsine transformed), Kruskal-Wallis test did not reveal any significant difference in growth between the control and treated plants, Results are the mean values with SD indicated by error bars from seven independent replicates.
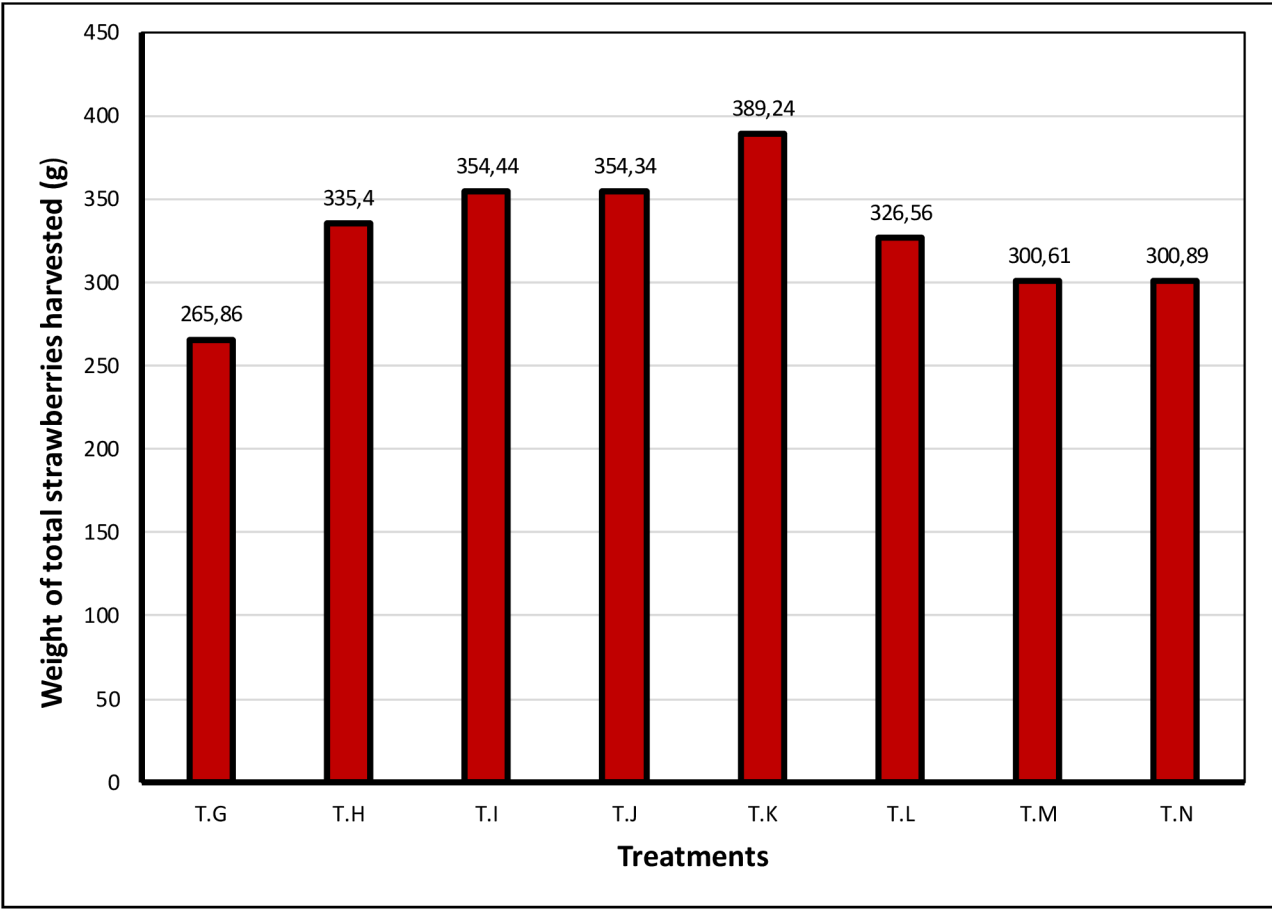
Figure 32: Biocontrol part: Total production of strawberries (g) with different concentrations of CBE
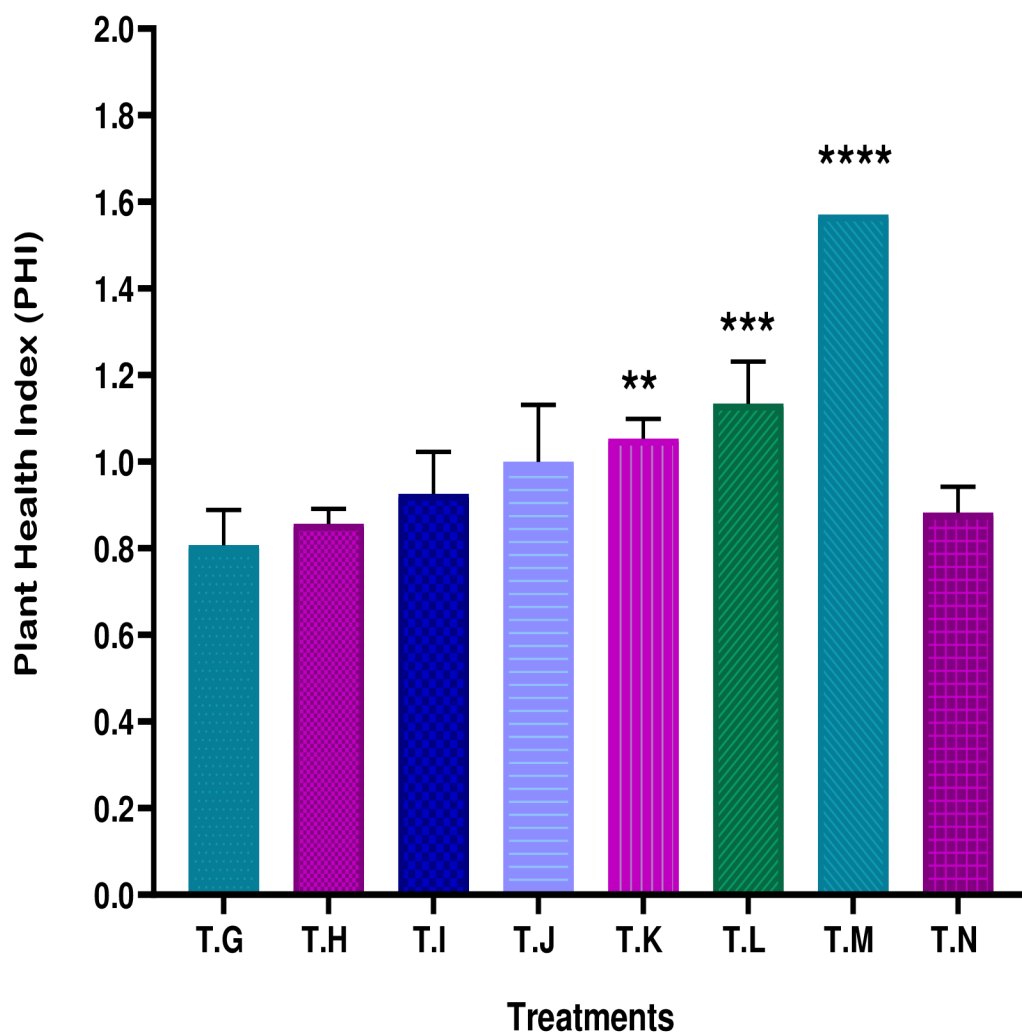
Figure 33: Plant health index (%) of biocontrol experiments with treated concentrations of CBE, T.G represents control without any treatment. Data was normalized (arcsine transformed), Kruskal-Wallis test revealed a significant difference in growth between the control and treated plants, Results are the mean values with SD indicated by error bars from seven independent replicates. The asterisks **, ***, and **** denote the significant values $P < 0.01$, $P < 0.001$, and $P < 0.0001$, respectively.

Chapter 6: Discussion

6.1 Strawberries: Economic Importance, Demands and Limitations

Worldwide strawberry production was valued at 14 billion USD in 2020 (FAO. 2022. FAOSTAT n.d.) and the global production soared 41% from 2004 to 2020 which indicates the demand and growing economic importance of the crop. Strawberries are produced in 76 countries and China is the largest producer worldwide, accounting for 5 billion USD, over 3 times the value of the second-largest producer, the United States of America. Within the European Union, strawberries are grown in 26 countries but Spain, Poland, and Germany account for 58% of the production with a contribution of 28%, 17%, and 13% respectively. The Average per capita consumption in the EU is 5 kg slightly higher than the second largest producer USA i.e., 4.5 kg (Simpson 2018). Because of the global demand and popularity among consumers strawberries need to be transported to different markets. Despite adopting cool chain marketing and improved shelf-life techniques, this fruit remains perishable and needs to be produced close enough to the consumer market. Among the biggest threats to commercial strawberry production are extreme weather and pressure from pests and diseases (Hernández-Martínez et al. 2023). One of the existential threats to strawberry plants is *P. cactorum* as it affects roots and fruits causing root rot and leather rot respectively.

6.2 Phytophthora Crown and Leather Rot in Strawberries: Importance and Management

Phytophthora crown rot and leather rot caused by *P. cactorum* are two of the most damaging diseases of strawberries worldwide causing up to 40% of production loss (Stensvand et al. 1999). Because of its soil-borne nature and formation of sexual oospores that survive for many years in the soil, *P. cactorum* is difficult to handle in infested fields. Yield loss due to soilborne diseases costs about 15 million USD each year in Florida (Sharma et al. 2022) and about 150 million USD all over the USA(Samtani et al. 2019).

Globally different strategies have been adopted to manage crown and leather rot which include the selection of good planting sites, appropriate root stock, resistant cultivars, raised beds for planting, proper drainage, avoiding flooded irrigation, crop rotation, soil solarization, heat treatment of plantings before plantation, use of straw mulches, application of composted animal manures followed by plastic mulching increased soil microbial activity and reduced

the incidence of *Phytophthora* crown and root rot (Núñez-Zofío et al. 2011).

The suppression of diseases i.e., Crown rot and leather rot, caused by *P. cactorum* mainly relies on the use of fungicides. Chemical fungicides like Azoxystrobin, Pyraclostrobin, Mefenoxam, potassium phosphite, Metalaxyl, Dimethomorph, and Fluopicolide are considered promising agents to control phytophthora diseases in strawberries. However extensive use of fungicides resulted in the rapid rise of resistance in the pathogen population against fungicides. After the commercial availability of various fungicides, resistance phenomenon was observed in different pathogen populations. Results from the current study also confirmed the presence of resistant strains of *P. cactorum* in Czech strawberry fields. Resistance against fungicides is distributed unevenly and the effect of various FRAC groups of fungicides is also different on various life stages of the pathogen.

 Variability in resistance against Metalaxyl has been documented in the isolates we tested. The isolates are either fully sensitive or highly resistant, which is due to the assumed monogenic nature of resistance to this fungicide (Randall et al. 2014; Chen et al. 2018). In our analyses, 24 isolates out of 141 which is 17% of the population, were resistant. All the resistant isolates originated from strawberry plants while the isolates isolated from woody plants were sensitive to Metalaxyl. This explains that resistance is either developed after using Metalaxyl continuously on strawberry plants, or the plants used were carriers of *Phytophthora* spores and were introduced to the field in a compromised phytosanitary environment. The Former statement can be nullified because the use of Metalaxyl on strawberries is banned in the Czech Republic. Another possibility is that the fields were used before for another crop and the use of metalxyl makes some strains resistant. The idea of emerging pathogens can also be not neglected in this scenario, and one of the probabilities is because of compromised phytosanitary practices these resistant isolates are unintentionally transferred from the country of origin of seedlings and introduced in the Czech Republic as happened in the past with the migration of P. infestans from Mexico to North America and then to Europe in 1845. Resistance against this fungicide is well documented in many *Phytophthora* spp (Molinero-Ruiz et al. 2008; Hobbelen et al. 2014; Marin & Peres 2021). The development of resistance against Dimethomorph has been considered improbable due to the polygenic nature of such resistance, although the Dimethomorph-resistant mutants were acquired in laboratory conditions (Chabane et al. 1996; Stein et al. 2004). The results of current analyses show the resistance formation in *P. cactorum* isolates in field conditions, although the number of such events is unclear, considering at least two different types of isolates resistant to this fungicide

are present together in one locality 18_07_2S12 is resistant to Dimethomorph + Metalaxyl; 18_07_6 is resistant only to Dimethomorph). Both the Dimethomorph and Metalaxyl resistant isolates that originate from *P. cactorum* populations in strawberry fields in the Czech Republic, are considered to belong to the genetically homogenous lineage "S" (Pánek et al. 2021). The variability in response to these fungicides is somewhat inconsistent with the low genetic diversity in that group.

The efficiency of fungicides varies between the different life stages of a pathogen and is probably determined by the various modes of action of the active ingredients. In the case of Metalaxyl, the mycelial growth phase and sporangia formation are the stages affected by this fungicide, because Metalaxyl inhibits the synthesis of ribosomal RNA by disrupting the activity of RNA polymerase (Kerkenaar 1981; Randall et al. 2014; Wang & Ji 2021). Since the growth of hyphae is necessarily associated with RNA synthesis, Metalaxyl significantly affects such regions but does not have an impact on zoospore release, probably because such a process is not directly determined by RNA synthesis. A similar principle could also be considered for the inefficiency of Dimethomorph against zoospore release, although the mode of action of Dimethomorph is different. This fungicide inhibits cell wall deposition in actively growing regions of hyphae; therefore it is efficient against mycelial growth or sporangia formation, but not against zoospore release. Azoxystrobin, which we showed to be the most effective against zoospore release, relies on the inhibition of the ATP supply by mitochondria. This is achieved by the disruption of the activity of cytochromes which determine oxidative respiration (Kuhn et al. 1991). Since respiration is necessary for energetically demanding metabolic processes, such as active movement, zoospores are affected by this fungicide. However, mitochondria are also present in growing hyphal tips in high numbers, with a high probability that they do not respire or synthesize ATP there but play other roles which could explain the inefficiency of Azoxystrobin against mycelial growth. The only fungicide efficient against all three tested life stages of *P. cactorum*, Fluopicolide, causes the disruption of cytoskeletal proteins such as actins, integrins, tubulins, and spectrins. These structures are necessary for all living cells, so their damage affects all of the life stages we tested.

Differences in resistance against fungicides on various levels, i.e., between host-specific groups, between diverse life stages, and between isolates, were observed and this should be carefully taken into consideration in the further use of these fungicides in plant protection. Among the tested compounds, only Fluopicolide was effective against all tested life stages of *P. cactorum*. Although no isolate resistance against Fluopicolide was found, the entire

population probably has a somewhat increased resistance against this fungicide. Even in the case of the careful use of fungicide rotation, some *P. cactorum* strains acquire resistance against the fungicides used, because some life stages are insensitive to a particular fungicide and survive the treatment. Similar accidental contacts of a pathogen with a non-lethal fungicide application gradually increase the resistance level of the population. The use of anti-resistant strategies formulated to prevent the development of resistance should be the necessary minimum basis for the sustainability of plant protection.

6.3 Relation of RPA190 Gene and Metalaxyl Resistance in *P. cactorum*

The study of Metalaxyl resistance in oomycetes ahead of *P. cactorum* is predominantly focused on *P. infestans* because of its importance in food production security. The transfer of the Metalaxyl resistance gene is well documented in *P. infestans*, *P. sojae,* and *P. capsica* and is considered a Mendelian mode of heritability. According to these findings resistance to Metalaxyl is of monogenic nature and the resistant gene is incompletely dominant. The suggested molecular mechanism of resistance to Metalaxyl in *P. infestans* is due to point mutations in the RPA190 gene (Randall et al. 2014). However, according to Matson et al. (2015), the presence of multiple mutations in RPA135 and RPA190 genes could be responsible for Metalaxyl resistance in *P. infestans*. Wang et al. (2021) recently revealed that the RPA190 gene is responsible for resistance to Metalaxyl in *P. capsici* both in vitro and in vivo conditions. This gene encodes a major subunit of RNA polymerase I, and Metalaxyl strongly inhibits RNA polymerase activity in sensitive isolates compared to resistant ones. Multiple point mutations were also detected in Metalaxyl resistant *P. infestans* isolates by (Chen et al. 2018) in the RPA190 gene. These findings initiate a debate about the involvement of the RPA190 gene in Metalaxyl resistance in *P. cactorum* isolates. In the current study, the relation between the RPA190 gene and Metalaxyl resistant *P. cactorum* isolates was explored. Three-point mutations mentioned by Chen et al. (2018) in Metalaxyl resistant *P. infestans* isolates were not detected in our findings. This led to two different arguments. Either there are some other point mutations responsible for Metalaxyl resistance in *P. cactorum* or the genetic makeup of RPA190 differs in *P. infestans* and *P. cactorum.* But the first argument can be nullified because a recent study conducted by Marin et al. (2023) also didn't find any mutation in RNA polymerase subunit genes in Mefenoxam resistant *P. cactorum* isolates while 4 mutations were reported in the coding region and 2 in the noncoding region which further supports that RPA190 is not related to Mefenoxam resistance.

It is also necessary to highlight that Metalaxyl contains two enantiomers (R and S), and they differ in their activity. The R-enantiomer is known as Mefenoxam which is 1000 times more efficient in vitro and 3-4 times more efficient in vivo than S-enantiomer, Metalaxyl (Zadra et al. 2002).

Another interesting finding in the current study is about variation in gene expression of RPA190 of Metalaxyl resistant and sensitive *P. cactorum* isolates. One of the Metalaxyl resistant isolates i.e., R2 has no differences in gene expression level when compared with Metalaxyl treated and control which suggests there is no relation of the RPA190 gene with resistance phenomenon. In the case of two isolates i.e., R1 and R3 where the gene expression values for control are higher than the treated ones. It highlights the downregulation of the RPA190 gene. This might indicate that the pathogen might have developed alternative mechanisms of resistance that are not reflected in gene expression. However, in the case of R4, almost 10x high expression levels were detected for the treatment, as compared to the control. This indicated that the expression of the RPA190 in this isolate was induced by the application of fungicide. In conclusion, gene overexpression can be assumed to be associated with fungicide resistance in the case of R4. Similar findings were reported by (Wang et al. 2021) where they concluded that the expression of RPA190-pc is involved in the regulation of *P. capsici* resistance to Metalaxyl. However, to date, no other specific study about the interaction of RPA190 of *P. cactorum* and Metalaxyl resistance has been reported and this generates a missing gap for further research.

6.4 Biological Control of *P. cactorum*: Paradigm Shift

Global environmental issues especially groundwater contamination, aquatic life disturbance, bioaccumulation, and the burden of chemical fungicides in the environment, ignite the exploration of green and sustainable solutions to control phytopathogens. In this regard, a lot of biocontrol agents i.e., Viral, bacterial, or fungal, are introduced. Exploring the potential of microalgae and specifically marine microalgae is comparatively new to aforementioned biocontrol agents. In current research, marine, brown microalgae i.e., *T. lutea* was screened for its antifungal potential against *P. cactorum*. Importantly this is the first time that *T. lutea* has been evaluated for its antifungal potential against *P. cactorum*. Mycelial growth inhibition results are promising, possibly reducing the use of synthetic fungicides in strawberry production if pursued further.

The reduced mycelial growth rate of *P. cactorum* isolates during a confrontation with *T. lutea*

crude extracts dissolved in V8 juice agar, compared to the control, suggest that *T. lutea* crude bioextract has a mycelial inhibitory effect. The exact mechanism behind this inhibitory effect is unknown. However, when crude bioextract was dissolved in selected organic solvents i.e. n-Hexane, chloroform, methanol, and Milli Q, and extracted metabolites were checked for its antifungal effect, it was concluded that the compound/s exhibited an inhibitory effect are highly polar and water soluble. One point of discussion is also whether *T. lutea* may release some extracellular enzymes, which can be nullified because we didn't expose the algal culture to stress and neither extracellular enzymes are harvested. As the cells were disrupted and dried biomass was analysed, the exhibited antifungal potential is most possibly intracellular. Taking into consideration *T. lutea* as microbial cell factories, there are different classes of compounds as the cells contain lipids, carbohydrates, proteins, free amino acids, pigments, and small peptides, and with current investigations, it couldn't be specified yet. However, after UHPLC-HRMS analysis of crude bio extract, it was observed that the majority of the compounds are amino acid derivates and alkaloids.

After examining the growth pattern of mycelia in the presence of CBE, it was hypothesized that probably another stage of the pathogen life cycle is affected in the presence of CBE because slight growth was observed initially but after a few days mycelial growth was completely halted, and Petri dishes were observed for 3 months and there was no progress in mycelial growth. To observe the interaction of *P. cactorum* and crude bio extract at the ultra-level, SEM was performed, and it was concluded that sporangia formation is completely inhibited in *P. cactorum* treated with CBE compared to control. From a phytopathological perspective if the sporangia or spore formation ability of other fungal pathogens can also be halted by the application of crude bio extract then there is a possibility to employ *T. lutea* based biological control strategy to cope with phytopathogens. Biocontrol of *P. cactorum* with microalgal species is recently reported by (Jokel et al. 2023).

 Biostimulant potential of crude bio extract was also observed in the greenhouse experiment. The Plant health index calculated for strawberry plants treated with different concentrations of CBE revealed that 8 g/l concentration has a maximum effect on plant growth. As the concentration increases up to 10 g/l slight decline was observed in plant growth and it was concluded that 8 g/l is the sweet spot to employ CBE as a bio-stimulant. Similar results were also reported by (Supraja et al. 2020) in a study conducted on tomato cultivation where they used 20%, 40%, 60%, 80%, and 100% of microalgal extracts to do seed pretreatment, results showed that maximum shoot length was obtained when 60% of microalgal extracts were used,

but after that, any further increase in the amount of microalgal extracts became a reason for decrease in shoot length.

The observed sweet spot was also confirmed after the detection of a higher amount of growth phytohormone i.e., Auxin in strawberry plants treated with CBE 8 g/l. From an evolutionary perspective, higher plants evolved from lower vascular plants and microalgae, keeping this in mind it was hypothesized that *T. lutea* should also contain some phytohormones which are present in higher plants. Detection of phytohormones in *T. lutea* makes it a potential candidate to be used as a biostimulant agent, as plants need phytohormones for their optimum metabolism and growth. Phytohormones are detected in various microalgal species that support the evolutionary lineage of microalgae and higher plants (Han et al. 2018). Partial characterization of CBE confirms the presence of Phosphate, ammonium, nitrate, and nitrite and this could also be a reason for the increased health index and then decline at 10 g/l probably because of nutrient toxicity or overdosage, which needs to be further elucidated (Supraja et al. 2020).

In the biocontrol experiment, strawberry plants were artificially infected with *P. cactorum,* and different concentrations of CBE i.e. 0, 2, 4, 6, 8, and 10 g/l were applied. It was observed that 10 g/l has a maximum plant health index (PHI) compared to control. Root neck analysis shows that *P. cactorum* attack was not completely inhibited by CBE, but the plant health is better compared to the control. When compared with commercially available fungicide (Fo AL) treatment it was realized that CBE has also up to some extent improved plant immunity against *P. cactorum.* PHI of FoAL treatment was maximum, and control was lowest, considered as baseline.

Chapter 7: Conclusion

Variation in sensitivity displayed against various fungicides by the *P. cactorum* population of the Czech Republic was observed. Out of eight fungicides used against *P. cactorum*, Dimethomorph displayed the best inhibitory potential in inhibiting mycelial growth and sporangia formation. Dimethomorph was not effective against zoospore release by *P. cactorum*. While in the greenhouse experiment, Dimethomorph alone was not too effective but when mixed with Pyraclostrobin, they had efficient control of *P. cactorum,* and maximum plant health index (PHI score) was recorded. Fluopicolide was efficient at all three stages of *P. cactorum* i.e., mycelial growth inhibition, sporangia formation, and zoospore release, and no resistant isolate was found against this fungicide, but this fungicide seems to be fungistatic because after one week of collection of data, steady growth of pathogen was observed. EC_{50} values recorded against Fenamidone range from 76.1 to 369.6 µg/ml but its effect was also fungistatic as mycelial growth of isolates was observed, at the highest concentration of 100 µg/ml, even after one week of collection of data. Azoxystrobin was ineffective in mycelial growth inhibition but effective in inhibiting sporangia formation and zoospore release. Metalaxyl was effective in mycelial growth inhibition and sporangia formation inhibition but the presence of Metalaxyl resistant isolates is a big challenge and alarming for future disease management. When Metalaxyl and Azoxystrobin were used in the mixture, in the greenhouse experiment, it was effective and a PHI score of 105.54 was recorded. In overall isolates of *P. cactorum* from the Czech Republic 17% of isolates were recorded as resistant to Metalaxyl while 83% were sensitive. Currently registered plant protection products against *Phytophthora* root rot of strawberries in the Czech Republic and almost all of the member states of European Union are Aliette, Lalstop G46 WG, Polyverse, and Serifel with Fosetyl-AL, *Clonostachys rosea* strain J1446, *Pythium oligandrum* M1, and *Bacillus amyloliquefaciens* strain MBI 600 as active ingredients respectively. A paradigm shift can be observed from chemical fungicides to biocontrol products which highlights the importance of alternate strategies in plant protection.

To manage resistance issues in *P. cactorum*, *T. lutea* crude bio extract exhibits inhibitory potential and can be used as a biocontrol agent. Crude bio extract also has a biostimulant effect and can potentially improve plant immunity. To further improve the toolbox in disease management, *T. lutea* can be employed as a potential biocontrol and biostimulant agent in an integrated pest management strategy in the future. Benefits of these green agents include decreased chemical use, greenhouse gas emissions, and biocontrol of plant diseases. However,

the profitability of their biomass production determines their level of success. Taking into consideration the global sustainable development goals, environmental pollution, and sustainable agriculture it is necessary to further develop and commercialize algae-based biocontrol and biofertilizers.

Chapter 8: Publications

- Evaluation of the Ability of Seven Active Ingredients of Fungicides to Suppress *P. cactorum* at Diverse Life Stages, and Variability in Resistance Found among Isolates

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- Use of Metalaxyl against some soil plant pathogens of class Peronosporomycetes – review and two case studies

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- Characterization of the molecular mechanisms of resistance against DMI fungicides in *Cercospora beticola* populations from the Czech Republic

Ram Kumar [1], Jana Mazáková [1], Asad Ali 1, Vishma Pratap Sur [2], Madhab Kumar Sen [3], Melvin D. Bolton [4], Marie Manasová[1], Pavel Ryšánek[1] and Miloslav Zouhar[1], *

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List of Tables

List of Figures

List of Abbreviations

AHLs N-acyl-L-homoserine Lactones

CBE Crude Bio Extract

CBEL Cellulose-Binding Elicitor Lectin

CRN CRinkling and Necrosis

CWDEs Cell Wall-Degrading Enzymes

DMSO Dimethyl Sulfoxide

DNA Deoxyribonucleic Acid

EC Effective Concentration

FRAC Fungicide Resistance Action Committee

HR Hypersensitive Reaction

IAA Indole Acetic Acid

ISR Induced Systemic Resistance

MAMPs Microbe-Associated Molecular Patterns

PCR Polymerase Chain Reaction

PHI Plant Health Index Score

PIP1 Phytophthora Inhibited Protease 1

PRRs Plant Recognition Receptors

PTI Pattern-Triggered Immunity

qPCR Quantitative Polymerase Chain Reaction

RNA Ribonucleic acid

SAR Stramenopiles (heterokonts), Alveolates, and Rhizaria

SAR Systemic Acquired Resistance

SNPs Single Nucleotide Polymorphism

WT Wild Type

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Supplementary Data

Supplementary Table 1: Top 20 Strawberry Producers

Area	Item	Year	Value	Unit
China, mainland	Strawberries	2019	3212814	tonnes
United States of America	Strawberries	2019	1021490	tonnes
Mexico	Strawberries	2019	861337	tonnes
Turkey	Strawberries	2019	486705	tonnes
Egypt	Strawberries	2019	460245	tonnes
Spain	Strawberries	2019	351960	tonnes
Russian Federation	Strawberries	2019	208800	tonnes
Republic of Korea	Strawberries	2019	192971	tonnes
Poland	Strawberries	2019	185400	tonnes
Morocco	Strawberries	2019	167827	tonnes
Brazil	Strawberries	2019	165440	tonnes
Japan	Strawberries	2019	158443	tonnes
Germany	Strawberries	2019	143980	tonnes
United Kingdom of Great Britain and Northern Ireland	Strawberries	2019	141594	tonnes
Italy	Strawberries	2019	125130	tonnes
Belarus	Strawberries	2019	81887	tonnes
Netherlands	Strawberries	2019	75590	tonnes
Greece	Strawberries	2019	74430	tonnes
Australia	Strawberries	2019	68534	tonnes
Ukraine	Strawberries	2019	62620	tonnes

Supplementary Table 2: Top 20 strawberry Importers

Area	Item	Year	Value	Unit
United States of America	Strawberries	2019	186499	tonnes
Germany	Strawberries	2019	128105	tonnes
Canada	Strawberries	2019	96912	tonnes
France	Strawberries	2019	64357	tonnes
United Kingdom of Great Britain and Northern Ireland	Strawberries	2019	57379	tonnes
Russian Federation	Strawberries	2019	43885	tonnes
Italy	Strawberries	2019	42314	tonnes
Belgium	Strawberries	2019	33325	tonnes
Netherlands	Strawberries	2019	30113	tonnes
Portugal	Strawberries	2019	20315	tonnes
Austria	Strawberries	2019	20138	tonnes
Spain	Strawberries	2019	18519	tonnes
Poland	Strawberries	2019	17698	tonnes
Saudi Arabia	Strawberries	2019	17115	tonnes
Mexico	Strawberries	2019	16960	tonnes
Czechia	Strawberries	2019	16368	tonnes
Switzerland	Strawberries	2019	14088	tonnes
United Arab Emirates	Strawberries	2019	10388	tonnes
Belarus	Strawberries	2019	10023	tonnes
Romania	Strawberries	2019	9124	tonnes

Supplementary Table 3: Top 20 Strawberry Exporters

Area	Item	Year	Value	Unit
Spain	Strawberries	2019	300036	tonnes
Mexico	Strawberries	2019	137393	tonnes
United States of America	Strawberries	2019	130784	tonnes
Netherlands	Strawberries	2019	57071	tonnes
Greece	Strawberries	2019	45175	tonnes
Belgium	Strawberries	2019	45069	tonnes
Egypt	Strawberries	2019	38543	tonnes
Turkey	Strawberries	2019	25352	tonnes
Morocco	Strawberries	2019	21439	tonnes
Yemen	Strawberries	2019	18800	tonnes
Italy	Strawberries	2019	12990	tonnes
Germany	Strawberries	2019	12229	tonnes
Serbia	Strawberries	2019	8896	tonnes
France	Strawberries	2019	8732	tonnes
Lithuania	Strawberries	2019	7448	tonnes
Portugal	Strawberries	2019	6468	tonnes
Poland	Strawberries	2019	6411	tonnes
Guatemala	Strawberries	2019	6383	tonnes
Belarus	Strawberries	2019	5849	tonnes
Republic of Korea	Strawberries	2019	5259	tonnes

Supplementary Figure 1: Amino acid sequence alignment of Primer 1 for *P. cactorum* Resistanat (R) and Sensitive (S) Isolates with previously identified SNPs in *P. infestans*

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Phytophtho  PPLEVQSQLQLMWQNEDGLMEMLYGDRNIASGRVSGRKP-------DGW------RKFFL
R1_Primer1  ----------------------YGDRNIASGRVSGRKP-------DGW------RKFFL
R2_Primer1  -------------------GLLYGDRNIASGRVSGRKP-------DGW------RKFFL
S4_Primer1  -------------------GLLYGDRNIASGRVSGRKP-------DGW------RKFFL
R4_Primer1  -------------------GLLYGDRNIASGRVSGRKP-------DGW------RKFFL
S3_Primer1  ----------------------NGDRNIASGRVSGRKP-------DGW------RKFFL
R3_Primer1  ----------------------NGDRNIASGRVSGRKP-------DGW------RKFFL
S2_Primer1  ----------------------NGDRNIASGRVSGRKP-------DGW------RKFFL
S1_Primer1  ------------------------------------RRPQHCLAAVSSWPARRLAQVLSL
                                              *:*          ..*       :  : *


Phytophtho  NVIPVAPSRFRPPVFMGDKQFEHAQNSHLSKIMTYSESIVQSDYYKRQAATTSDEDDAEK
R1_Primer1  NVIPVAPSRFRPPVFMGDKQFEHAQNSHLSKIMTLSESIVQGDYYKRQAATTSDEDDAEK
R2_Primer1  NVIPVAPSRFRPPVFMGDKQFEHAQNSHLSKIMTLSESIVQGDYYKRQAATTSDEDDAEK
S4_Primer1  NVIPVAPSRFRPPVFMGDKQFEHAQNSHLSKIMTLSESIVQGDYYKRQAATTSDEDDAEK
R4_Primer1  NVIPVAPSRFRPPVFMGDKQFEHAQNSHLSKIMTLSESIVQGDYYKRQAATTSDEDDAEK
S3_Primer1  NVIPVAPSRFRPPVFMGDKQFEHAQNSHLSKIMTLSESIVQGDYYKRQAATTSDEDDAEK
R3_Primer1  NVIPVAPSRFRPPVFMGDKQFEHAQNSHLSKIMTLSESIVQGDYYKRQAATTSDEDDAEK
S2_Primer1  NVIPVAPSRFRPPVFMGDKQFEHAQNSHLSKIMTLSESIVQGDYYKRQAATTSDEDDAEK
S1_Primer1  NVIPVCAFTF-PPAFSGDKQFEHAQNSHLSKIMTLSESIVQGDYYKRQAATTSDEDDAEK
            *****..   *  **.* ***************** ****** *****************


Phytophtho  EEQVNLSRKLALWTELQNAVNLLVDSSKAKPGTDVAQGIKQVIEKKEGLFRKHMMGKRVN
R1_Primer1  EQQVNLSRKLALWTELQNAVNLLVDSSKAKPGTDVAQGIKQVIEKKEGLFRKH-------
R2_Primer1  EQQVNLSRKLALWTELQNAVNLLVDSSKAKPGTDVAQGIKQVIEKKEGLFRKHMMGK---
S4_Primer1  EQQVNLSRKLALWTELQNAVNLLVDSSKAKPGTDVAQGIKQVIEKKEGLFRKHMMGK---
R4_Primer1  EQQVNLSRKLALWTELQNAVNLLVDSSKAKPGTDVAQGIKQVIEKKEGLFRKH-------
S3_Primer1  EQQVNLSRKLALWTELQNAVNLLVDSSKAKPGTDVAQGIKQVIEKKEGLFRKHIG-----
R3_Primer1  EQQVNLSRKLALWTELQNAVNLLVDSSKAKPGTDVAQGIKQVIEKKEGLFRKHMEWVK--
S2_Primer1  EQQVNLSRKLALWTELQNAVNLLVDSSKAKPGTDVAQGIKQVIEKKEGLFRKHWIG----
S1_Primer1  EQQVNLSRKLALWTELQNAVNLLVDSSKAKPGTDVAQGIKQVIEKKEGLFRKHW------
            *:*******************************************************
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Supplementary Figure 2: Amino acid sequence alignment of Primer 2 for *P. cactorum* Resistanat (R) and Sensitive (S) Isolates with previously identified SNPs in *P. infestans*

```
Phytophtho  NAKALDAHMMGCVHGSNSDIIKTCLPSGQSKAFPKNNFSLMVLTGAKGSMVNHSQISCGL
R3_Primer2  ---XXXXXMMGCVHGSNSDIIKTCLPSGQSKAFPKNNFSLMVLTGAKGSMVNHSQISCGL
R4_Primer2  --------MMGCVHGSNSDIIKTCLPSGQSKAFPKNNFSLMVLTGAKGSMVNHSQISCGL
S4_Primer2  -------PMMGCVHGSNSDIIKTCLPSGQSKAFPKNNFSLMVLTGAKGSMVNHSQISCGL
S3_Primer2  ---XXXXXMMGCVHGSNSDIIKTCLPSGQSKAFPKNNFSLMVLTGAKGSMVNHSQISCGL
S1_Primer2  --------PMGCVHGSNSDIIKTCLPSGQSKAFPKNNFSLMVLTGAKGSMVNHSQISCGL
R1_Primer2  -------AMMGCVPGSNSDIIKTCLPSGQSKAFPKNNFSLMVLTGAKGSMVNHSQISCGL
S2_Primer2  ---XXXXXHDGLRAGSNSDIIKTCLPSGQSKAFPKNNFSLMVLTGAKGSMVNHSQISCGL
R2_Primer2  --------------XXXXXFLKTCLPSGQSKAFPKNNFSLMVLTGAKGSMVNHSQISCGL
                       ::***********************************************

Phytophtho  GQQALEGRRVPILCSGRSLPSFEPFDPAPRAGGYVTDRFLTGLRPQEYYHHCMAGREGLV
R3_Primer2  GQQALEGRRVPILCSGRSLPSFEPFDPAPRAGGYVTDRFLTGLRPQEYYHHCMAGREGLV
R4_Primer2  GQQALEGRRVPILCSGRSLPSFEPFDPAPRAGGYVTDRFLTGLRPQEYYHHCMAGREGLV
S4_Primer2  GQQALEGRRVPILCSGRSLPSFEPFDPAPRAGGYVTDRFLTGLRPQEYYHHCMAGREGLV
S3_Primer2  GQQALEGRRVPILCSGRSLPSFEPFDPAPRAGGYVTDRFLTGLRPQEYYHHCMAGREGLV
S1_Primer2  GQQALEGRRVPILCSGRSLPSFEPFDPAPRAGGYVTDRFLTGLRPQEYYHHCMAGREGLV
R1_Primer2  GQQALEGRRVPILCSGRSLPSFEPFDPAPRAGGYVTDRFLTGLRPQEYYHHCMAGREGLV
S2_Primer2  GQQALEGRRVPILCSGRSLPSFEPFDPAPRAGGYVTDRFLTGLRPQEYYHHCMAGREGLV
R2_Primer2  GQQALEGRRVPILCSGRSLPSFEPFDPAPRAGGYVTDRFLTGLRPQEYYHHCMAGREGLV
            ***********************************************************

Phytophtho  DTAVKTSRSGYLQRCLIKHLEDLNVGYDHTVRNSDGGVIQFLYGEDGIDPVQSAMLSGKD
R3_Primer2  DTAVKTSRSGYLQRCLIKH-----------------------------------------
R4_Primer2  DTAVKTSRSGYLQRCLI-------------------------------------------
S4_Primer2  DTAVKTSRSGYLQRCLIKHK----------------------------------------
S3_Primer2  DTAVKTSRSGYLQRCLIKQ-----------------------------------------
S1_Primer2  DTAVKTSRSGYLQRCLNQAQQ---------------------------------------
R1_Primer2  DTAVKTSRSGYLQRCLNQAQ----------------------------------------
S2_Primer2  DTAVKTSRSGYLQRCLIQAQK---------------------------------------
R2_Primer2  DTAVKTSRSGYLQRCLNXXXXXI-------------------------------------
            ***************
```

Supplementary Figure 3: Amino acid sequence alignment of Primer 3 for *P. cactorum* Resistant (R) and Sensitive (S) Isolates with previously identified SNPs in *P. infestans*

```
Phytophtho  DVESSRAQEVEQRLNQVALSELINNTNGVRVKDQFHSSENGILWVRDYQIRLSFFKLKEI
S1_Primer3  --------------------------------------------------------KEI
R1_Primer3  ------------------------------------------------TIRLTFFKLKEI
R3_Primer3  ----------------------------------------XXXXXITIRLTFFKLKEI
S3_Primer3  ------------------------------------------GDTIRLTFFKLKEI
S2_Primer3  -------------------------------------XXXXXDQIRLTFFKLKEI
S4_Primer3  ------------------------------------------GDQIRLTFFKLKEI
R2_Primer3  -------------------------------------XXXXXIRIRLTFFKLKEI
R4_Primer3  ----------------------------FHSSENGILWVRDYQIRLTFFKLKEI
                                                               ***


Phytophtho  KRVFGLSADQVFNSVGRGFVGKLLTLISREMKKSGVTVSAAAEKNNFKAPSGADKKKNDD
S1_Primer3  KRVFGLSADQVFNSFGRGFVGKLLTLISREMKKSGVTVSAAAEKNNFKAPSGADRKKT--
R1_Primer3  KRVFGLSADQVFNSFGRGFVGKLLTLISREMKKSGVTVSAAAEKNNFKAPSGADRKKTND
R3_Primer3  KRVFGLSADQVFNSFGRGFVGKLLTLISREMKKSGVTVSAAAEKNNFKAPSGADRKKTND
S3_Primer3  KRVFGLSADQVFNSFGRGFVGKLLTLISREMKKSGVTVSAAAEKNNFKAPSGADRKKTND
S2_Primer3  KRVFGLSADQVFNSFGRGFVGKLLTLISREMKKSGVTVSAAAEKNNFKAPSGADRKKTND
S4_Primer3  KRVFGLSADQVFNSFGRGFVGKLLTLISREMKKSGVTVSAAAEKNNFKAPSGADRKKTND
R2_Primer3  KRVFGLSADQVFNSFGRGFVGKLLTLISREMKKSGVTVSAAAEKNNFKAPSGADRKKTND
R4_Primer3  KRVFGLSADQVFNSFGRGFVGKLLTLISREMKKSGVTVSAAAEKNNFKAPSGADRKKTND
            **************.****************************************:**.


Phytophtho  DEDDDDEQGTLRFGSRGEVQGYGEMDEEDEKIRKAQMADDSDIDSYDETSGNQKNNGADT
S1_Primer3  ------------------------------------------------------------
R1_Primer3  D-DDDDEQGTLRFGSRGEVQGYGEMDEEDEKIRKAQMADDSDIDSDDETSGNKKKQR---
R3_Primer3  D-DDDDEQGTLRFGSRGEVQGYGEMDEEDEKIRKAQMADDSDIDSDDETSGNKKKQRR--
S3_Primer3  D-DDDDEQGTLRFGSRGEVQGYGEMDEEDEKIRKAQMADDSDIDSDDETSGNKKSNE---
S2_Primer3  D-DDDDEQGTLRFGSRGEVQGYGEMDEEDEKIRKAQMADDSDIDSDDETSGNKKSNE---
S4_Primer3  D-DDDDEQGTLRFGSRGEVQGYGEMDEEDEKIRKAQMADDSDIDSDDETSGNKKSNE---
R2_Primer3  D-DDDDEQGTLRFGSRGEVQGYGEMDEEDEKIRKAQMADDSDIDSDDETSGNKKSNE---
R4_Primer3  D-DDDDEQGTLRFGSRGEVQGYGEMDEEDEKIRKAQMADDSDIRS---------------
```