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**ECO FRIENDLY CONTROL STRATEGIES AGAINST *PHYTOPHTHORA*
SPECIES IN NURSERIES**

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Declaration

I hereby declare that this submission is my own work and that, to the best of my knowledge and belief, it contains no material previously published or written by another person nor material which to a substantial extent has been accepted for the award of any other degree or diploma of the university or other institute of higher learning and all sources of information have been listed and included on the reference sections and acknowledged.

Dania Hanna Tabet.

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List of Publications

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Detecting biocontrol agents against *Phytophthora* spp. with plant growth promoting activities amongst naturally occurring bacteria in the genus *Gluconobacter* spp.

The 9th Meeting of the International union of forest research organizations (IUFRO) Working Party 7.02.09: Phytophthora in Forests and Natural Ecosystems; La Maddalena, Sardinia, Italy; 18-25 October 2019.

Abstract

Phytophthora sp. is considered to be one of the most severe diseases in nurseries worldwide. While current synthetic fungicides are effective at controlling the disease, they are an environmental and economic burden.

In line with the International Plant Protection Convention's (IPPC) strategic framework for 2020-2030, which aims to protect global plant resources and facilitate safe trade while minimizing the impact of pests and diseases on food security, trade, economic growth, and the environment, and within the EU directives to reduce the use of synthetic pesticides and increase the use of sustainable alternative disease control strategies that can be part of integrated pest management systems, practical biological control solutions are urgently needed.

Here, we review the scientific literature journal paper on *Phytophthora* diversity in European nurseries, common plant extracts used to control *Phytophthora* disease, and focus on the most recent developments in the search for efficient and sustainable biological control of late blight diseases. Additionally, during this study, we evaluate the effectiveness of some plant extracts and biological control agents (BCAs) in reducing populations of *Phytophthora* sp., a major threat to agricultural, ornamental, and forest plants.

Results show that the incidence of *Phytophthora* diseases in nurseries has increased over the years in Europe, as well as in Italy, and provide strong evidence of the high diversity of alien invasive *Phytophthora* species presents in 51 European countries.

Furthermore, the plant extract literature survey, shows that over 200 articles, published until 2021 have tested a wide range of plants from 140 families, with *P. infestans*, *P. capsici* and *P. nicotianae* being the most studied species.

Despite a large body of scientific research into microorganisms and plant extracts with potential for the biological control of *Phytophthora* disease, relatively few commercial biocontrol products, licensed to control *Phytophthora* diseases exist, and their practical uptake in Europe is lower than expected, suggesting that such solutions are not yet feasible, or effective.

In *in vitro* tests, the antifungal nature of *Tulbaghia violacea* aqueous leaf extracts against *Phytophthora* sp. and other fungal pathogens, shown here for the first time, indicates the potential use of this extract as a biofungicide.

Additionally, during this study, we describe the possibility of *Gluconobacter kondonii* (G7), *Pseudomonas fluorescence* (G1) and other bacterial isolates (Cas, Pro, A1, Mk) as a promising, beneficial candidates with anti *Phytophthora* activity against several *Phytophthora* sp. and with a various plant growth promoting (PGP) traits. However, *in vitro* tests fail to translate the biocontrol effect to *in planta* applications.

The application of BCAs and plant extracts in an integrated management approach could be considered a satisfactory level for sustainable and environmentally friendly management strategies for *Phytophthora* disease. However, potential bottlenecks in application methods in *in planta* should be addressed early in the selection process.

Keywords: *Phytophthora* spp., Plant extracts, Nursery, Biological control agents, Biocontrol

Dedication



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List of Abbreviations

AAB	Acetic Acid Bacteria
ANOVA	One-Way Analysis of Variance
BCAs	Biological Control Agents
BCF	Bacterial culture filtrate
BPs	Biodegradable Pots
CA	Carrot Agar
CAGR	Compound Annual Growth Rate
CAS	Chrome Azurol S Agar
CFU/m	Colony Forming Units Per Milliliter of Diluted Soil Sample Plated
CIVI-Italia	Centro Interprofessionale Per Le Attività Vivaistiche
CMA	Corn Meal Agar
CMC	Carboxy Methyl Cellulose Agar
COVID-19	Coronavirus Disease
DAI	Days After Inoculation
DAS- ELISA	Double-Antibody Sandwich Enzyme-Linked Immunosorbent Assay
DES	Deep Eutectic Solvent
DI	Disease Incidence
DNS reagent	3,5-Dinitrosalicylic Acid
EAE	Enzyme-Assisted Extraction
EC ₅₀	Half Maximal Effective Concentration
ELISA	Enzyme-Linked Immunosorbent Assay
EPPs	Emergent Plant Pathogen
EU	European Union
FPA	Filter-Paper-Degrading Activity
GC-MS	Gas Chromatography–Mass Spectrometry
HCN	Hydrogen Cyanide
HDTMA	Hexadecyltrimethyl Ammonium Bromide
HIV/AIDS	The Human Immunodeficiency Viruses
hpi	Post Infiltration
HR	Hypersensitivity Response
hr	Hour
IAA	Indolic Acetic Acid
In-planta	Studies done in the host plant, under controlled conditions, for instance in greenhouse
IPM	Integrated Pest Management
ITS	Internal Transcribed Spacer
IU	International Units
KB medium	King's B Medium
LB	Luria-Broth
MAE	Microwave-Assisted Extraction
MEA	Malt Extract Agar
MFC	Minimum Fungicidal Concentration
MGI	Mycelial Growth Inhibition Rate
MICs	Minimum Inhibitory Concentrations

OD	Optical Density
PARP	Pimaricin, Ampicillin, Rifampicin, Pentachloronitrobenzene
PARPNH	Pymaricin, Ampicillin, Rifampicin, Pentachloronitrobenzene, Nystatin, Hymexazol
PCR	Polymerase Chain Reaction
PDA	Potato Dextrose Agar
PDB	Potato Dextrose Broth
PEF	Pulsed Electric Field Assisted Extraction
PGPB	Plant Growth Promoting Bacteria
PGPR	Plant Growth Promoting Rhizobacteria
pH	Potential of Hydrogen
PIPES	Piperazine-1,4-Bis (2-Ethanesulfonic Acid)
PKV	Pikovskaya's Agar Medium
PLE	Pressurized Liquid Extraction
PSAM	Pectinase Screening Agar Medium
PSAM	Pectinase Screening Agar Medium
RHS	Uk Royal Horticultural Society
SMA	Synthetic Mucor Agar
UAE	Ultrasound-Assisted Extraction
UHT	Ultra-High Temperature Processing
UN	United Nations
US- USA	The United States Of America
V8 agar	Medium Consists Of 8 Types Of Vegetables
VOCs	Volatile Organic Compounds
YPM	Yeast Peptone Mannitol

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CHAPTER 1: INTRODUCTION

1.1 Introduction

Ornamental nursery plants are an important pathway for alien pests and diseases worldwide (Jung et al., 2016; Kenis et al., 2018) and climate change will also improve the settlement for introduced species into a new geographic areas (Lilja et al., 2007).

It is estimated that a new invasive species cost the U.S. over \$120 billion a year (Pimentel et al., 2005) and Australia has suffered losses amounting to at least US\$ 298.58 billion until 2017 (Bradshaw et al., 2021).

Throughout history various *Phytophthora* sp., considered as an emergent plant pathogen (EPPs), was the major cause of catastrophic epidemics in the world in a wide variety of annual crops and woody perennials (Corredor-Moreno & Saunders, 2020).

Phytophthora sp., a genus in the Oomycetes of the Kingdom Stramenopila, morphologically resembles fungi but is phylogenetically close relatives of brown algae, currently comprising approximately 150 species (Park et al., 2013). Among other soil-borne fungal diseases pathogens, *Phytophthora* sp. is considered as one of the major threats to ornamental, agriculture plants, aquaculture, urban ecosystems and forest natural environment (Derevnina et al., 2016; Fry et al., 2013).

Phytophthora can attack any part of the plants at any age (Tsao, 1990). The soilborne species infect the fine roots and bark of the suberized roots and collar region with motile zoospores released from the sporangia under wet soil conditions and airborne species infect the leaves, shoots, fruits and bark of branches and stems with deciduous sporangia produced in moist conditions and dispersed by rain and wind splatter.(Jung et al., 2018).

On agriculture crop, the most important, is caused by *P. infestans* on potato production and tomato cultivation, where it causes 100% crop loss in potato foliage in the field and storage tuber (Goutam et al., 2018). Another most destructive alien *Phytophthora* species well-documented as epidemics of trees and forests are caused by *P. cinnamomi* that have been found associated with Jarrah dieback and littleleaf disease of pines (Jung et al., 2016) and which has been causing decline and root rot in *Quercus* species (Gutiérrez-Hernández et al., 2017). A further worldwide economic loss in the production of strawberries has been affected by *P. cactorum* the causative agent of crown rot disease and the responsible for extensive mortality on silver birch seedlings in forest nurseries (Lilja et al., 2007). Additionally, *P. ramorum*, recognized an alien forest pathogen (Hansen, 2008) causing sudden oak death in California and along with *P. kernoviae*, both pathogens, having a similar host range have been considered as invasive in Europe causing *Rhododendron* dieback (Harwood et al., 2009).

The floriculture and nursery industry are often responsible for being the cause of the introduction and subsequent spread of many invasive species (Parrella et al., 2015).

In Europe, a large-scale nursery survey for soilborne *Phytophthora* species, 59 of the 68 *Phytophthora* sp. detected in the nursery were probably introduced to Europe and the most commonly destructive and well-documented alien invasive species sampled were *P. plurivora*, *P. cinnamomi*, *P. cactorum*, *P. nicotianae*, *P. ramorum*, and *P. citrophthora* (Jung et al., 2016). Therefore, accidental introductions of a new invasive *Phytophthora* alien species will have a hefty economic toll, with a high magnitude of costs associated with direct loss and management interventions thus, having information on country and regional *Phytophthora* pathogen species

prevalence and relative host susceptibility will facilitate *Phytophthora* disease prevention by enhancing site management strategies for pathogen disease suppression (McKeever & Chastagner, 2016).

Clearly, in Europe, current hygiene and monitoring practices in nurseries are not sufficient to eliminate the risk of spreading this harmful pathogen (Simamora et al., 2018). Generally, it requires frequent applications of synthetic fungicides, or a resistance breeding plants.

Not all fungicides are generally used in a curative manner. Propamocarb, a commonly used chemical fungicides should be used preventatively (Hu et al., 2007). Additionally, it became more difficult to control diseases due to low durability in fungicides protective function (de Vries et al., 2018) and the appearance of several new genotypes showing different mating types and resistant to chemical fungicides (Matson et al., 2015; Silvar et al., 2006).

To date, for a sustainable and environmental-friendly management of this disease and for a reduction of problems created by intensive farming systems technology many studies have focused on finding biocontrol measures in substitution of chemical pesticides to protect or to induce a plant resistance under biotic stress (Lahlali et al., 2022).

The development of these sustainable measures is required because the new control strategy within the EU (REACH; EC 1907-2006) reduced the number of commercially available fungicides (Hultberg et al., 2010).

Actually, the biopesticide market is growing significantly with a new biopesticide products and it will record the compound annual growth rate (CAGR) of nearly 15.65% over the period from 2020 to 2026 (www.businesswire.com).

Very importantly, plants harbor a prominent biodiversity microbial community that colonize both the rhizosphere and phyllosphere. Some of these bacterial members have been shown to inhibit the growth of plant pathogens by direct mechanisms through parasitism and secretion of secondary metabolites and by enhancing host defense in an indirect mechanisms (Raymaekers et al., 2020). These microbes are known as biocontrol agents (BCAs). Highly interesting beside disease suppression that some plant growth promoting rhizobacteria (PGPR) or endophytes has proven ability to promote plant growth and can offer an interesting bio-fertilization beside reduction of abiotic and biotic stresses (De Vrieze et al., 2020; Lamsal et al., 2013).

Currently, the predominant genera isolate with biocontrol activity against *Phytophthora* species, are *Bacillus* spp. and *Pseudomonas* spp. that have been considered a biocontrol agents to control many other plant diseases (Bahramisharif & Rose, 2019; Dimkić et al., 2022; Hashemi et al., 2021; Youyou Wang et al., 2020).

Additionally, plants belonging to different plants families were evaluated as another alternative for conventional products against *Phytophthora* sp. and some species could have important roles in biologically based management strategies for this diseases (Bowers & Locke, 2004b). The increasing importance of plant extract as biocontrol triggered the need to analyze and find the best species for the future and besides contributing to *Phytophthora* disease management this work furthers the use of a leftover product and promoting a circular economy approach.

Finally, to mitigate the impact of invasive *Phytophthora* diseases, nurseries best management practices can be achieved by using the appropriate new innovative technologies for irrigation system, potting media, and containers, in addition for training employees to report early symptoms (Jung et al., 2018) will be the most helpful in reducing the risk of diseases.

1.2 Aims and objectives of the study

Hence, continual scientific effort in terms of biological control, is needed for an effective crop protection against *Phytophthora* pathogen where the control of this disease can be based on many natural plant products and bacterial antagonists.

The novelty of this study was the selection of new bacterial biological control agent, microbial metabolites, and plant extracts and to critically assess “*in vitro*” and “*in planta*” its anti-*Phytophthora* activity.

The broad objective of the Ph.D. project is to have an integrated sustainable eco-friendly control method to manage and control *Phytophthora* infestations in nurseries, the place where plants must be provided with the proper balance of nutrients and essential management for an optimum seedling growth and vigor.

Thus, this PhD thesis project will be directed:

(a) To quantify *Phytophthora* infestations in nurseries

This research was conducted to gain an understanding of the prevalence of *Phytophthora* species in European countries, with a focus on Italian nurseries. The goal was to determine the spread of invasive harmful alien *Phytophthora* species to new region, and to raise awareness among managers and scientists on the need to implement biosecurity measures to minimize the ecological and economic threat posed to forest, food chains, natural ecosystems, and urban areas. The research also provided best management practices and recommendations for growers (Appendix H).

(b) To test several natural and synthetic plant products for controlling *Phytophthora*

To prevent *Phytophthora* diseases in nurseries, it is crucial to implement protective measures in nurseries by developing new eco-friendly control methods, such as plant extract. This will aid in improving environmental sustainable management protocols for controlling *Phytophthora* disease. To achieve this goal, a literature survey has been conducted on the main publications of plant extract for controlling *Phytophthora* diseases. This will help to screen and investigate the effects and assess the inhibitory efficacy of some selected by-product plants extract on the vegetative and reproductive growth of *Phytophthora*. The results may propose an efficient sustainable alternative approach for controlling *Phytophthora* on trees and ornamental plants in nurseries.

(c) To develop integrated eco-friendly control/management methods against alien *Phytophthora* species in nurseries

Among the biological protection strategies suggested for *Phytophthora* disease management, the use of biological control agents is one of the most eco-sustainable control methods.

To further explore this approach, a collaborative scientific review paper entitled “The hunt for sustainable biocontrol of oomycete plant pathogens, a case study of *Phytophthora infestans*” was published in a special issue of the journal “Fungal Biology Reviews”. The paper reviews the scientific literature and focuses on the most recent developments in the efficient and sustainable biological control of late blight disease. This was an opportunity to gain a deeper knowledge of oomycete pathogens and biological control agents and to work in a collaborative group.

Additionally, to gain a better understanding of the interaction of bacterial biological control agents with different *Phytophthora* species and the environment, six bacterial isolates were screened, and the persistence of bacteria in soil after multiple irrigation cycles was investigated inside two different types of pots: Plastic and cellulose biodegradable carton pot. This activity could represent a first step towards the use BCAs formulated inside carton pots for an eco-friendly control/management and a durable sustainable method for growers.

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CHAPTER 2: LITERATURE REVIEW

2.1 PHYTOPHTHORA IN EUROPEAN NURSERIES: A CASE STUDY OF *PHYTOPHTHORA*'S DISTRIBUTION IN ITALY

2.1.1 Introduction

Over the last few years, a rapid diffusion of invasive and exotic species of *Phytophthora sp.* has been observed worldwide causing an economic loss to the horticulture and forest industry (Hansen, 2008; Moralejo et al., 2009). In many cases, plant trade represents the highest environmental risk for the spread of this pathogen resulting its outbreaks in gardens, orchards and natural ecosystems where it causes a major threat and risks for native plant (Riolo et al., 2020).

The horticultural industry has experienced significant growth within the European Union (EU) in recent years, with the Netherlands having the highest ornamental plant sales, followed by Italy and France (Eurostat, 2021). In Italy, nursery plant production increased by 41% (approx. 591 million euros) between 2000 and 2019 (Eurostat, 2021). However, the COVID-19 pandemic disrupted the distribution of horticultural products (Bulgari et al., 2021).

Moreover, to prevent the introduction and spread of *Phytophthora sp.* within the (EU), surveillance strategies and phytosanitary measures based on specific import requirements and traceability via “plant passport” certification system has been adopted for regulating plant trade (Prospero et al., 2013). In Italy, fruit crops, olive, citrus, strawberry propagation material follows a certification program under the supervision of “CIVI-Italia” a national association, officially recognized by the Italian Ministry of Agriculture and the coordination and control of the regional phytosanitary service. However Italy still relies on imports from other countries for live plant material, for ornamental, forestry, and horticultural crops that went up by 20% compared to 2019 (Trendeconomy, 2021; Cacciola & Gullino, 2019). Although national and international policies aim to prevent the spread of *Phytophthora* pathogens through the trade of potted plants, recent studies have shown that there is a high diversity of rare and new cryptic *Phytophthora* species in European ornamental nurseries and in Italy. This diversity is linked to intensive trade in living plants within and between countries, which increases the rapid spread of soil-borne pathogens (Bregant et al., 2021; Jung et al., 2016).

Given the importance of nurseries for an ideal place for the reproduction and dissemination of oomycetes pathogens, in addition to the constant discovery of new or rare *Phytophthora* species introduced mainly through trade and transport of plant material, a comprehensive literature web survey has been conducted until 2021 for studying the distribution of *Phytophthora sp.* in European countries. Focusing on Italy, the aim of this report was to provide an insight about the diversity of *Phytophthora sp.* in nurseries with the main host species affected.

2.1.2 Material and method

A comprehensive literature survey on *Phytophthora sp.* diversity in European countries was conducted using the following academic search engines: *Google science Scholar* (scholar.google.com), *Web of science* (webofknowledge.com), and *Scopus* (scopus.com) database. The combinations of search terms such as “*Phytophthora*”, “Nurseries”, “The country’s name” and/ or “First report” were attempted in each database to search for the relevant publications that characterize the main *Phytophthora* species associated in European countries and more specifically on species distributed in Italian nurseries.

The first screening process articles have been done according to Title and Abstract contents that allowed us to discard irrelevant papers from the collection and to find from the online data literature database of *Phytophthora sp.* in European nurseries published till 2021, a total of 316 articles including around 80 articles from Italy. Moreover, publications from 20 European countries out of 52 have been compared with the large-scale analysis study of *Phytophthora* infestation in European nurseries by Jung et al., 2016.

2.1.3 Results

In European nurseries

The current study presents an updated data for all scientific publications on *Phytophthora* species present in European plants nurseries. Studies have shown that the incidence of *Phytophthora* diseases has increased over the years in Europe as well in Italy and the results of this online literature data survey confirming results from previous study (Jung et al. 2016) and provide strong evidence on the high diversity of *Phytophthora* species presents across 51 European countries.

Phytophthora species have been recorded in 29 European countries ((Table 2.1), (Appendix C: Figure 1)). Italy has the greatest number of recorded species followed by Germany and Netherlands with more than 25 detected species.

Table 2. 1 Number of *Phytophthora* species reported in nurseries by European country.

Country	Species	Country	Species	Country	Species
Austria	1	Greece	3	Romania	1
Belgium	2	Hungary	8	Russia	1
Bulgaria	6	Italy	44	Serbia	12
Croatia	15	Netherlands	26	Slovenia	1
Czechia	13	Ireland	13	Spain	27
Finland	6	Lithuania	6	Sweden	17
Denmark	1	Norway	6	Switzerland	8
Estonia	1	Macedonia	4	Turkey	10
France	6	Poland	16	United Kingdom	15
Germany	34	Portugal	7		

The number of described species has risen dramatically from 49 species in 18 European countries (Jung et al, 2016) to a total number of 77 *Phytophthora sp.* Among these species *P. ramorum* and *P. cinnamomi* were the most frequent, isolated from 19 European countries, followed by *P. cactotum*, *P. plurivora*, *P. cambivora*, *P. cryptogea*, *P. citrophthora* and *P. nicotianae* isolated from more than 10 European countries. Almost all *Phytophthora sp.* found in this study have been previously reported in European nurseries (Jung et al. 2016) except the following one: *P. acernia*, *P. gallica*, *P. italica*, *P. mediterranea*, *P. meadii*, *P. crassamura*, *P. hydropathica*, *P. pachyplora*, *P. pistachiae*, *P. pseudocryptogea*, *P. bisheria*, *P. inflata*, *P. richardiae*, *P. gregata*, *P. polonica*, *P. undulata*, *P. rosacearum*, *P. kernoviae*, *P. austrocedri*, *P. occultans*, *P. eriugena* and *P. colocasiae*.

Some of the detected *Phytophthora sp.* are likely endemic however quarantine species were also detected in most countries with exception of Denmark, Portugal, Russia, Austria, Hungary, Romania, Bulgaria, Macedonia, and Turkey.

Interestingly, some of the *Phytophthora* sp. were less common and isolated from a single country for example, *P. acerina*, *P. asparagi*, *P. bilorbang*, *P. hydropathica*, *P. italica*, *P. meadii*, *P. mediterranea*, *P. megasperma sensu stricto*, *P. pachyplora*, *P. pistachiae*, *P. pseudocryptogea* from Italy, *P. bisheria*, *P. multivesiculata* and *P. terminalis* from Netherlands, *P. eriugena* from Ireland, *P. foliorum*, *P. kelmania* and *P. infestans* in Spain, *P. gregata* and *P. polonica* from Serbia, *P. mexicana*, *P. primulae* and *P. uliginosa* from Germany, and *P. quercetorum* from Portugal.

In addition, it is important to note that few studies have reported members of *Phytophythium* sp. where it was only mentioned by few journal articles in Check republic, Spain, Sweden, and Turkey.

Except for clade 5, all ten phylogenetic clades of *Phytophthora*, corresponding to both soil- and airborne species, occurred in all three major climatic regions of Europe, infecting forest,

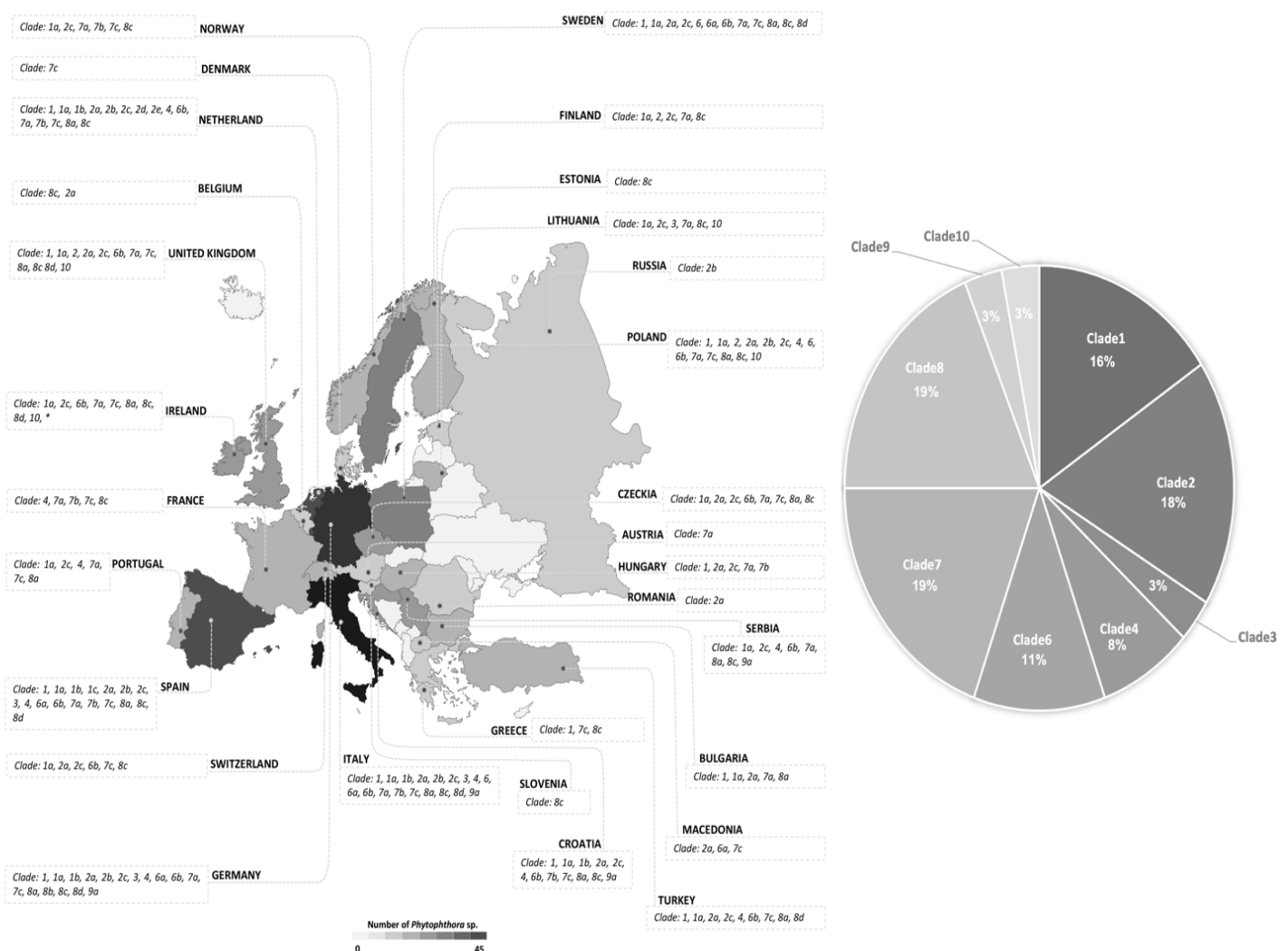


Figure 2. 1 *Phytophthora* Clade diversity according to European countries (X. Yang et al., 2017). Increasing intensity of color indicates higher number of *Phytophthora* sp.

**P. eriugena*: Doesn't include in a clade

Horticultural, and ornamental European nurseries.

While the most common *Phytophthora* sp. from clade 1, 2, 7 and 8 were predominantly present in most infected European countries, accounted for over 15%, the proportion of clade 6, which is usually found in healthy natural ecosystems and is less common in European nurseries (Jung et al., 2016), represented 11%. In contrast, the less recovered *Phytophthora* species belonging to clades 3, 4, 9 and 10 affected fewer than 10% of European countries.

Interestingly, clade 10 was found to be more concentrated in the northern part of Europe (Figure 2.1).

Phytophthora sp. has been reported on more than 250 plants genera. The highest host range was recovered from *P. cinnamomi* and *P. ramorum*, which infected 59 and 25 plant genera, respectively. Among the 14 infected plant families, 36% of ornamental plants genera affected by *P. ramorum* belong to the family Ericaceae, with *Rhododendron* and *Viburnum* being the most commonly reported plant species.

Many forest plant species typical of the Mediterranean region, such as *Arbutus unedo*, *Laurus* spp., *Quercus* spp., and many others, have been found to be most affected with more than ten *Phytophthora* species (Figure 2.2). This can facilitate the introduction and spread of *Phytophthora* disease by increasing the probability of movement of aggressive species to new forests locations.

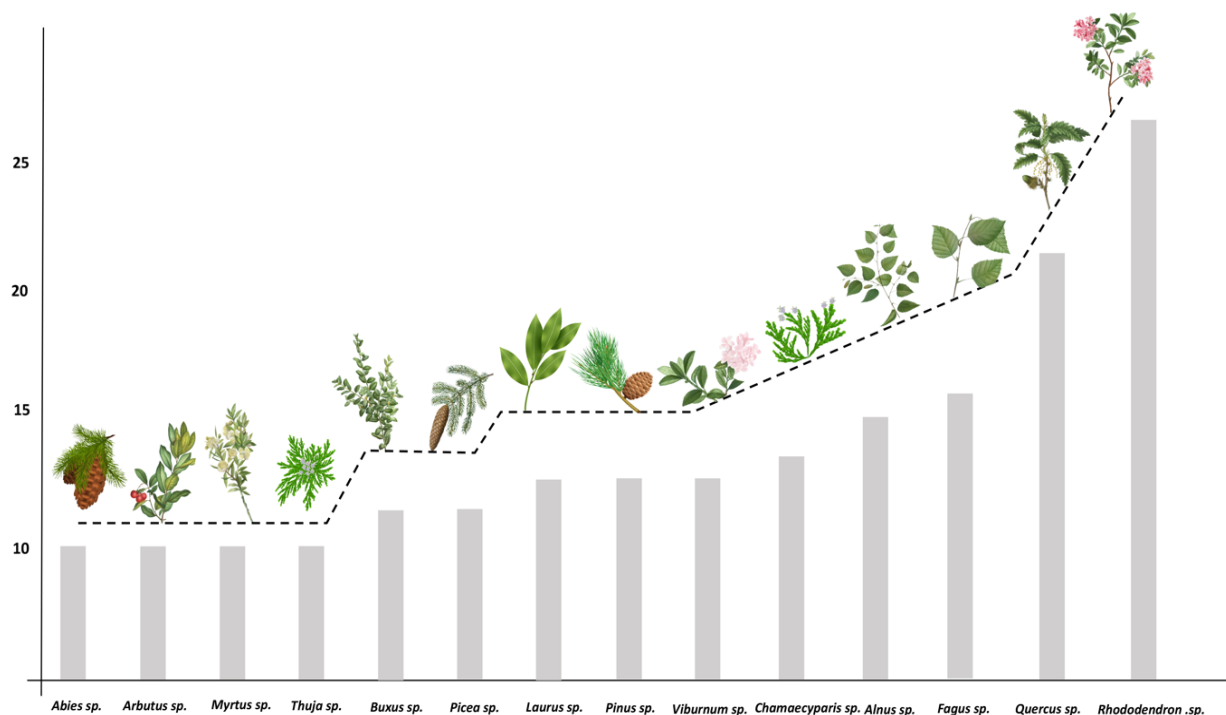


Figure 2. 2 Number of *Phytophthora* sp. according to plant host genera.

This cover has been designed using images from Freepik.com

In Italian nurseries

In Italy, articles have been reviewed with some descriptive statistics and a summary of *Phytophthora*-host interactions. The dataset includes the year of infection and the main geographical locations, as shown in (Appendix A).

Between 1986 and 2003, there was a slow increase in the number of journal articles per year regarding *Phytophthora* infestation in Italian nurseries, with an average of 2 papers per year for this period. However, in 2004 there was a dramatic increase in the number of journal articles published, resulting in a total of 86 publications by the end of 2020 (Figure 2.3).

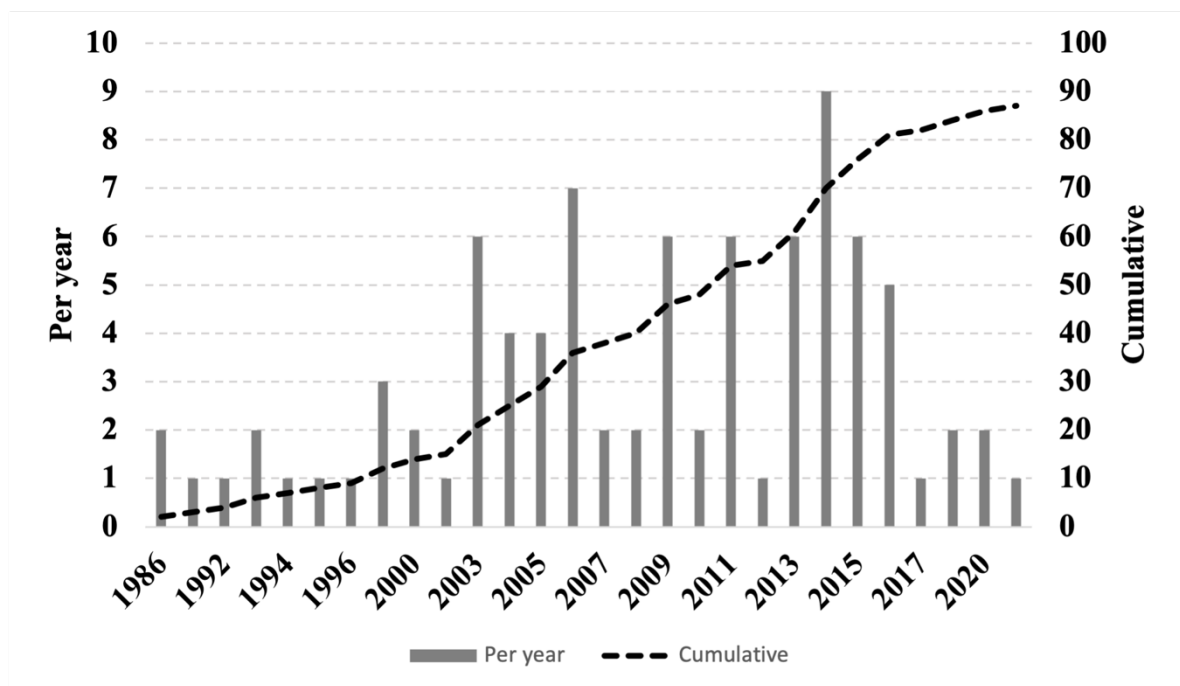


Figure 2. 3 Number of scientific journal articles published per year from 1986 till 2021 about *Phytophthora*'s infestation in Italian nurseries and total accumulated publications over time.

In Italy, over the past few decades, 53% of the articles reported new disease outbreaks, 25% represented nursery survey studies, and the remainder related to molecular or general research studies.

Many regions have been severely impacted by *Phytophthora* diseases, and the number of known species has doubled during the past decade due to extensive regional surveys. Some of the oldest *Phytophthora* species detected in Italian nurseries include *P. nicotianae*, *P. citricola*, *P. palmivora*, *P. citrophthora*, and *P. cinnamomi*, which were first reported in 1977 and 1982 (Jung et al., 2016).

Seven new *Phytophthora* species, including *P. italica*, *P. crassamura*, *P. parvispora*, *P. mediterranea*, *P. cinnamomi-like*, *P. meadi-like* and *niederhauserii-like*, have been identified in Italian nurseries for the first time and considered as representatives of novel *Phytophthora* taxa. In Addition, five *Phytophthora* species with a global host range have also been reported in Italy for the first time including: *P. asparagi*, *P. hydropathica*, *P. cinnamomi-parvispora*, *P. lacustris*, and *P. tentaculate* (Appendix B).

On the other hand, for the first time, 26% of *Phytophthora* -host combinations represented a new host record for Italy, while 43% of pathogen-host combinations were recorded as new host worldwide (Figure 2.4).

In addition, *P. alni subsp. uniformis*, *P. lateralis*, *P. × pelgrandis*, *P. hedraiandra*, *P. niederhauserii*, *P. ramorum*, *P. pistachiae*, *P. pachypleura*, *P. multivora*, *P. meadii*, *P. palmivora* and *P. taxon pgchlamydo* were, for the first time, reported in Italy as isolated from new hosts in infected plant nurseries (Appendix B).

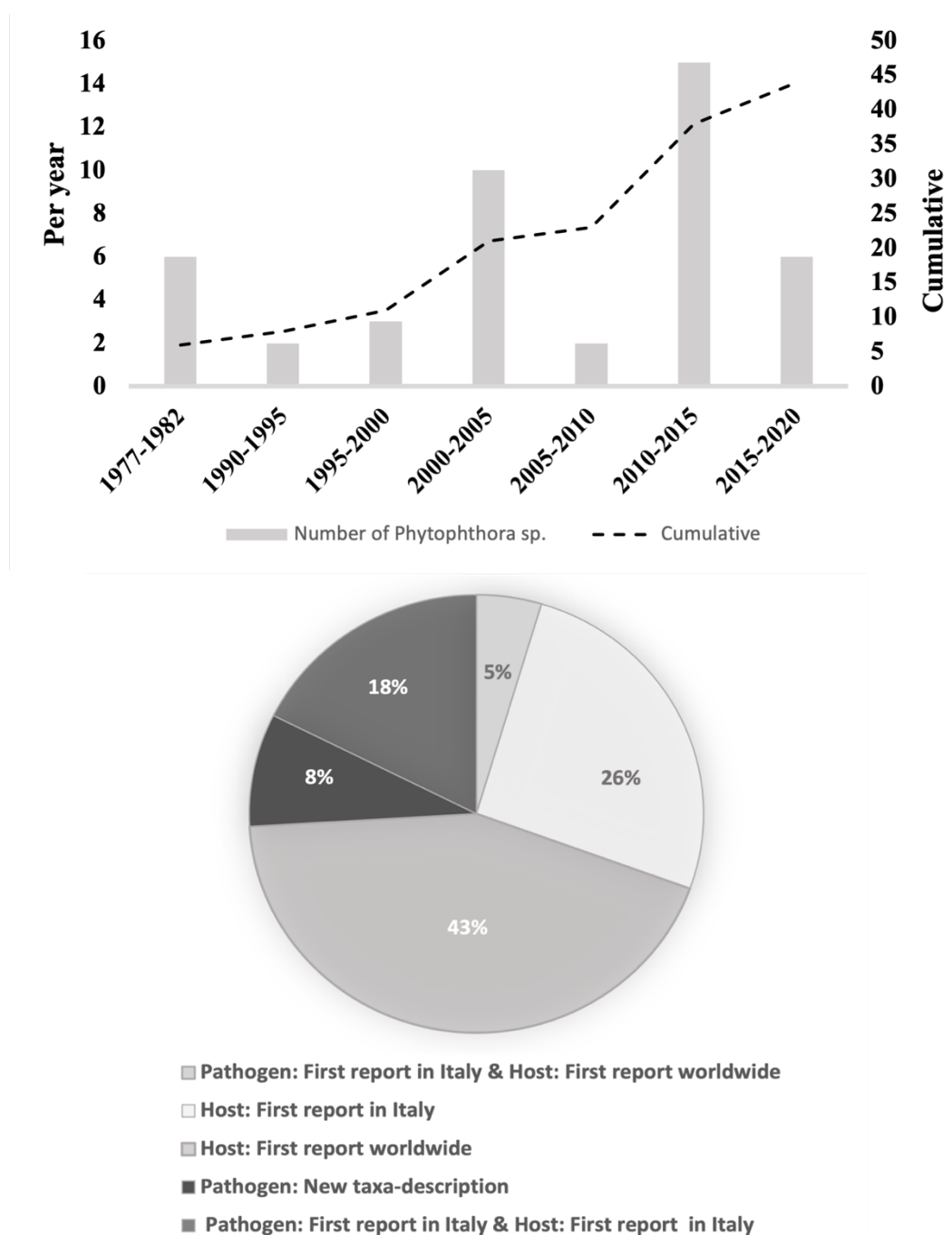


Figure 2. 4 *Phytophthora* sp. first time reported in Italian nurseries B) Number of first-time reported *Phytophthora* species in Italian nurseries per year and total accumulation over time. B) Pie chart representing the percentage of first report of *Phytophthora* -host combination.

The incidence of *Phytophthora* spp. diseases among nurseries ranged from 2% to 100%. The highest incidence with a sudden collapse and over 50% of symptomatic or dead plants was reported on *Salvia* sp. affected by *P. cryptogea* (Garibaldi et al., 2015), *Celtis australis* infected with *P. megasperma sensu stricto* (Luongo et al., 2015), *Daphne odora* and *Buxus sempervirens* affected by *P. nictotianae* (Garibaldi et al., 2009; Luongo et al., 2013), *Rosa* sp. infected with *P. citrophthora* (Salamone et al., 2011), *Pistacia lentiscus* infected with *P. pistaciae* on (Linaldeddu et al., 2020a), *Origanum* sp. infected with *P. tentaculate* (Martini et al., 2009), and *Dodonaea viscosa* infected with *P. palmivora* (Aiello et al., 2011).

Phytophthora sp. were isolated from potted ornamental, fruit and forest tree samples in all regions of Italy except for Lombardy, Trentino, Aosta valley, Molise, and Abruzzi (Figure 2.5).

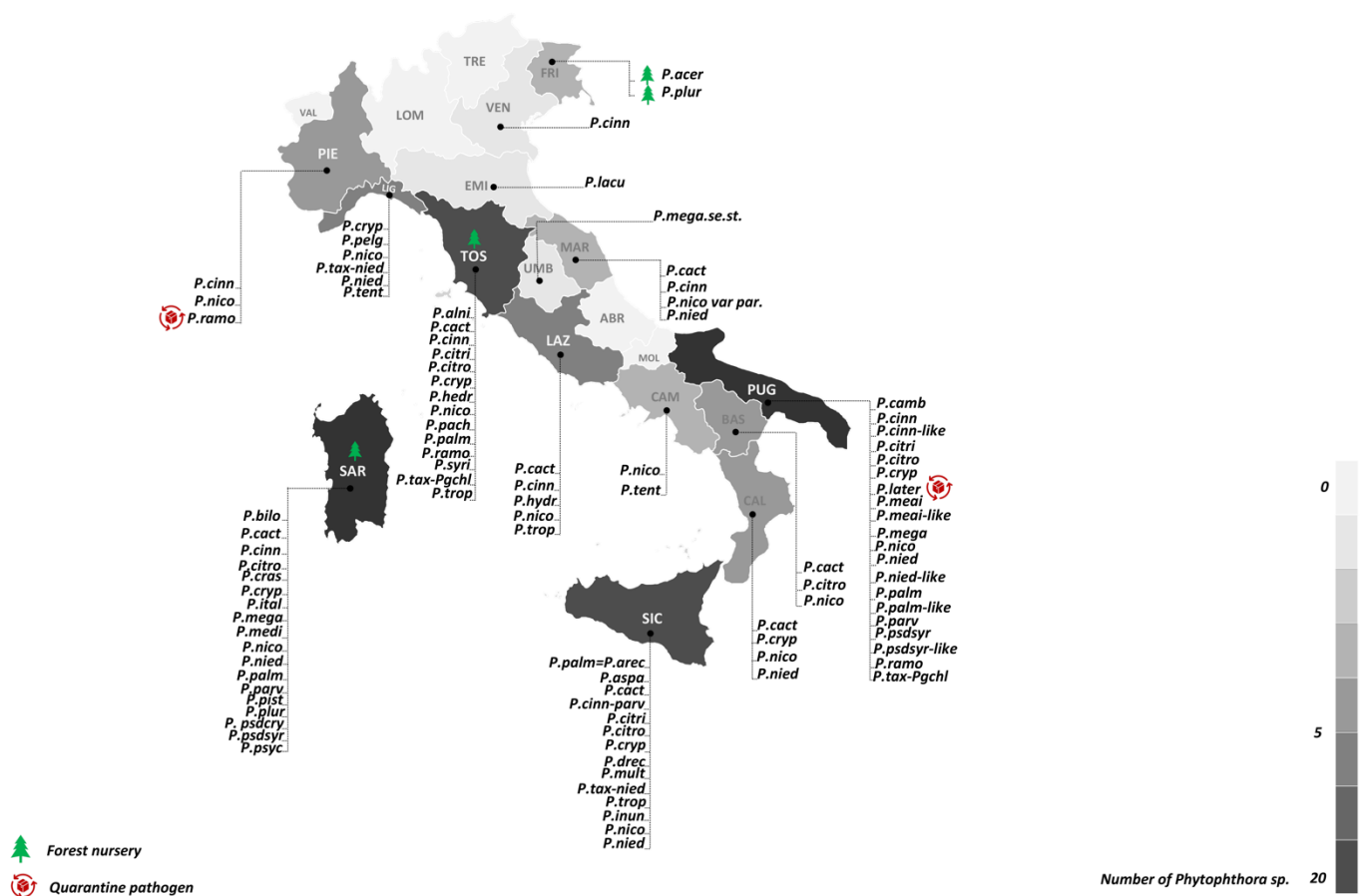


Figure 2. 5 Figure 5: Map of *Phytophthora* sp. distribution according to Italian region.

P. acerina = *P. acer*, *P. alni subsp. uniformis* = *P. alni .unifo*, *P. asparagi* = *P. aspa*, *P. bilorbang* = *P. bilb*, *P. cactorum* = *P. cact.*, *P. cambivora* = *P. camb.*, *P. cinnamomi* = *P. cinn.*, *P. cinnamomi-like* = *P. cinn like*, *P. citricola* = *P. citr.*, *P. citricola sensu stricto* = *P. citr s.st.*, *P. citrophthora* = *P. citro.*, *P. cryptogea* = *P. cryp.*, *P. crassamura* = *P. crass.*, *P. drechsleri* = *P. drech.*, *P. gonapodyides* = *P. gona.*, *P. hedraiandra* = *P. hedra.*, *P. hydropathica* = *P. hydro.*, *P. inundata* = *P. inun.*, *P. italica* = *P. ital.*, *P. lacustris* = *P. lacu.*, *P. lateralis* = *P. latera.*, *P. meadii* = *P. meadii*, *P. meadi like* = *P. meadi like*, *P. megasperma* = *P. mega.*, *P. mediterranea sp.* = *P. medit.*, *P. megasperma sensu stricto* = *P. meg s.st.*, *P. multivora* = *P. mult.*, *P. nicotianae* = *P. nico.*, *P. niederhauserii* = *P. niede.*, *P. niederhauserii-like* = *niede. like*, *P. palmivora* = *P. palm.*, *P. plurivora* = *P. pluri.*, *P. pachyplora* = *P. pachy.*, *P. parvispora* = *P. parvis.*, *P. × pelgrandis* = *P. ×pelgr.*, *P. pistachiae* = *P. pista.*, *P. pseudocryptogea* = *P. psd.cryt.*, *P. pseudosyringae* = *P. psd.syr.*, *P. psychrophila* = *P. psych.*, *P. taxon pgchlamydo* = *P. pgchl.*, *P. ramorum* = *P. ramo.*, *P. syringae* = *P. syri.*, *P. tentaculata* = *P. tenta.*, *P. tropicalis* = *P. trop.*,

The distribution of different *Phytophthora* species in various regions is shown in Figure 2.5. The highest number of *Phytophthora* species was found in Apulia, Sardinia, Sicily, and Tuscany. These areas are known for their high concentration of nurseries, which are some of the most important in Europe and sell plants all over the world (Ginetti et al., 2015).

In total, 44 *Phytophthora* species were recovered from 175 different hosts in sites with a range of climatic conditions, from Mediterranean to alpine. Most of the recovered species were thought to be exotic, with only three species likely being native to Europe (Jung et al., 2016; Redondo et al., 2018).

According to the EPPO A2 list of quarantine pests, two quarantine species were detected: *P. lateralis*, which poses serious threat to new environment (Bregant et al., 2021), was found in Apulia in two Italian nurseries on four different hosts (Prigigallo et al., 2015) and *P. ramorum*, which was eradicated in the Piedmont and Tuscany regions (Gullino et al., 2003; Ginetti et al., 2014a).

Phytophthora nicotianae, from clade 1 was the most frequently found species, with most isolates shared worldwide origin and a wide range of hosts (Biasi et al., 2016; Mammella et al., 2011; Panabieres et al., 2016; Prigigallo et al., 2015). The second most common species was *P. cactorum* (clade 1), *P. cryptogea* (clade 8), *P. cinnamomi* and *P. niederhauserii*, both of which have caused devastating *Phytophthora* epidemics in Europe.

Other less frequently isolated species included *P. acernia*, *P. alni*, *P. asparagi*, *P. bilorbang*, *P. cambivora*, *P. crassamura*, *P. cinnamomi*-like, *P. drechsleri*, *P. hydropathica*, *P. hydropathica*, *P. inundata*, *P. italica*, *P. gonapodyides*, *P. lacustris*, *P. lateralis*, *P. meadi*, *P. meadi* like, *P. mediterranea*, *P. multivora*, *P. niederhauserii* -like, *P. pachypleura*, *P. psychrophile*, *P. pistachio*, *P. pseudocryptogea*, *P. × pelgrandis* and *P. syringae* (Figure 5).

Many of the species and taxa detected in this study have been previously reported by Jung et al. (2016) in the large-scale study across 23 European countries.

However, 16 new *Phytophthora*–host associations were identified in this study:

P. acerina/ *Alnus incana*; ***P. alni* subsp. *uniformis***/ *Alnus cordata*; ***P. crassamura***/ *Picea abies*; ***P. cinnamomi*-like**/ *Grevillea* spp., *Cercis siliquastrum*, *Bougainvillea glabra*; ***P. hydropathica***/ *Viburnum tinus*; ***P. italica***/ *Viburnum tinus*; ***P. meadii***/ *Mentha* sp., *Petunia parviflora*, *Lobelia erinus*, *Impatiens hawkeri*; ***P. meadi* like**/ *Dahlia campanulata*; ***P. mediterranea***/ *Myrtus communis*; ***P. megasperma sensu stricto***/ *Celtis australis*; ***P. niederhauserii* -like**/ *Cyclamen persicum*; ***P. pachypleura***/ *Aucuba japonica*; ***P. pistachiae***/ *Pistacia lentiscus*; ***P. pseudocryptogea***/ *Laurus nobilis*; ***P. pseudosyringae***/ *Ilex aquifolium*, *Cercis siliquastrum* and ***P. psychrophila***/ *Ilex aquifolium*.

More than twenty isolated *Phytophthora* species have been described as first reports of host-pathogen combinations for Italy and elsewhere:

P. asparagi on fox tail agave, *P. italica* (Belisario et al., 1993) and *P. mediterranea* affecting *Myrtus communis* (Bregant et al., 2021); *P. lacustris* on *Prunus persica* (Nechwatal et al., 2013); *P. cactorum*, *P. cinnamomi* (Belisario et al., 2004) and *P. hydropathica* (Vitale et al., 2014) on *Viburnum tinus*; *Photinia fraseri* affected by *P. cactorum* (Vettraino et al., 2006); *P. cryptogea* on Lantana plants (Cacciola et al., 2005), and Dwarf Banana (Pane et al., 2018); *P. citricola* and *P. drechsleri* on *Cytisus scoparius* (Pane et al., 2005); *P. tentaculata* on *Gerbera* sp. (Cristinzio et al., 2006) and on *Origanum vulgare* (Martini et al., 2009); *P. cinnamomi* var. *parvispora* on *Mandevilla* sp. (Pane et al., 2010); *P. palmivora* on *Dodonaea viscosa* cv. *Purpurea* (Aiello et al., 2011) and *Phyllirea latifolia* (Bregant et al., 2021); *P. megasperma sensu stricto* on *Celtis australis* (Luongo et al., 2015); *P. megasperma* on *Cytisus scoparius* (Vettraino et al., 2004) and on *Laurus nobilis* (Bregant et al., 2021); *P. niederhauserii* on *Mimosa* (R Faedda et al., 2013), *Callistemon* (Cacciola et al., 2009), *Diospyros kaki*, *Fuchsia*

magellanica, *Cyclamen persicum*, *Convolvulus cneorum*, *Salvia sp.*, *Olea europaea* (Prigigallo et al., 2015) and on *Pistacia lentiscus* (Scanu et al., 2011); *P. nicotianae* on *Forsythia sp.* (Cacciola et al., 1994), *Edgeworthia papyrifera* (Garibaldi et al., 2010), *Chamaerops humilis var. argentea* (Roberto Faedda et al., 2011) and on *Armeria maritima*, *Convolvulus mauritanicus*, *Tagetes erecta*, *Allium schoenoprasum*, *Lobelia erinus* and *Dahlia campanulata* (Prigigallo et al., 2015); *P. tropicalis* on *Prunus armeniaca* (Pane et al., 2009), *Albizia julibrissin* (Luongo et al., 2016) and *Cuphea* (Cacciola et al., 2006); *P. inundata* on Dwarf Banana (Pane et al., 2018); *P. psychrophila* and *P. pseudosyringae* on *Ilex aquifolium*; *P. pseudocryptogea* and *P. citrophthora* on *Laurus nobilis*; *P. bilorbang* on *Phyllirea latifolia* (Bregant et al., 2021). *P. crassamura* on *Picea abies* (Scanu et al., 2015); *P. cambivora* on *Rosmarinus officinalis*, *Dahlia campanulata*, *Prunus mahaleb* and *Impatiens hawkeri*, (Prigigallo et al., 2015). In addition to simultaneous Infections with *P. palmivora* and *P. nicotianae* on *Trachycarpus fortune* (Cacciola et al., 2011) (Figure 1, Appendix B).

The species found on the most hosts were *P. nicotianae* (67 hosts), *P. palmivora* (21 hosts), *P. cinnamomi* (18 hosts), *P. cryptogea* (17 hosts), *P. niederhauserii* (15 hosts) and *P. citrophthora* (14 hosts).

Accordingly, *Phytophthora species* were frequently detected from a typical cultivated plant species of the Mediterranean region: *Prunus sp.*, *Citrus sp.*, *Olea europea*, *Quercus sp.*, *Myrtus communis*, *Grevillea sp.*, *Cyclamen sp.*, *Convolvulus sp.*, *Arbutus unedo* and from other two widely sold ornamentals plants: *Viburnum sp.* and *Rhododendron sp.* that were affected by seven *Phytophthora* taxa (*P. cactorum*, *P. cinnamomi*, *P. citricola*, *P. hedraiandra*, *P. hydropathica*, *P. ramorum*, and *P. taxon Pgchlamydospora*) and five taxa (*P. cactorum*, *P. cryptogea*, *P. nicotianae*, *P. ramorum*, and *P. tropicalis* respectively).

Additionally, five trees' species of the genus *Prunus*: *Prunus armeniaca*, *Prunus laurocerasus*, *Prunus lusitanica*, *Prunus mahaleb*, *Prunus persica* were the most affected plants, infected with a nine well-known highly damaging *Phytophthora sp.*

Beside infecting dicotyledons, *Phytophthora cryptogea* and *P. inundata* has been also found infecting *Musa sp.* a monocotyledon plants species (Pane et al., 2018).

Furthermore, from this study detected *Phytophthora* species belongs to eight clades (Clade 7, 2, 8, 6, 1, 3, 4 and 9) out of the twelve known phylogenetic clades and corresponding to terrestrial and aquatic species of *Phytophthora* (Figure 2.6).

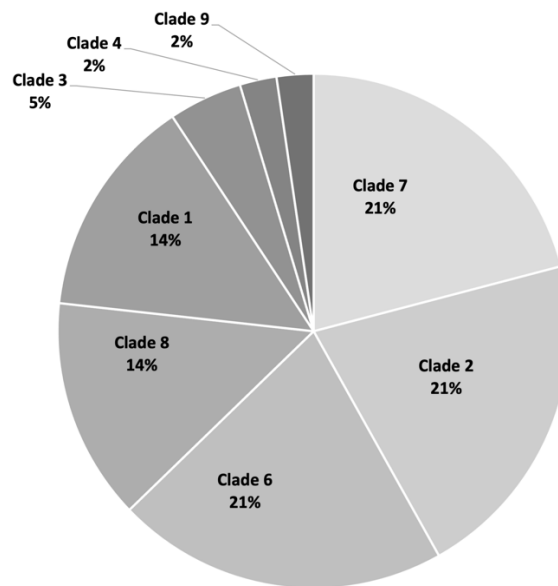


Figure 2. 6 Frequency of *Phytophthora* Clade in Italian nurseries

Nine of the *Phytophthora* species detected belonged to clade 7 (*P. alni subsp.uniformis*, *P. xcambivora*, *P. cinnamomi*, *P. cinnamomi-like*, *P. mediterranea sp*, *P. niederhauserii*, *P. niederhauserii-like*, *P. Parvispora*, *P. pistachiae*), and to species in clade 2 (*P. acerina*, *P. citricola*, *P. citrophthora*, *P. meadii*, *P. meadi like*, *P. multivora*, *P. pachypleura*, *P. plurivora*, *P. tropicalis*) and to species in clade 6 (*P. asparagi*, *P. bilorbang*, *P. crassamura*, *P. gonapodyides*, *P. inundata*, *P. lacustris*, *P. taxon Pgchlamydo*, *P. megasperma*, and *P. megasperma senso stricto*). Moreover, six species in clades 8 (*P. cryptogea*, *P. drechsleri*, *P. lateralis*, *P. pseudocryptogea*, *P. ramorum*, *P. syringae*,) and clade 1 (*P. cactorum*, *P. hedraiandra*, *P. italica*, *P. nicotianae*, *P. xpelgrandis*, *P. tentaculata*.) respectively. However, each of clades 4 and 9 includes only one species: *P. palmivora* and *P. hydropathica*, respectively (Figure 2. 6).

Interestingly, *P. pistachiae*, *P. mediterranea sp.* and *P. alni subsp.uniformis* from clade 7 were mostly detected from forests nurseries and a limited geographic distribution has been noticed in South Italy for clade 3 including only two *Phytophthora* species, *P. pseudosyringae* and *P. psychrophile*.

The typical symptoms of *Phytophthora* including chlorosis, root and crown rot, dieback, stem canker, trunk gummosis, foliar blight and leaf lesions, stunting, wilting and death of plants. These symptoms have been observed in most isolated plants. However, some plants exhibit specific symptoms, such as leaf reddening on *Pistacia lentiscus* (Linaldeddu et al., 2020a) and premature defoliation on high-bush blueberry (Tamiatti, 2003). In some species, wilting and death of plants without leaf drop has been observed (Garibaldi et al., 2004), as well brown to black foliar lesions, necrosis of the petioles and browning of the stems (Ginetti et al., 2014a). In some case, *Phytophthora* was recovered from asymptomatic plants where above-ground plant organs, appeared visually healthy. Migliorini et al., (2015) convincingly demonstrated the weakness of a visual inspection of symptoms as a primary method for detecting *Phytophthora* infestations. Similarly, Cacciola et al., (2011) isolated the wide host range pathogens *P. palmivora* and *P. nicotianae* from asymptomatic *Trachycarpus fortune* in Sicily.

In addition to potting soil and irrigation water, different species were consistently isolated from a variety of plants parts (petiole base, cambium, roots, leaves, collar, basal stem, and rhizosphere soil) of symptomatic plants. Isolation was done by baiting with Ericaceous leaves

mainly *Rhododendron* sp. and *Camellia* sp. and less frequently with leaves of *Quercus* sp., *Hedera helix*, *Capsicum annuum*, *Sambucus nigra* and apple fruits (e.g. *Golden delicious*). This was done on a different type of selective medium containing the following ingredients (P: Pymaricin, A: Ampicillin, R: Rifampicin, B: Benomyl, N: Nystatin; P: Pentachloronitrobenzene, H: Hymexazol): Corn Meal Agar (CMA; (Belisario et al., 1997); Potato Dextrose Agar (PDA), Synthetic Mucor Agar (SMA; (C. G. Elliott et al., 1966), PARPNH (Erwin & Ribeiro, 1996); P₅ARPH (Jeffers & Martin, 1986), BNPRAH (Masago et al., 1977), PDA +BNPRAH; PARBhy (Vettraino et al., 2001), PARP (Jeffers & Martin, 1986), SMA+Carrot juice+ MBC (benomyl hydrochloride) + 1 M NaOH + Pimaricin + Rifamycin (Brasier et al., 2005), Acidified Malt Extract Agar (MEA) (Tamiatti, 2003), PDA + Carrot juice + Pimaricin+ Hymexazol (Linaldeddu et al., 2020a), PDA + Nistatin + Rifampicin +Ampicilin (Pilotti et al., 2014).

Phytophthora's identifications were initially made through morphological and cultural observations of the pathogen growth on various media: carrot agar (CA), corn meal agar (CMA), malt extract agar (MEA), potato dextrose agar (PDA), and V8 agar. These observations were followed by microscopic studies of morphological characteristics such as size and shape of sexual and asexual reproductive structures, as well as cardinal temperatures of growth.

Moreover, in most articles, sporangia formation was induced on V8 agar, carrot agar, or PDA using various methods, such as saline solution of Chen and Zentmyer (Erwin & Ribeiro, 1996), sterile soil extract (Garibaldi et al., 2009), non-sterile soil extract (Pane et al., 2018), infected pepper seeds on water agar flooded with pond water (Ilieva et al., 1995), sterile water (Roberto Faedda et al., 2013), unsterile pond water (Scanu et al., 2015), filtered pond water (Ginetti et al., 2015) and clarified V8 broth (Aiello et al., 2018).

Protein and molecular phylogeny-based methods were also used for identification. Until 2011, many articles electrophoretic analysis of total mycelial proteins with some of the following isozymes (alkaline phosphatase, esterase, fumarase, NAD-glucose dehydrogenase, malate dehydrogenase, and superoxide dismutase) on sodium dodecyl sulfate-polyacrylamide gel electrophoresis in combination with molecular diagnostics detection methods.

PCR-based technologies using specific primers (*ITS6* /*ITS4*; *Ph1/Ph4*, *TS1* /*TS4*) were commonly used to amplify the internal transcribed spacer (ITS) regions of the nuclear ribosomal RNA gene cluster. However, for some species this region may not be the best choice due to variance among phylogenetically closely related species or intraspecific divergence (Schroeder et al., 2013).

In these cases, other regions that were used in this study include nuclear encoded genes coding for translation elongation factor 1 α (*EF-1 α*), β -*tubulin*, as well as the mitochondria-encoded cytochrome c oxidase subunits I and II (*Cox 1*, *Cox 2*) and less commonly, the phenol acid decarboxylase (*Pheca*) gene.

The need to fulfill Koch's postulates is an absolute requirement when reporting a new host for *Phytophthora* sp., a culturable oomycete.

While *Phytophthora* includes soilborne and aerial species, different pathogenicity test methods have been performed in 25% of articles reporting a new disease outbreak. *Phytophthora* sp. was evaluated by soil inoculation with zoospores suspension to the root crown (Pane et al., 2010), infestation by chlamydospores suspension (Cacciola et al., 2003) or with inoculum produced on autoclaved kernel seeds (Pane et al., 2009). In contrast, other authors have verified the virulence of *Phytophthora* species by spraying the canopy with a zoospore suspension (Aiello et al., 2018), inoculating with a mycelial plug on leaves or under twigs bark in a wet chamber, or incubating a cutting shoot in a test tubes with sterile water (Ginetti et al., 2015; Vitale et al., 2014). However, some inoculation has been performed under bark at the collar of

plants seedlings (Bregant et al., 2021) and in only one case, pathogenicity was tested on apple fruit and potato tuber (Belisario et al., 1997). As well as, *Phytophthora*'s pathogenicity tests have been performed on a variety of plant species in some studies, Luongo et al., (2016) inoculated *Phytophthora* on bell pepper plants, an agricultural crop, and on *Albizia julibrissin* the main host infected by *P. tropicalis*.

2.1.4 Discussion

A widespread occurrence of *Phytophthora sp.* with a wide host range has been documented in infested nursery stock worldwide, causing damage to ornamental and forest plant species. International trade has also played a significant role in the introduction and spread of these diseases to new environments and hosts, as seen in the introduction of *P. plurivora* into the US from Europe (Schoebel et al., 2014).

Considering the importance of nurseries in Europe, a *Phytophthora* literature survey and mapping of species could provide a valuable information for limiting the spread of these pathogens. In addition, a better understanding of *Phytophthora* communities present in Europe is crucial for the development of effective mitigation and management strategies for these diseases.

The occurrence of *Phytophthora* in European ornamental nurseries is well documented, with a high diversity of species present. Jung et al., (2016) identified 49 different species in almost 732 nurseries, and other studies such as (Prigigallo et al., 2015) have also documented the presence of these pathogens in European nurseries. This study presents updated data on all scientific publications on *Phytophthora* mainly present in European ornamental plants nurseries, and more specifically in Italy.

Increased surveys specifically for quarantine *Phytophthora sp.* and continuous visits per year from researchers and the government plant health inspectors (Lane et al., 2003; Schlenzig et al., 2015) were considered as a helpful management strategy in estimating the magnitude of the occurrence and trends of *Phytophthora* disease over time.

For example, in Italy, most collected samples have been taken after a visual symptomatic incidence and 25% from the total research articles have been dedicated from a survey study.

Even though surveys are more likely to report *Phytophthora* disease, it can also result in the frequent discovery of previously unknown *Phytophthora sp.* (Brasier et al., 2005).

During this study the number of detected species has been increased a high number of different formally described species has been isolated in addition to the re-evaluation of previously informally designated isolates as example: '*Phytophthora citricola* complex', *P. citricola* to *P. plurivora*, *P. taxon pgchlamydo* to *P. chlamydospora*, *P. megasperma* complex, *P. taxon Salixsoil* to *P. lacustris*, *P. italica* identified as *P. iranica*.

There is a need for a specific molecular primer for the detection of informally designated *Phytophthora* taxa or difficulty related species to separate such as those from the *P. megasperma* and *P. citricola* complex since it encompasses different subgroups some of which are recognized as host-specific or pathogenic to a wide range of hosts (Luongo et al., 2015; O'Hanlon et al., 2016).

In Italian nurseries, some provisionally named species (Cleary et al., 2021) have been isolated and of note is the detection of *P. ×pelgrandis*, *P. ×cambivora*, *P. alni subsp. uniformis* a stable hybrid and *P. meadii* an expected one (Sansome et al., 1991). These hybrids can potentially pose a serious threat to nurseries and natural ecosystems by infecting a wider host range, displaying an increased epidemiological risk and enhanced vigor compared to their progenitors (Poucke et al., 2021). Moreover, asexual propagation and cultivation systems of different crop

in the same nursery would contribute to the spread of aggressive pathogen of this type of hybrid whereas the propagation by seed results in a geographic isolation of lineages (Biasi et al., 2016). On the other hand, potted ornamental nurseries with the occurrence of both mating type in heterothallic *Phytophthora* taxa play an important role in increasing genetic variation within the species allowing pathogen to rapid adapt to a new host populations and environment (Scanu et al., 2014). Indeed, both mating types of *P. cryptogea*, *P. nicotianae*, and *P. palmivora* have been recovered in Sicilian nurseries (Pane et al., 2010; Cacciola et al., 2005, Aiello et al., 2011, Pane et al., 2018).

Additionally, in Italy, the occurrence of homothallic species (20) were generally higher than the heterothallic ones (16) (Appendix B). Moreover, nine of homothallic species except for *P. Mediterranean*, the heterothallic new discovered *Phytophthora* sp., are adapted to a specific host. This in agreement with Prigigallo *et al.*, (2015) and Mammella *et al.*, (2011) which revealed that isolates of asexually propagated clones were frequently associated with a single host in samples.

Furthermore, even with the possible introduction or the presence of one mating types, the hazard represented by some *Phytophthora* species is evident as they were found on a wide range hosts in addition to some biological traits such as abundant production of resistant spores (chlamydospores) or hyphal aggregates contributing to a long-term survival strategy allowing *Phytophthora* to survive adverse soil conditions even in the absence of host plants an example reported of *P. nicotianae* chlamydospores that can survive for up to 6 years (Panabières et al., 2016). (Appendix C). In addition, for *P. megasperma*, *P. plurivora*, *P. asparagi*, *P. syringae*, *P. crassamura* and *P. inundata* that can produce oospores with thick walls. In general, the ability to produce resting structures is a common adaptive strategy of these species to cope with adverse environmental conditions and to increases their competitiveness in terrestrial environments (Jung et al., 2019; Luongo et al., 2015; Riolo et al., 2020). On the other hand, another important survival strategy is the ability of some *Phytophthora* species, such as *P. drechsleri*, *P. inundata*, *P. lacustris*, *P. hedraindra* and other polyphagous species *P. meadii*, *P. palmivora* and *P. tropicalis* to spread easily once introduced to a new environment due to their caducous sporangia and abundant zoospores that are disseminated by rain and irrigation water (Aiello et al., 2011b; Luongo et al., 2016a).

Due to current global warming, several *Phytophthora* sp. found in nurseries might pose a threat to natural ecosystems especially when they have the potential to survive in very different environments including high and low temperature habitats (Appendix C) and the ability to colonize different hosts, an example of *P. cinnamomi* in clade 7 that causes severe damage and decline on holm and cork oaks in the southwest of the Iberian Peninsula and Southern Italy (Brasier et al., 1993; Frisullo et al., 2018; Seddaiu et al., 2020b). Recently, it has been detected in cold regions on a blueberry stand in Germany and in a mountain nurseries in Italy (Bregant et al., 2021; Nechwatal & Jung, 2021). Similarly, a closely related two new species, *P. parvispora* and recently described one *P. mediterranea*, that are highly temperature-tolerant 37°C (Bregant et al., 2021; Scanu et al., 2014) favorable for their establishment in new environments and climate change is likely to increase this possibility.

Indeed, some other species like *P. hydopatica*, within clade 9, occasionally found in Lazio region on *Viburnum tinus* (Vitale et al., 2014), has been considered also as serious threat to agricultural and natural environment due their high optimal growth temperatures (35°C) that are favorable for disease development.

The Invasion potential of *Phytophthora* species in future climates will depend also on their ability of some species to escape from nurseries. So far, few *Phytophthora* sp. have been found in association with nursery crops. These include species from clade1 (*P. tentaculata*, *P. ×*

pelgrandis, *P. italica*, *P. hedraiaandra*), clade 2 (*P. meadii*, *P. pachyplora*, and *P. tropicalis*), clade 7 (*P. alni*, *P. mediterranea*, *P. niederhauserii*, *P. pistachiae*), clade 8 (*P. drechsleri*, *P. lateralis* *P. ramorum*) and clade 6: (*P. taxon Pgchlamydospora*).

In general, most of the detected species have been found infecting forest tree, but some species should be carefully monitored, as they have the ability to colonize agriculture crops or have had a limited association with fruit crops. For example *P. palmivora* has been isolated from olive (Cacciola et al., 2000) and pomegranate tree (Linaldeddu et al., 2020b); *P. nicotianae* has infected tomato plants (Pane et al., 2000), *P. megasperma* and *P. multivora* have recently appeared on white mulberry (Pane et al., 2016) and *P. pseudosyringae* has been isolated from *Castanea sativa* (Scanu et al., 2010).

Phytophthora sp. in clade 6 are of particular interest because they are mostly isolated from natural habitats and are less commonly found in fruit and ornamental nurseries (Nowak et al., 2015).

According to a study by Jung et al., (2016), these species were found in 3.8% of the European nursery stands. However, from this study, it represented 11% of infected European nurseries. In Italy, nine aquatic isolates from clade 6 have been mostly found in Southern Italy, the result is in accordance with Riolo et al., (2020), their presence in water courses and irrigation systems highlights the importance of these sources in the dissemination and transport of these pathogens to nurseries. Some members of clade 6 may not pose a threat to nurseries, as they can be opportunistic or sometimes aggressive tree pathogens (Santilli et al., 2020).

It is unusual to find such a high diversity of *Phytophthora* species (18 species) in a small area like Sardinian nurseries. These species were predominantly found in forest nurseries, and some, such as “*P. mediterranea*, *P. crassamura*, *P. parvispora* and *P. italica*” are new discoveries. However, other detected species, such as *P. acerina*, *P. pseudocryptogea*, and *P. plurivora*, have already been reported on *Alnus glutinosa* and pose a serious threat to riparian alder stands in Sardinia (Seddaui & Linaldeddu, 2020a). This highlights the importance of monitoring and managing the presence and spread of these plant pathogens in order to protect forests and agricultural systems from potential damage.

The detection of *Phytophthora* species can be influenced by the isolation methods used. In Italy, the use of baiting with *Sambucus nigra* and or *Quercus suber* leaves has led to the discovery of new species, such as *P. mediterranea* and *P. crassamura* and the identification of rarely described species such as *P. pistachiae*, *P. pseudocryptogea*, *P. psychrophile*, and *P. acerina*. This suggests the use of different types of baiting leaves in addition to the traditional method of using *Rhododendron* leaves, may be useful in the detection of these species (Bregant et al., 2021; Linaldeddu et al., 2020; Scanu et al., 2014).

The rapid advances in molecular diagnostics techniques, such as 454-pyrosequencing and metabarcoding approach (Prigigallo et al., 2016) have contributed to the discovery of a large diversity of *Phytophthora* species, such as in the Apulia region of Italy. These techniques can also help in the characterization of invasive species and the detection of the hybrids (Poucke et al., 2021) but it is not always possible to test the pathogenicity of these species on plants because living cultures cannot be obtained. These molecular techniques are powerful tools for understanding the diversity and potential impacts of *Phytophthora* species, but it is important to also consider other factors, such as the biology and ecology of the species, in order to fully understand their potential impacts and manage their presence and spread.

Performing pathogenicity test on *Phytophthora* sp. after they have been detected is important for understanding their biology, evolution, and aggressiveness. These tests can also provide insights into the origin of the pathogens, as they can help determine whether they are native or

alien invasive species (Jung et al., 2016). It is important to perform pathogenicity tests not only on the newly detected host, but also on similar plants in forests and natural ecosystems (Bregant et al., 2021), as this can help to identify potential impacts and inform the development of appropriate management strategies. In order to obtain reliable results, it is important to follow a standardized protocol for pathogenicity testing that takes into account the age, type of host material, and environmental conditions.

The increasing number of described *Phytophthora* species has raised important concerns about the accuracy of detection methods and the management practices in nurseries.

In this study, the most commonly used methods for detecting *Phytophthora* in nurseries were traditional morphological methods, followed by molecular characterization methods which are time-consuming and labor-intensive (Figure 2.7).

The spread of *Phytophthora* through nurseries particularly through the movement of infested plant material, is a major concern. Phytosanitary inspection agencies may not always detect infested nursery stock because infected plants may have been removed or pruned or may not yet be showing symptoms (X. Xu et al., 2009).

In some cases, *Phytophthora* has been isolated from asymptomatic plants. According to Jung et al., (2016), this may be due to the suppression of *Phytophthora* by chemical fungicides, or the presence of a primary inoculum in early soil infestation (Cacciola et al., 2011), where the plants in a controlled environment still have a stable microbiota structure (Tkacz et al., 2020) that suppresses the pathogen.

Despite strict quarantine regulations and national phytosanitary inspections, several quarantine species of *Phytophthora*, have been detected in imported infested plants stock. For example, *P. pachypleura* has been isolated from *Acuba japonica* imported from France (Ginetti et al., 2015). *P. niederhauserii* was found in infected *Grevillea* plants imported to the UK from Italy (Brasier, 2008) and *P. ramorum* was detected in infested *Viburnum* imported from Italy to the Czech Republic (Běhalová, 2006), and in a plant stock distribution between two local nurseries in 2013 (Ginetti et al., 2013).

Detection of *Phytophthora* is typically based on visual inspection for symptoms, but this can be complicated when multiple *Phytophthora* species affecting common host plants for quarantine pathogens have similar symptomology. This can make it difficult for phytosanitary inspection services to control the spread of these pathogens (Ginetti et al., 2014) and prevent them from affecting different host plants, especially when there are no visible symptoms (Migliorini et al., 2015). For this reason, it is important for plant pathologists, phytosanitary inspection services, and growers to have accurate, non-destructive, in-situ analysis technologies and plant biosensors for detecting *Phytophthora*. Researchers should also have a common molecular-based detection protocol (Jung & Blaschke, 2004) for re-identifying and confirming *Phytophthora* species isolated in nurseries (Figure 2.7).

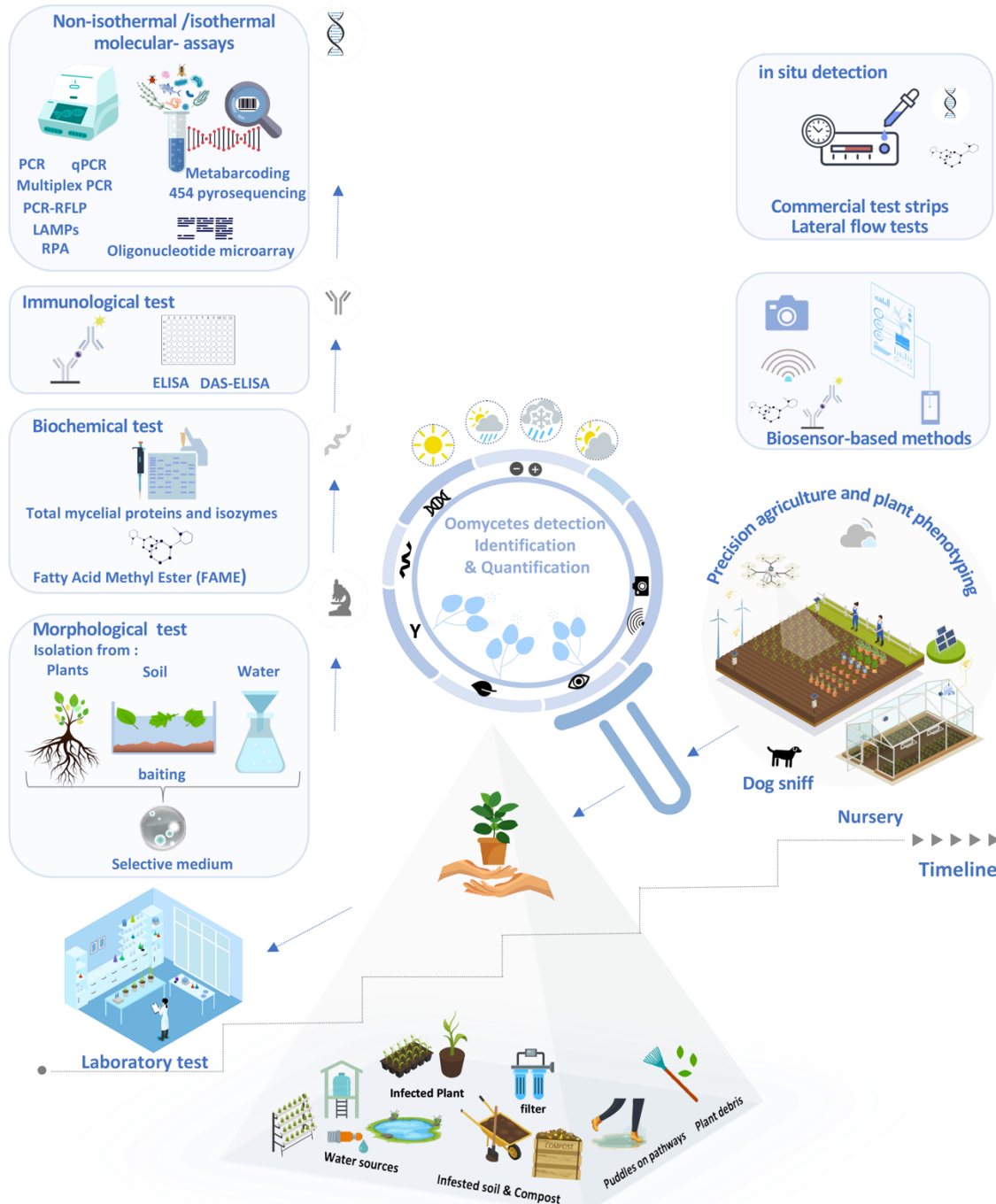


Figure 2. 7 Different *Phytophthora* detection methods. The detection and identification of *Phytophthora* sp. isolated from water, soil and plants have been achieved by using traditional method (baiting on selective media, morphological characteristic) as well as biochemical (polyacrylamide gel electrophoresis of total mycelial proteins and isozymes, Fatty acid methyl ester), Immunological (enzyme-linked immunosorbent assay (ELISA), the double-antibody sandwich enzyme- linked immunosorbent assay (DAS- ELISA)) and molecular (Sequences of DNA, Metabarcoding) methods. However, over the past years, advances in detection procedures have been attempted by using in situ lateral flow diagnostic kit. Moreover, to expedite *Phytophthora* pathogen detection, future trends on the use of (bio)sensors might demonstrated a great potential.

Additionally, the widespread infestation of *Phytophthora* in nurseries is often due to poor nursery practices (Jung et al., 2016).

To prevent the establishment of *Phytophthora* diseases, it is important to implement protective measures in nurseries, while also considering the critical thresholds for each management practice (Parke et al., 2014).

Some of the best measures to reduce the risk of *Phytophthora* development include (Figure 2.8):

1) Good cultural practices, such as maintaining good sanitation, avoiding the movement of soil and contaminated plant material, preventing direct contact between soil and plant containers, and disinfecting seeds, trays, and containers and removing plant debris. The effectiveness of disinfectants in reducing the spread of disease can vary depending on the concentration and treatment time used (James et al., (2012).

Additionally, infestation levels of nurseries can be influenced by the ability of *Phytophthora* sp. to form enduring resting structures, which allow the pathogen to survive in adverse environmental conditions (Jung et al., 2018). To address this, it can be helpful to apply biological control agents (Funahashi & Parke, 2016), use of fungicides with different modes of action in alternation (Aiello et al., 2018), and control pesticides losses (Hinz et al., 2020). The use of host plant defense inducers (Neupane et al., 2022) and cover crops as a potential tool for suppression of soil borne diseases (Parajuli et al., 2022) may be useful.

2) Irrigation water can be a major source of *Phytophthora* diversity in nurseries. To reduce the risk of pathogen infection, it can be helpful to treat irrigation water through filtration and disinfection with chemicals such as sodium hypochlorite or calcium chlorite (Parke et al., 2019). This can help to reduce the diversity of *Phytophthora* species present in the nursery and prevent the spread of infection.

3) It is important also to avoid soil compaction by using a sustainable and healthy potting medium with a diverse soil biota (Panth et al., 2020), and by avoiding the use of disinfected soil or the reuse of plant growth substrate (Aiello et al., 2018). In the case of composting, it is important to ensure that temperatures was achieved and sufficient to kill the pathogens (Parke et al., 2019).

4) The use of healthy plant material and proper management of nutrients can be a high priority to reducing *Phytophthora* infection. It is also essential to check for the importation of timber and to prohibit the importation of soil, or growing medium from countries with a high risk of *Phytophthora* infection, as has been done by several countries such as Australia, New Zealand, Canada and the USA (Migliorini et al., 2015). Plant exchanges and plant hunting can also contribute to the spread of *Phytophthora* diseases (Schlenzig et al., 2015).

Therefore, it is important to constantly update and enforce regulatory measures such as quarantine laws (Panth et al., 2020), with stricter regulations needed for the movement of specific plants (such as Azalea and Viburnum) that may be infected with different quarantine species.

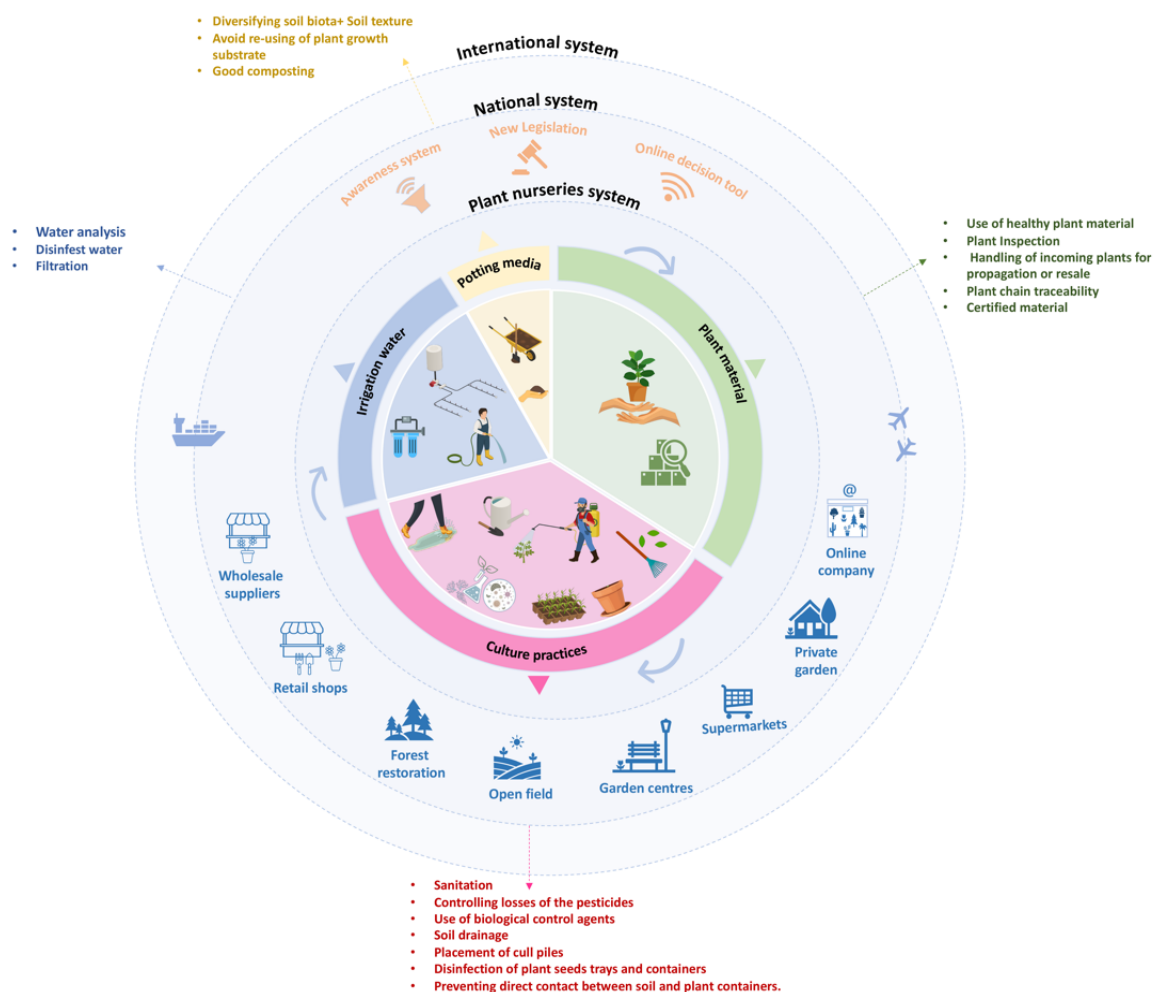


Figure 2. 8 *Phytophthora* diseases management in nurseries production system: For a successful management of *Phytophthora* in nurseries (First bubble) , greater emphasis should be placed on preventing disease introduction into natural ecosystem (Second bubble) or the dispersal to a new area through international trade and travel (Third bubble) where the management goal may be to interrupt the life cycle of a pathogen circulate through a nursery systems (Arrow around first bubble), by different systems approaches regarding 1) Potting media, 2) Plant material 3) Cultural practices and 4) Irrigation water in which the nursery management program seek to prevent pathogens flow with the help of an updated and enforced regulations and an online farmers awareness program.

2.1.5 Conclusion:

Nursery plants are significant vectors for *Phytophthora* plant pathogens on a global scale, and there is a significant problem with the failure to detect these pathogens within international and national plant distribution and certification systems. To prevent the introduction of these pathogens, it is necessary to develop integrated systems approaches and adopt biological control strategies through management programs.

This study may not provide a comprehensive understanding of the *Phytophthora* community in Italian nurseries, and further surveys should be conducted in vegetable, cut flower, fruit

nurseries, and soilless horticultural cultivation where the presence of *Phytophthora* could potentially pose a risk to agriculture crops in the future.

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2.2 NATURAL PLANT EXTRACT TARGETING *PHYTOPHTHORA* SP. AS BIOLOGICAL CONTROL METHODS: EXISTING AND RECENT FINDING

Abstract

Over the last decades, synthetic fungicides have become a major environmental and health concern. To address this issue, the development of new fungicidal compounds has become urgent. Natural plant products have been a source of several antifungals used in clinical settings and agriculture. This review focuses on the discovery of plant extracts that show the ability to inhibit *Phytophthora* sp..

A comprehensive systematic search for plant extracts against different *Phytophthora* sp. was conducted using articles published until July 2021 from the following scientific databases: NCBI PubMed, Scopus and web of science. The plant family species and *Phytophthora* target species are listed. Results show that garlic species are the most widely used and have demonstrated efficacy in various *in vitro* and *in planta* properties against different *Phytophthora* sp. based on their traditional uses and scientific evaluation. In addition, this report provides an overview of other natural antifungals that target *Phytophthora* activities and discusses recent discoveries of natural products that may be used as starting points for the development of potential selective and non-toxic anti-*Phytophthora* fungicides.

2.2.1 Introduction

Conventionally fungicides are the most effective strategy for preventing and protecting crops from *Phytophthora* pathogens (Le et al., 2019). The main products used to manage various foliar and soil-borne oomycota pathogens include copper-based fungicides (Lamichhane et al., 2018), the phenylamides group (e.g. Mefenoxam, Metalaxyl), phosphonate-based compounds (e.g. Potassium phosphite, Fosetyl-Al), and the new oomycota fungicides with different mode of action (e.g. Ethaboxam, Thiazole carboxamide, Fluopicolide, Mandipropamid and Oxathiapiprolin) (Belisle et al., 2019; Hao et al., 2019). However, these synthetic fungicides can take a long time to degrade completely, causing toxicity to living organisms and impact the growth of BCAs, in addition to the selection of resistant *Phytophthora* strains (Ons et al., 2020; Pompimon et al., 2009). With increasing interest in environmentally friendly, sustainable agriculture and the public demand for organic products, natural plant extracts and other biological strategies have been suggested as alternative to synthetic fungicides for controlling crop diseases (Al_Samarrai et al., 2012; Boiteux et al., 2014; Gulzar et al., 2022; W. Wang et al., 2004).

Plant extract are a complex of secondary metabolites isolated from plants. The extraction processes of these biomolecules is based on their solubility with various solvents, which are chosen based on the polarity of the bioactive compounds of interest. The solvents used range from highly polar solvents such as distilled water, methanol ethanol and acetone to less polar solvents like hexane, chloroform and ethyl acetate (Naboulsi et al., 2018).

There have been advances in the extraction of plant bioactive compounds using non-conventional methods such as the subcritical water extraction (SWE) (Zakaria & Kamal, 2016), supercritical carbon dioxide (CO₂) technique (Molnar et al., 2017), ultrasound-assisted extraction (UAE), enzyme-Assisted extraction (EAE), microwave-Assisted Extraction (MAE),

pulsed Electric Field assisted extraction (PEF) and pressurized Liquid Extraction (PLE) (Azmir et al., 2013). These methods are considered to be more effective in terms of the quality and properties of plant extract bioactive compounds and are more environmentally sustainable, reducing operational time and the use of synthetic and organic chemicals (Zakaria & Kamal, 2016). Many of these new, environmentally friendly extraction methods have been developed as alternatives to traditional methods such as Soxhlet organic solvent extraction, maceration, and hydro-distillation traditional methods (Zakaria & Kamal, 2016; Azmir et al., 2013).

Plant extracts have been used in various commercial sectors such as the pharmaceutical (Patra et al., 2018), chemical, and food industries (Balasubramaniam et al., 2022). In agriculture, they are still of high interest, with several authors investigating their application in sustainable agriculture for a wide range of purposes, such as biofertilizers or as a natural plant biostimulants that enhance crop productivity (Rouphael & Colla, 2020), seed treatment (Mbega et al., 2012), synthesis of silver nanoparticles for use as a biopesticide (Bapat et al., 2022) and as resistance inducer in plants (Krzyzaniak et al., 2018; Latha et al., 2009). In addition, the potential of plant extract has been explored as botanical fungicides (Bhuyan et al., 2013), insecticides (Mantzoukas et al., 2022), nematicides (Zaidat et al., 2020), and herbicide (El-Mergawi & Al-Humaid, 2019). Some plant extracts have also been shown to contain phytochemicals such as azadirachtin, pyrethroids, and carvone that have been successfully formulated as botanical pesticides and used in integrated pest management (Nguyen et al., 2009).

While it is known that certain plants have antibacterial and antifungal properties in laboratory studies, little is still understood about which specific botanical taxa have exhibited the most promising activity against *Phytophthora sp.* to date. This review presents a comprehensive analysis of the literature on plants used as anti-*Phytophthora* activities, with a focus on their reported growth inhibitory activity.

2.2.2 Materials and Methods

Online literature survey

This study is a literature review that was conducted online. It focuses on scientific articles that were published between 1957 and 2021 (July 2021) in academic journals. The articles were obtained from the following databases: ISI Web of Science (webofknowledge.com), PubMed (pubmed.ncbi.nlm.nih.gov) and Scopus (scopus.com). We used a combination of search terms, including “*Phytophthora sp.*”, and “Plant extract” and or “Biocontrol” to find relevant articles about the use of plant extracts to control *Phytophthora* diseases.

A screening process has been conducted to eliminate irrelevant papers, first based on the title and abstract, and then by excluding articles related to algae, essential oils, and compost of the 604 papers retrieved. The remaining selected publications were organized and reviewed according to plant families, plant parts, the type of solvent used, and the major chemical components. All reported plant families were cross-checked for accuracy in accordance with The Plant List (<http://www.theplantlist.org/>) and Phylogenetic trees were viewed and edited using iMetaLab Phylo (<https://shiny1.imetalab.ca/phyloexplorer/>).

2.2.4 Results and discussion

Due to public and environmental awareness for safer alternatives to synthetic pesticides, the growth rate of scientific publication concerning plant extract used to control *Phytophthora*

diseases has been steadily and continuously increasing in the last few years, to a total of 229 screened articles until July 2021 (Figure 2.2. 1a).

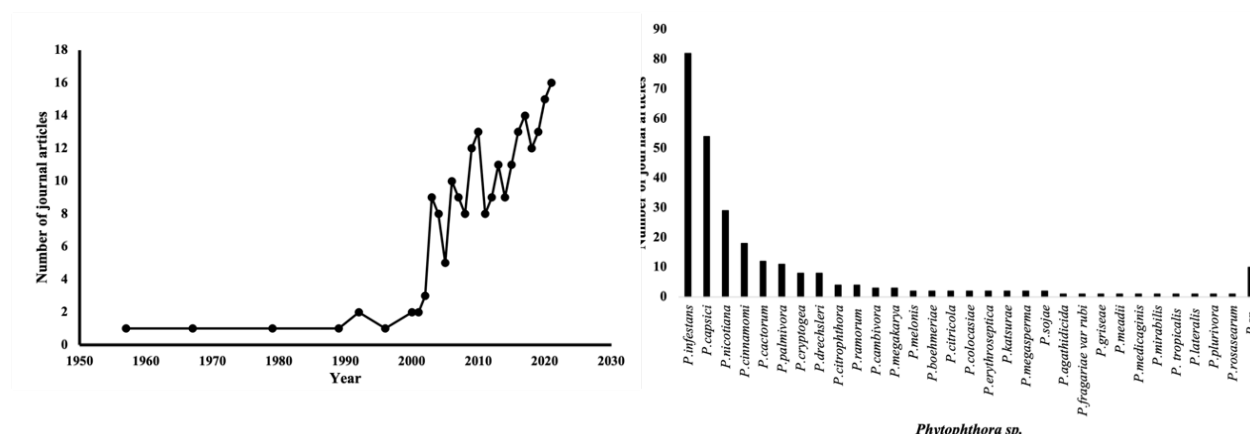


Figure 2.2. 1 Number of journal articles concerning plants extract for controlling *Phytophthora* sp. over the year (a) and Number of articles per *Phytophthora* sp. (b)

The tested natural plant extract exhibited anti *Phytophthora* activities against 30 *Phytophthora* sp. where *P. infestans* followed by *P. capsici* and *P. nicotiana*, the most studied species with scientific publications exceeded 30 publications in 2021 (Figure 2.2. 1b), this could be due to factors that these species are destructive and costly plant pathogen to date and *P. infestans* is considered as a major threat to food security (Goss et al., 2014).

This study reported that 700 plants species were evaluated and tested as alternative sources for chemicals fungicides. These plant species belong to 140 different families, with the most studied being the Asteraceae, Fabaceae, Lamiaceae, Poaceae, Amaryllidaceae, and Myrtaceae (Figure 2.2. 2 (a)), (Appendix D: Table 1).

More than ten scientific publications for each of these families have reported on the diverse plant genera within them. For example, the Asteraceae family, which accounts for more than 39 plant genera tested on different *Phytophthora* sp. has been reported in 52 scientific journal articles (Figure 2.2. 2(b)).

This is likely due to the widespread of the Asteraceae family, which is one of the largest flowering plant families with over 1600 genera and 25,000 species worldwide (Rolnik & Olas, 2021).

Several studies have also confirmed the presence of high levels of chemical bioactive compounds in different plant species belonging to the Asteraceae family, including essential oils such as borneol, camphor, and cis-verbenol that have shown potential in inhibiting the growth of certain *Phytophthora* sp. (Salgado-garciglia et al., 2010). In addition, these plant species contain polyphenolic compounds, lignans, saponins, sterols, polysaccharides and phenolic acids, the total of phenolic and flavonoid content of extract ranging from 8.035 to 90.305 mg GAE/L (milligrams of gallic acid equivalent of plant extract) and 18.031 to 185.437 mg QE/L (milligram of quercetin equivalent of plant extract) respectively (Shuang Li et al., 2021; Petropoulos et al., 2018; Rolnik & Olas, 2021).

Artemisia sp. is the dominant genus in the Asteraceae family, and extensive studies have been conducted on seven *Artemisia* species for their potential as alternative sources for chemical fungicides. With the exception of *Artemisia viridissima* (Yanar et al., 2011) and *Artemisia capillaris* (Nguyen et al., 2009), these studies have shown that some of the most the tested *Artemisia* species, including *Artemisia vulgaris* (Stephan et al., 2005; Yanar et al., 2011),

Artemisia iwayomogi kitamura (Kim et al., 2004), *Artemisia annua* (Dorn et al., 2007) and *Artemisia ludoviciana* (Salgado-garciglia et al., 2010), have some effect in inhibiting

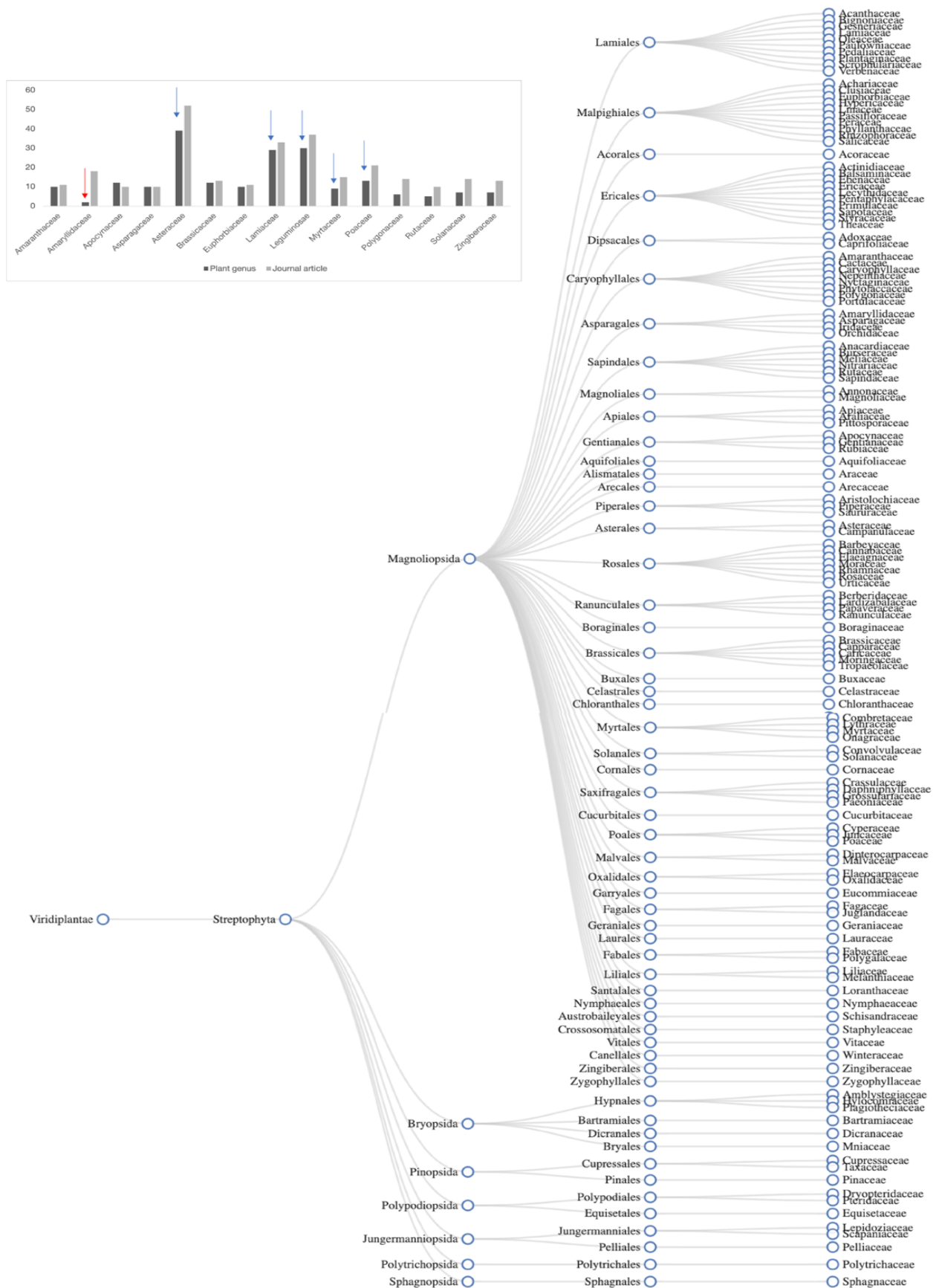


Figure 2.2. 2 Summary of most Plant extract families to control *Phytophthora* disease reported till 2021: a) Plants family phylogenetic trees iMetaLab Phylo (<https://shiny1.imetalab.ca/phyloexplorer/>). b) Histogram representing both number of Plant genera and the number of journal articles of each family

Phytophthora sp.. Interestingly, *Artemisia absinthium* was identified as the most promising species for use as anti-*Phytophthora* biological control, and the potential of silver nanoparticles (AgNPs) synthesized with aqueous extract of *Artemisia absinthium* was investigated for the first time *in vitro* and *in planta* on various developmental and reproductive stages of seven *Phytophthora* spp. (Ali et al., 2015a).

Moreover, except for one plant species *Lycoris aurea*, *Allium* sp. is the most widely tested genus in the Amaryllidaceae family, with 17 scientific publications until 2021. Bulbs or leaves of four species (*Allium sativum*, *Allium eriophyllum*, *Allium fistulosum* and *Allium cepa*) have been extracted with water or using methanol and ethanol organic solvents and have been extensively studied for their effectiveness against seven different *Phytophthora* sp.. The use of garlic plant extract as anti-*Phytophthora* agent is due to the presence of allicin and other organosulfur compounds that are found in high abundance among cultivars (Hayat et al., 2016). Several studies have reported significant success in using these extracts *in vitro* and *in planta* tests to protect plants against *Phytophthora* and other phytopathogenic fungi (Sealy et al., 2007). Garlic plant extract has been shown to effectively reduce zoospore germination and sporangia of *P. infestans* (Bekepe et al., 2006; Curtis et al., 2004) and inhibit mycelium growth (Ngadze, 2014) by damaging the hyphal cell wall or significantly inhibiting the production of cell wall degrading enzymes in various fungi (Hayat et al., 2016). In addition, at lower concentration, garlic extract can enhance cucumber seedlings growth (Hayat et al., 2016) and reduce *Phytophthora* infection in some *in planta* tests. For example, Demirci & Dolar, (2006) reported a reduction of *Phytophthora* blight severity on pepper by 39.8% with ethanolic garlic extract. In some cases, prophylactic treatment with garlic extract was more effective than curative treatment (Curtis et al., 2004; Su Li & Zhihui, 2008). However, in other cases, commercial formulations of garlic extract have not provided adequate *in planta* control of foliar blight on tomato plants. For example, in Envirepel, a 0,5% concentration of garlic extract did not provide adequate control (Dorn et al., 2007), and oomycetes were recovered from both sand and peat-based root substrates after one application of a 10% commercial garlic extract, although the extract was effective at killing the pathogen in *in vitro* tests (Sealy et al., 2007) (Appendix E: Table 1).

Therefore, the effectiveness of plant extracts in controlling *Phytophthora* under field conditions may be influenced by several factors, including the number of applications, the type of root substrate (Sealy et al., 2007), the extract concentration (Bowers & Locke, 2004a), and the stability of the extract under field environmental conditions (Dorn et al., 2007). Industrial formulation may be necessary to improve the efficiency of plant extracts for use in farmers' fields (Bowers & Locke, 2004a). Nanoparticles have also been shown to have potential for improving *Phytophthora* disease management (Ali et al., 2015a; Matei et al., 2018; Velmurugan et al., 2015; Velu et al., 2017).

For example, in a study by Matei et al., (2018), silver nanoparticles and polyphenol inclusion compounds prepared with chitosan oligomers or deep eutectic solvent (DES) completely inhibited the mycelial growth of *Phytophthora cinnamomi* at concentrations of 250 and 500 $\mu\text{g}\cdot\text{mL}^{-1}$, with the highest sensitivity ($\text{EC}_{50} = 0.1 \mu\text{g}\cdot\text{mL}^{-1}$) observed when using the DES-based treatments and gallic acid. Silver nanoparticles (AgNPs) synthesized using agricultural waste like the peanut shells showed a moderate activity, with a 5 to 6 mm zone of inhibition against *Phytophthora infestans* and *Phytophthora capsici* (Velmurugan et al., 2015). However,

the use of *Saccharum officinarum* leaf biomass extract resulted in a 10 mm zone of inhibition against *Phytophthora capsici* at a concentration of 250 µg/well according to Velu et al., (2017). These plant-based, synthesized silver nanoparticles that contain biological molecules are more environmentally friendly, less toxic and cheaper than other methods of synthesis (Ahmed et al., 2016; Velmurugan et al., 2015).

The incorporation of nanotechnology significantly improves the anti-*Phytophthora* properties of plant natural compounds. However, further extensive research is needed to understand the efficiency of nanoparticles in agricultural fields application (Sahoo et al., 2022; Velmurugan et al., 2015).

This represents another constraints for plant extract commercialization in addition to the registration process that need a huge cost and a prolonged procedures for evaluation specially in Europe where the regulatory policies of botanical-based pesticides have the same regulatory framework as chemical pesticides (Arora et al., 2022).

According to EU plant extract database, there are very few commercialized products based on plant extracts and other natural compounds. Of the list of plant extract products registered in the EU, only 23 % have been approved. Azadirachtin and garlic extract have been approved for use in more than 10 European countries (Figure 2.2. 3).

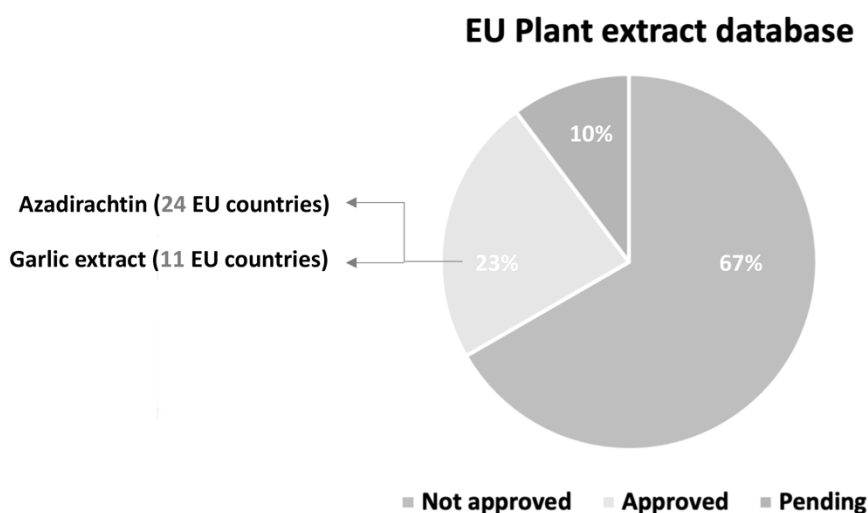


Figure 2.2. 3 Pie chart representing the percentage of plant extract for approval according to EU legislation. Azadirachtin and Garlic extract represent the most registered product in EU countries (https://ec.europa.eu/food/plants/pesticides_en).

Currently, botanical extracts are produced in large quantities by a variety of industries despite the gap between recognising plant extract chemical compounds and developing them into functional products (Arora et al., 2022). This is due to their potential as natural sources of fungicides, as well as their sustainable, easily biodegradable properties (Pham et al., 2021) and low-cost, environmentally friendly alternatives (Persaud et al., 2021). In addition, many researchers have studied the use of plants extracts as broad-spectrum botanical fungicides (Liao et al., 2021; Nguyen et al., 2009).

For example, extract of *Phyllostachys pubescens* showed a 100.00% inhibitory rate against *Phytophthora capsici* with anti-fungal activity against other plant pathogens such as *Fusarium graminearum*, *Valsa mali* Miyabe et Yamada, *Botryosphaeria dothidea*, *Venturia nashicola*,

and *Botrytis cinerea* Pers (Liao et al., 2021). This could help in the commercialization of the product, as a broad spectrum of action is more desirable for application (Matrose et al., 2021). Similarly, several researchers have explored the potential use of plant extracts to induce plant defense reactions (Benouaret & Goupil, 2015) or enhance plant growth (Hayat et al., 2016), in addition to their compatibility with other fungicides (Liao et al., 2021). However, little research has been done to determine the potential for phytotoxicity under a specific conditions. (Sealy et al., 2007).

2.2.3 Conclusion

Over the last two decades, many studies have been conducted to develop new biocontrol products from plant extracts to manage *Phytophthora* disease. One common method is through the use of plant essential oils or the application of plant-derived compounds. However, the efficacy and safety of using plant extracts as a control method against *Phytophthora* pathogens is still under investigation, and more research is needed.

Additionally, there are fewer reports available on the effects on beneficial soil organisms, and very little attention has been given to phytotoxicology evaluation on plants, which could become a limiting factor during registration. More research is needed to understand the mechanisms of action of the active compounds in plant extracts, and to use advanced proteomics and metabolomics approaches to understand the interaction between plant extracts and *Phytophthora* biology. Research should also consider the root substrate and soil type to which the treatment is applied.

It is important to highlight that the performance and efficacy of plant extracts vary, likely due to differences in extraction protocols, collected plant cultivars, harvesting seasons, and the effects of environmental conditions on plant secondary metabolism.

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2.3 THE HUNT FOR SUSTAINABLE BIOCONTROL OF OOMYCETE PLANT PATHOGENS, A CASE STUDY OF *PHYTOPHTHORA INFESTANS*

Summary

A review article have been published in the Fungal Biology Reviews special edition on oomycetes entitled “The hunt for sustainable Biocontrol of oomycete plant pathogens: A case study of *Phytophthora infestans*”.

This paper reviews the most recent publications on the mechanisms and applications of biological control for late blight diseases of tomato and potato.

Late blight diseases pose a significant fungicide burden worldwide and threaten food security, so there is a pressing need for more environmentally sustainable control measures, such as biological control. However, these measures are not yet widely used.

Additionally, the authors discuss current knowledge from mechanistic and applied biological control studies conducted *in-vitro*, *in-planta* and *in-agro*, and evaluate the prospects for the use of such studies in an integrated disease management perspective for the future.

To the authors' knowledge, a comprehensive review of the state-of-the-art in terms of prospects for biological control of *P. infestans*, one of the most devastating oomycete phytopathogens has not been carried out before.



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Review

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

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ABSTRACT

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

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

1. Introduction

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

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

Biological control of plant diseases can be defined as the application of beneficial micro, (or macro) living organisms to control aerial or soilborne plant pathogens. We consider biological control in the strictest sense of the definition, i.e., always involving a living organism that targets the pest directly or indirectly (Heimpel and Mills, 2017; Stenberg et al., 2021). The term biocontrol agent (BCA) refers to the organism (typically a bacterium, fungus, oomycete, nematode, insect, virus or occasionally plant) that is used to control the disease-causing agent (Stenberg et al., 2021). Mechanisms of biocontrol are broadly divided into four categories (Köhl et al., 2019). 1) Competition for resources, including nutrients, space, and/or water and often for rare nutrients such as iron. Biocontrol agents that use this mode of action are termed competitive saprophytes. Here, disease suppression by mixed communities of microbes or other organisms in the soil in combination with physiochemical soil properties that discourage the growth of disease-causing microbes may also occur (e.g., through the action of suppressive soils) 2) Mycoparasitism, whereby the biocontrol agent is termed a facultative hyperparasite and where direct infection of the plant pathogen (prey species)

occurs. 3) Antibiosis, also a feature of facultative hyperparasites, where antimicrobial compounds or toxins and lytic enzymes are produced to destroy the prey (plant pathogen). 4) Induced host resistance, whereby plant hormone mimics or precursors are produced and/or innate immunity is triggered by a facultative plant symbiont to confer resistance to incoming pathogens. Biocontrol ability, and mode of action, is generally strain-specific depending on the host, plant, pathogen, and environmental factors and most BCAs employ more than one mode of action to control pathogens (Köhl et al., 2019). The methods through which biocontrol is used can be classified into four main categories depending on whether native BCA species are utilized, with or without targeted human intervention (conservation biological control and natural biological control, respectively) or if BCAs are added into the agroecosystem for permanent or temporary establishment (classical biological control and augmentative biological control, respectively) (Stenberg et al., 2021).

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

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

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

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

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

In the hunt for new or improved biocontrol agents, most studies firstly employ *in vitro* assays of growth inhibition or death of the prey species, typically in confrontation assays (e.g., Fig. 1 lower panel). This approach allows mechanistic understanding of direct interactions between BCAs and their prey to be elucidated under laboratory settings. In the case of *P. infestans*, this allows researchers to test potential BCAs for direct control of the vulnerable pre-infection stages of

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

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

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

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

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

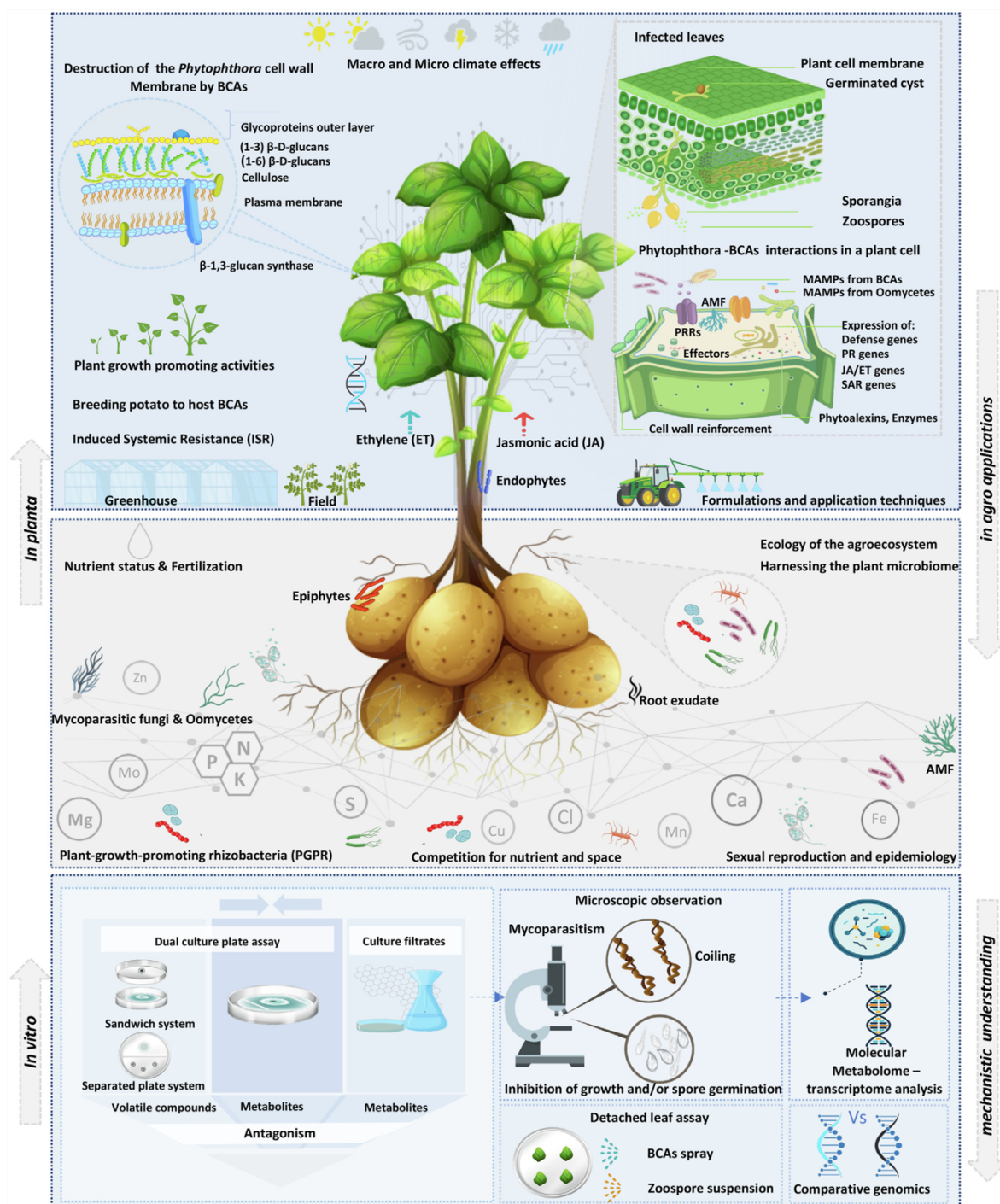


Fig. 1 – Towards both a mechanistic understanding and practical use of biocontrol agents against potato late blight disease. *In vitro* assays may lead to the identification of metabolites, volatiles or direct mycoparasitic effects of BCAs, which, combined with molecular tools such as metabolomics, transcriptomics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

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

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

Numerous other bio-active metabolites are produced by bacterial BCAs, and those from species of *Bacillus* and *Pseudomonas* have been particularly well studied. For example, cyclic lipopeptides produced by several *Pseudomonas fluorescens* strains have specific activity against the vulnerable wall-less zoospores of *P. infestans* (De Vrieze *et al.*, 2020; Zachow *et al.*, 2015) and siderophore production has also been associated with anti-oomycete activity, in the *Pseudomonas* genus (De Vrieze *et al.*, 2020). Iron acquisition genes including those linked to the production of Pyoverdines have been linked to both the ability of *Pseudomonas* strains to survive in the soil and colonise plant roots, as well as to direct antagonism of *P. infestans* (De Vrieze *et al.*, 2020) suggesting iron competition could be one of the mechanisms used by some *Pseudomonas* species to inhibit *P. infestans* development. Although members of both the *Pseudomonas* and *Bacillus* genera are known to be prolific producers of numerous bioactive compounds including those with anti-oomycete activities, the *in vitro* activities of such compounds are not easily transferable to *in planta* or *in agro* assays (Caulier *et al.*, 2018), thus new methods for selection of BCAs need to be developed. One possibility for the screening of potential

bacterial BCAs could be to screen for the production of biosurfactants and siderophores, since in their large-scale analysis of over 2800 bacterial isolates (Caulier *et al.*, 2018), identified that the strains that were effective *in planta* at controlling late blight were prolific producers of both, and indeed the production of such compounds might be the significant factor contributing to the success of these strains as BCAs. As microbial competition for nutrients and ecological niches, on, or within plants, contributes to the antagonistic activity of competent bacterial strains (Vorholt, 2012), isolates naturally associated with potato plants, such as some of these *Bacillus* and *Pseudomonas* species, have the highest chance to be artificially reintroduced to a crop for control purposes, through classical biocontrol strategies.

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

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

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

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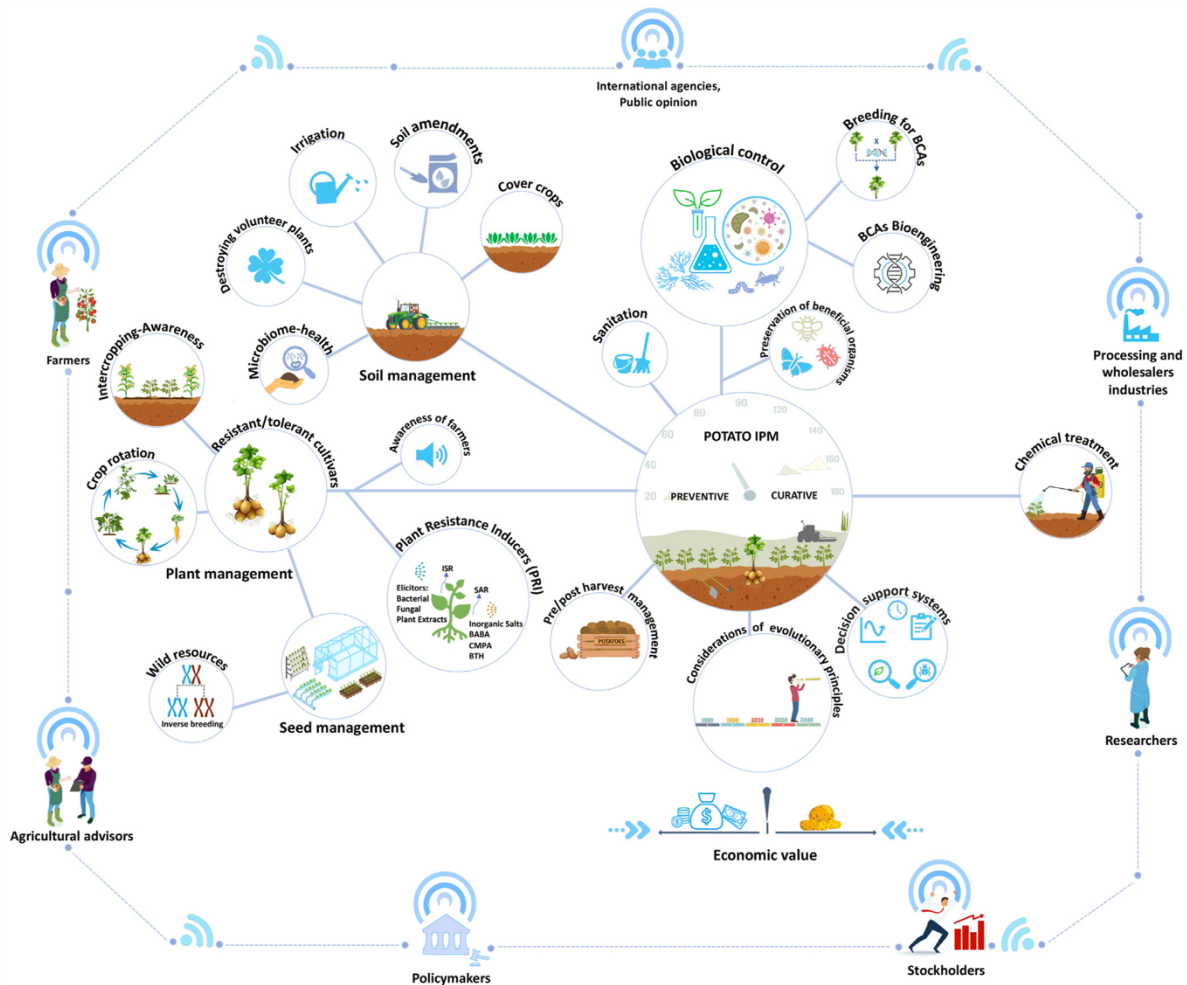


Fig. 2 – Integrated Pest Management (IPM) in potato. A schematic of the potential components of an IPM system for the protection of potato from pests such as late blight disease. For successful implementation of IPM in potato, greater emphasis should be placed on preventing pest problems (traveling left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

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

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

A substantial number of studies have tested BCAs against late blight *in planta*, either in controlled environment detached leaf assays, or in whole plant greenhouse bioassays (Table S1),

although the efficacy of control varies. Since commercial tomato cultivation in Northern Europe is almost exclusively carried out in greenhouses, it is relatively easy to mimic this production system to accurately analyse BCA efficacy in a research setting. Valuable knowledge that might also trans-late into potato cultivation systems can also be gained in this manner. In addition to direct disease control measures, *in planta* assays have the advantage of evaluating bio-stimulation, for example Kariuki et al. (2020) observed that both *T. harzianum* and *T. asperellum* stimulated growth of

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

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

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

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

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

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

Many *Trichoderma* species have been tested for potato late blight disease control in *agro* (Al-Mughrabi, 2008; de Souza *et al.*, 2015; El-Naggar *et al.*, 2016; Wharton *et al.*, 2012; Yao *et al.*, 2016). For example, Al-Mughrabi (2008) showed a significant effect on late blight incidence when tubers were pre-treated with *Trichoderma* in field trials. The commercial product Planter-Box® containing *Trichoderma harzianum*, is effective against late blight seed pierce incidence on potato

tubers, and microscopic investigations confirm *T. harzianum* coiling around *P. infestans* hyphae and thus exhibiting classical mycoparasitic behaviour in *agro* (Wharton *et al.*, 2012). Yao *et al.* (2016) showed that *Trichoderma* can significantly reduce late blight disease severity in potato field trials and, most recently, Lal *et al.* (2021) tested Neem, a bioextract, in combination with *Trichoderma viride* and found a significant reduction of potato late blight in *agro*. However these studies did not determine if secondary metabolites also played a role in the efficiency of these treatments.

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

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

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

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

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

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

3.1. Licence to kill: registration of BCAs

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

The EU Commission Regulation No 546/2011 of 10 June 2011, implementing directive (EC) No 1107/2009, specifically regulates the use of plant protection products, including biological control agents, in agriculture. Requirements for registration to ensure safe usage are important and needed. However, these regulations also create an unwanted effect, namely that it is a long and potentially expensive process to get a product approved in the EU (Hauschild, 2017). The process is more extensive in the EU than in other parts of the world, (taking on average 65,7 months in the EU compared to 15,7 months in the USA) (Balog et al., 2017; Frederiks and Wesseler, 2019; Kiewnick, 2007). In general the US approval

process is much faster since microbial BCAs are registered under the regulatory framework of Biopesticides, and the US is accustomed to such. To formally have a microbial BCA approved in the EU, it needs to pass two steps within the legislation. The first step is the evaluation of the active substances. This step consists of three phases, the rapporteur member state phase, the risk assessment phase and the risk management phase (Frederiks and Wesseler, 2019). Generally, during the first step applicants provide a dossier of documents regarding the active substance to a member state. After evaluation of the dossier, the European Food Safety Authority starts assessing the risks in all aspects of food safety and the risk management is then carried out by the European Commission. The second step is to approve and register the plant protection product at a member state level. BCAs used in field crops are usually registered and approved to be used within specific zones of Europe, however country-specific or cross-zonal approval occurs too. EU Approved active substances and BCAs can be found here: https://ec.europa.eu/food/plant/pesticides/eu-pesticides-db_en.

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

3.2. The armoury: formulation of microbial BCAs

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

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

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

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

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

Trichoderma species have been harnessed for novel plant biotechnology approaches. One notable example is the use of *T. atroviride* as a “cell factory” to produce selenium nanoparticles (SeNPs) as an eco-friendly plant protection product. These fungal-derived SeNPs have an inhibitory effect on *P.*

infestans growth and spore production *in vitro* (Joshi et al., 2019) and *in planta* when used as a seed coating in tomato, where they prime the plant defence responses and stimulate induced systemic resistance (ISR) (Joshi et al., 2021). The SeNP treated tomato plants showed a significant deposition of callose and lignin as well as elevated H₂O₂ consistent with an ISR response, and an upregulation of general defence enzymes such as lipoxygenases, and cell wall degrading enzymes, with 72% of the primed plants showing complete disease protection against late blight (Joshi et al., 2021). Such bioengineering approaches are highly promising for the future of plant protection.

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

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

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

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

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

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

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

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

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

Given the adaptability of *P. infestans* towards fungicides with only one mode of action, it is worth considering combining more than one BCA to combat late blight, or at least using a BCA with several different modes of action. Synergistic effects of combining two or more BCAs against late blight in

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

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

3.7. The magic bullet? IPM solutions in practice

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

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

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

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

3.8. Conclusions

Large-scale *in vitro* screening approaches have had some notable successes in the identification of BCAs that reduce late blight disease severity in *agro* (Caulier et al., 2018), however these successes are often down to the fact that large numbers of isolates have been screened to find a single successful BCA, or that the microbes tested were identified as potato or tomato endophytes initially. Whilst *in vitro* approaches are very useful for the identification of the modes of action of BCAs, new

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

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Declaration of competing interest

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

Appendix A. Supplementary data

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

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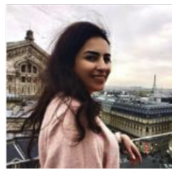
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Dania Tabet is originally from Lebanon where she completed her bachelor's degree in Agricultural engineering specialization in plant protection at The Lebanese University, faculty of agriculture. She completed her master's degree in Integrated Pest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



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



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



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



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



Laura Grenville-Briggs was educated at the Universities of Bath and Birmingham (PhD, 2003). After a post-doctoral period at the University of Aberdeen, UK, and a Marie-Curie Intra-European Fellowship at the Royal Institute of Technology, Stockholm, Laura joined the Swedish University of Agricultural Sciences (SLU) as an independent researcher in 2014 and was appointed Professor of Integrated Plant Protection in 2018. Her research group focuses on the

molecular and genetic determinants of disease in oomycete and fungal phytopathogens and their interactions with microbial biocontrol agents. The goal is to develop more durable, environmentally sustainable integrated pest management strategies for plant diseases.

CHAPTER 3: AQUEOUS PLANT EXTRACTS FOR ANTI-*PHYTOPHTHORA* ACTIVITIES

Abstract

The potential of some by-product aqueous plant extracts for controlling *Phytophthora* disease has been evaluated in *in vitro* tests on the vegetative and reproductive growth of different *Phytophthora* species. Based on the results of screening experiments with zoospore germination, five out of 17 extracts significantly inhibited the growth of most of the tested *Phytophthora* sp. in a concentration-dependent manner, with a significant effect observed at concentrations higher than 12,5 mg.ml⁻¹. The most active plant extract treatments were those of *Viburnum tinus* and *Chlorophytum* sp., leaves, *Citrullus lanatus* rind, and *Rose* sp. fruits, which have been selected for further investigation. *Viburnum tinus* and Rose fruits showed the highest of zoospores at different concentrations and were tested for their effect on the mycelium growth of *P. cryptogea*. However, none of the treatments were effective. In addition, the phytotoxicity of the extracts was assessed using watercress (*Lepidium sativum*) as a test plant. With the exception of *Tulbaghia* extract, most of the tested extracts showed an inhibition of seed germination and a reduction in seedlings growth.

Towards the goal of finding a new plant extract to control *Phytophthora* sp., we also studied the effect of 12 different varieties of garlic extract on the percentage of germinated zoospores of two isolates of *P. nicotianae*. The results of the experiments indicated a similar direct effect on both *Phytophthora* isolates, with a diversity in inhibition among cultivars.

3.1 Introduction

Plant extracts have enormous potential for use as fungicides in agriculture (Yoon et al., 2013). The use of plant-derived compounds is considered an eco-friendly alternative management and sustainable control strategy against *Phytophthora* disease. For instance, many natural products have shown good effects in controlling *Phytophthora* sp., one of the most serious and economically impactful diseases that continue to cause devastating impacts on natural ecosystems and crop losses worldwide (S. Zhang et al., 2022).

Recently, researchers have focused largely on the development of green technologies to reduce the environmental impact, of extracting natural products (Chemat et al., 2012; Donno et al., 2020).

Green extraction technologies can be defined as methods that reduce energy consumption, use alternative solvents and renewable natural products, and ensure a safe and high-quality extract/product (Chemat et al., 2012).

Additionally, many studies have evaluated the importance of eco-sustainable extractions, known as green technologies, in various field such as food processing (Soquetta et al., 2018), medicine (Kumar et al., 2021), pharmacological activities, cosmetic, and chemical industry (Chemat et al., 2012).

However, despite the increasing interest in using plant extracts as antifungals for plant protection, green technology is still limited, with the exception of a few studies in which novel applications approaches are based on nanoparticles synthesized through green methods

(Ahmed et al., 2016), in general, most studies continue to use conventional extraction techniques.

Therefore, there is a need for improved techniques of plant extraction to be used in the agriculture sector, targeting specific biocompounds. These methods should be ecologically sustainable, free of hazardous chemicals, and take into consideration strategies for reusing processing waste to create new biofungicidal compounds.

This study proposed preliminary screening experiments to assess anti-*Phytophthora* activity from different parts of various ornamental plant species, using plant derivative production waste, known as “By-products”, which are the remains after processing fruits and vegetables in the food processing industry (Lapornik et al., 2005).

The plants were selected based on results of a previous literature survey on plant extracts used to control *Phytophthora* disease (Chapter 1), in which a dataset of plant extracts was established, containing information *Phytophthora* sp., plants species, plant parts, solvents used, and extract composition. This allowed for the first time to screen several plant products with a potential antimicrobial activity against *Phytophthora* sp.

Therefore, the objective of the present study is to propose an efficient alternative approach for controlling different *Phytophthora* sp. by assessing in an *in vitro* experiment, on vegetative and reproductive growth, the inhibitory efficacy and potential use as fungicides aqueous extract of ornamental foliage and fruit remains extracted by one of the most environmentally sustainable solvents (Castro-Puyana et al., 2017).

3.2 Materials and Methods

3.2.1 Plant Material and Preparation of Extracts

Leaves, fruits, or seeds of various plants species were collected in 2020 from Viterbo, located in the central part of Italy (Viterbo, 42.4207° N, 12.1077° E). The collected material was taxonomically identified in (Table 3. 1) and were those available over each season.

The first set of experiments consisted of infusion plant extracts and testing their effect on *Phytophthora* mycelium using a solid agar bioassay.

However, the second set of experiments consisted of testing the plant extracts on *Phytophthora* zoospores, following this extraction procedure:

Plant extracts were prepared according to (Khan et al., 2018) with some modifications.

Leaves and fruits were thoroughly washed with distilled water, and immediately air-dried at 60 °C.

The dried plants were ground to a fine powder in a high-speed blender (Johnson, Annabella, Italy). For the preparation of (10%) organic crude extracts, 10 g of the dried plant powder was boiled for 20 min in 100 ml distilled water, followed by separation of the residue through a Büchner funnel and centrifuged at 5 000 rpm for 10 minutes (BECKMAN -J2-21M/E Centrifuge). Finally, aqueous extracts were lyophilized using a (VirTis, USA) Lyophilizer and the dry extracts were stored at -20°C until use.

Table 3. 1 Plant species selected for extraction

Species	Common name	Family	Plant part	Experiment
<i>Cucurbita maxima</i>	Squash	Cucurbitaceae	Seed	2nd screening
<i>Citrullus lanatus</i>	Watermelon	Cucurbitaceae	Fruit rind	2nd screening
<i>Musa acuminata</i>	Banana	Musaceae	Peel	2nd screening
<i>Pittosporum tobira</i>	Japanese cheesewood.	Pittosporaceae	Leaves	2nd screening
<i>Cucumis melo</i> (Inodorus Group) 'Canary'	Melon	Cucurbitaceae	Fruit rind +Seed	2nd screening

<i>Chlorophytum comosum</i>	spider plant	Asparagaceae	Leaves	1st -2nd screening
<i>Rosa sp.</i>	Rose	Rosaceae	Rosehip	1st -2nd screening
<i>Viburnum tinus</i>	Laurustine	Adoxaceae,	Leaves	1st -2nd screening
<i>Tulbaghia violacea</i>	wild garlic	Amaryllidaceae	Leaves	1st screening
<i>Tropaeolum majus</i>	Nasturtium	Tropaeolaceae	Leaves	1st screening
<i>Tilia sp.</i>	Linden	Malvaceae	Fruits	1st screening
<i>Lemna sp.</i>	Duckweed	Aracea	Whole plants	1st screening
<i>Photinia sp.</i>	Chinese hawthorn	Rosaceae	Leaves	1st screening
<i>Eruca vesicaria</i>	Rocket	Brassicaceae	Seedlings	1st screening
<i>Cynara cardunculus</i>	Globe artichoke	Asteraceae	Fruits	1st screening
<i>Cucurbita maxima</i>	Squash	Cucurbitaceae	Leaves	1st screening
<i>Arachis hypogaea</i>	Peanut or ground nut	Fabaceae	Seed shell	1st screening
<i>Helianthus sp.</i>	sunflower	Asteraceae	Seed shell	1st screening

3.2.2 *Phytophthora* isolates and culture conditions

Six different species of *Phytophthora*, with two isolates for some of them, were obtained from the Department of Plant Pathology, DIBAF department, University of Tuscia.

P. cryptogea (P189, P187), *P. multivora* (Ph15), *P. plurivora* (Ph30), *P. meadii* (P4), *P. cactorum* (P69) and *P. citrophthora* (P66, P6) were routinely cultured at 25 °C on carrot agar medium (CA) in darkness and all strains were maintained on potato dextrose agar (Oxoid, Basingstoke, UK) and water at 8°C room temperature.

3.2.3 *in vitro* assessment of the effect of plant extract on the activity of different *Phytophthora* sp.

Antimicrobial activities of plant extracts by zoospore Germination assay

For zoospore production, carrot agar plates for all tested *Phytophthora* sp. were cut into small square pieces and then flooded with sterile soil extract. Dishes were incubated under light for 2 days to several weeks at 25 °C, depending on *Phytophthora* sp. until sporangia formation. Zoospores release from sporangia was induced after treatment with ice-cold sterile water for 30 minutes at 4 °C, followed by one hour at room temperature until a sufficient number of zoospores had been released. Zoospores were harvested by pipetting from the water surface and counted using Thoma cell counting chamber (BLAU BRAND, Germany). Zoospore densities were approximately 10⁴ zoospores/ml.

Initial screening was done in 96-well plates, with each well containing 75 µL of carrot broth (CB) and then received 25 µL of zoospore suspension at a density of 10⁴ zoospores per mL. A two-fold serial dilutions of test plant extracts (100 mg/ml) dissolved in distilled water was carried out resulting in a final concentration of (50 mg/ml, 25 mg/ml, 12.5 mg/ml, 6.25 mg/ml and 3.125 mg/ml) test compound per well. Control wells were as above but were amended with distilled water instead of the test compound. Plates were incubated for 3 hours to one day, depending on *Phytophthora* sp., at 25°C in darkness. Approximately 50-100 zoospores per treatment were counted and the examination of germ tube length and morphology in the extracts tested in comparison to commercially available “Volare®” (Fluopicolide & Propamocarb-Bayer) fungicides and negative control were observed under a light microscope.

Antimicrobial activities of plant extracts by solid agar bioassay

Two isolates of *P. cryptogea* (P189, P187) were grown on CA plates at 25°C for 5 days. Individual agar disks (6 mm in diameter) were removed from the edge of an actively growing culture and were placed on the top of sterilized microscopic slide amended with PDA medium, supplemented at different concentrations (25, 12 and 6 mg.ml⁻¹) with one of the two tested

extracts, *Rose* and *Viburnum* (2 ml). The extracts were added to PDA after autoclaving when the agar had cooled to approximately 50°C. PDA without extracts was used as negative control, and the experiment was repeated once. Eight slides for each treatment and isolate, representing eight replicates, were placed inside sterilized glass petri dish, and the culture slides were then incubated at $25 \pm 1^\circ\text{C}$ for seven days. After the incubation period, the mycelial growth was determined as the mean colony radius area.

3.2.4 Assessment of the phytotoxicity of aqueous extract

The aim of the acute phytotoxicity test was to obtain data on the effects of plant extract on seed germination and development of seedlings.

The phytotoxic effect was performed *in planta* on *Lepidium sativum* according to (Himanen et al., 2012) with some modification.

Three concentrations (12.5, 6.25 and 3.12 mg.ml^{-1}) were used with distilled water as the control. Petri dishes ($\varnothing$ 90 mm) were lined with filter paper and moisturized with 1 ml of aqueous extract or water as the control. Each plate was seeded with 20 seeds of garden cress *Lepidium sativum*, covered with the lid, the Petri dishes were kept vertically inside humid chamber in an incubator at $25 \pm 1^\circ\text{C}$ for 5-6 days. After incubation, the number of germinated seeds was counted, and the average seedlings length was determined for each dish. An experiment was considered successful when the germination of cress in controls was over 95%.

3.2.5 Statistical analysis

Data were statistically analyzed using R program with a factorial experiment as a completely randomized design (CRD). All data were expressed as the mean $\pm$ SE and were analyzed using analysis of variance (one-way ANOVA) followed by a Tukey test LSD (least significant difference) for the zoospore and mycelium inhibition experiment, and Dunnett test for the phytotoxicity assay to test the significance of differences between means obtained among the treatments at the 5% level of significance.

3.3 Results and Discussion

In the present study, the anti-*Phytophthora* activity of crude plants extracts was evaluated using a green extraction method and a re-use strategy of plant by-products.

The plant extracts were initially screened for *Phytophthora* inhibition by a first trial test conducted using an aqueous infusion of fresh plants extracts on an agar disk diffusion assay (Sumarah et al., 2008) (Table 3. 1; Experiment 1). This initial screen revealed three plant extracts: *Chlorophytum comosum*, *Rosa* sp., and *Viburnum tinus* showed a significant difference between treatment and control (data unpublished) and were selected for further studies on zoospores germination length and mycelium inhibition.

The second screening was carried out using a microtiter plate-based assay to test the inhibition effect of aqueous plants extract on zoospore germination of six *Phytophthora* sp. (*P. cryptogea* (P189, P187); *P. citrophthora* (P66, P6); *P. multivora* (Ph15); *P. plurivora* (Ph30, P135); *P. meadii* (Ph4) and *P. cactorum* (P69)).

From these screens, of the eight tested natural products (Table 3. 1; Experiment 2), four aqueous extracts: *Chlorophytum comosum*, *Citrullus lanatus*, *Rosa* sp., and *Viburnum tinus*

were effective in suppressing zoospore germination of most tested *Phytophthora* species by at least one concentration ranging from 50 to 3.12 mg.ml⁻¹.

As shown in (Figures 3.1; 3.2; 3.4 and 3.6), the length of germinated zoospores, evaluated in the presence of four aqueous plant extracts, gradually increased with the decrease in extracts concentration, and the anti-*Phytophthora* activities exhibited inhibition rates greater than 50% with 25 mg.ml⁻¹ concentration for most tested extract.

The Plant extracts varied in their anti-*Phytophthora* activity among different *Phytophthora* species and responded differently even between tested isolates.

For example, *P. cactorum* and *P. citrophthora* was highly affected by *Rose* and *Viburnum* extract even at lower concentration, however, no inhibitory effect was recorded when tested with watermelon and *Chlorophytum*.

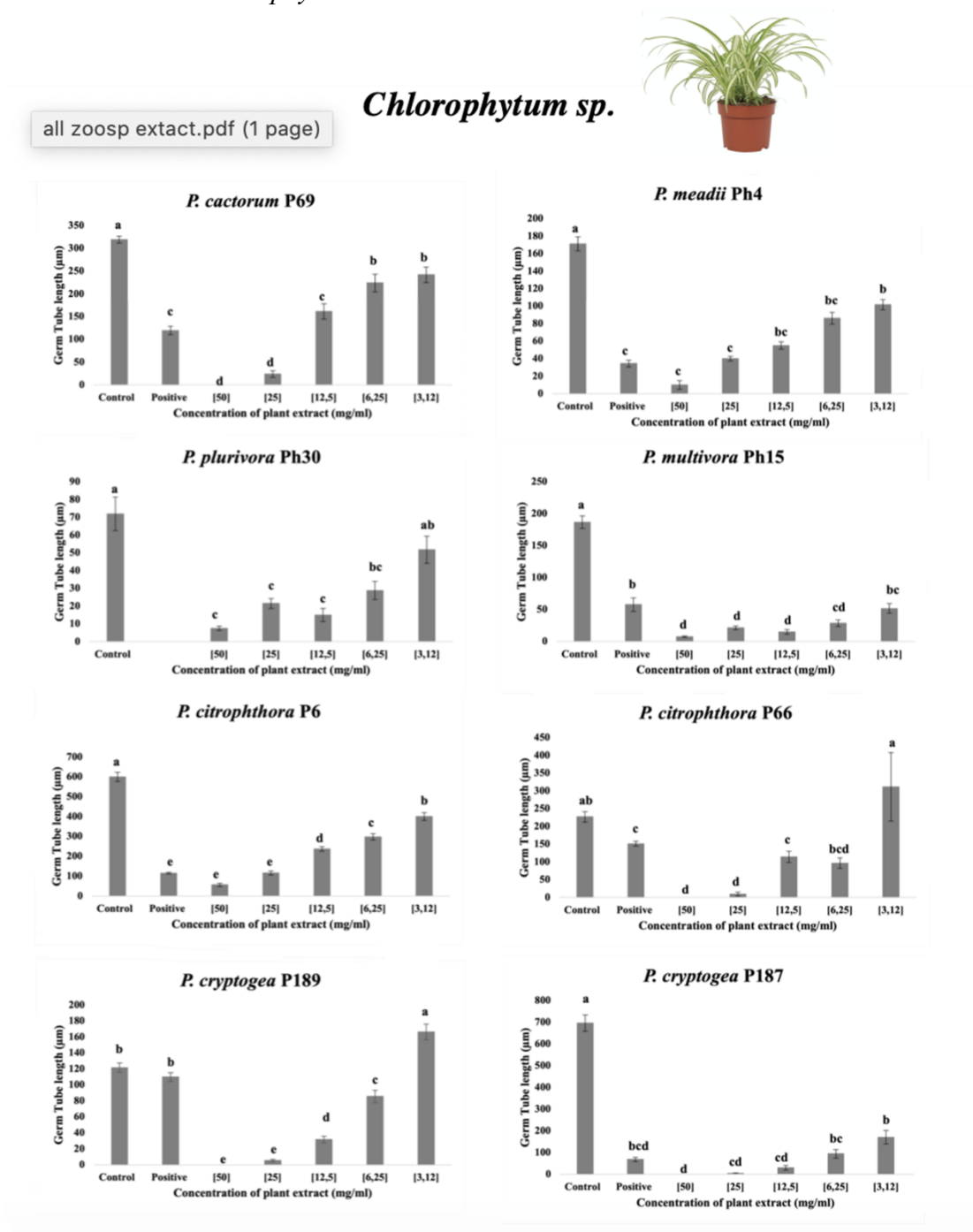


Figure 3. 1 Effect of *Chlorophytum* sp. plant extract on germ tube elongation of six different *Phytophthora* sp. at different concentration of plant extract, Positive = Volare[®] fungicide; Control = Water.

Data presents mean $\pm$ standard error. Bars labeled with the same letter are not significantly different from each other according to Tukey's HSD at $p < 0.05$.

The effectiveness of *Chlorophytum* botanical extract on oomycete pathogens has not been reported, even though the *Chlorophytum* genus has been widely studied in medicine research (Rzhepakovsky et al., 2022; Sharma et al., 2021) due to its antimicrobial activities against human fungal pathogens such as *Aspergillus niger* and *Candida albicans*, as well as other infectious bacterial clinical diseases (Chakraborty, 2009; GhorpadeD. & Amaravati, 2014; Sharma et al., 2021).

Moreover, few studies have been conducted on phytopathogenic fungal diseases. A previous study on *Chlorophytum comosum* water extract demonstrated antifungal effects against *Colletotrichum dematium* with minimum inhibitory concentrations (MICs) of 12.5 mg.ml⁻¹ (Masangwa et al., 2013). However, during this study, for most *Phytophthora* species used in the *in vitro* test, 50 mg.ml⁻¹ of *Chlorophytum* aqueous extract was the minimum inhibitory concentration for zoospores growth inhibition (Figure 3.1).

Aqueous leaf extracts of different *Chlorophytum* species have been found to contain various secondary metabolites. The phytochemical screening of leaves reveals the presence of starch, proteins, sugars, tannins, flavonoids, alkaloids, saponins and mucilage (GhorpadeD. & Amaravati, 2014), which may be responsible for the observed antifungal property against *Phytophthora* species *in vitro*.

Additionally, according to Sharma et al., (2021) botanical methanolic crude extract of *Chlorophytum borivillianum* contain saponin metabolites, which have interesting biological activities against different pathogenic microorganisms, with a MIC in the range of 0.5 mg.ml⁻¹ to 0.0625 mg.ml⁻¹.

This study aimed to investigate the anti-*Phytophthora* activities of various agricultural by-products, with a focus on watermelon rinds which are commonly discarded and can contribute to environmental issues and biomass loss (Du & Ramirez, 2022).

Results showed that aqueous watermelon rind displayed moderate inhibitory activity against *Phytophthora* sp. The inhibitory properties were most effective at a concentration of 25 mg.ml⁻¹, with the exception of *P. plurivora*. However, at lower concentration of 6, 12 mg.ml⁻¹ and below, it did not show inhibition for some *Phytophthora* species, such as *P. multivora*, *P. citrophthora* and *P. cryptogea* (P187).

A previous study reported similar observation regarding the non-effect of aqueous seeds water extract of *Citrullus colocynthis* against *P. capsici*. However, methanolic extract results revealed a slightly inhibition rate (Thavamani et al., 2012). Harrison et al., (2012) also reported that zoospore germination of *P. capsici* was affected by methanol (70%) seedcoat extract of *Citrullus lanatus*, and the amount of inhibition varied among *Citrullus* genotypes. Additionally, he showed that seed exudates can highly inhibit the bacterial pathogen *Acidovorax avenae* subsp. *citrulli*.

In a similar vein, the effect of aqueous watermelon rind extract with a concentration of 2-6 g L⁻¹ has also been documented as causing growth inhibition and bacterial cellular damage on the growth of *Aphanizomenon flos-aquae*, one of cyanobacterial that can lead to the deterioration in aquatic environments (Zhang et al., 2020).

Agricultural waste products have been identified as potential sources for managing soil-borne diseases in several studies. Lujan et al., (2021) used pecan byproducts rich in phenolic compounds to control *P. capsici* on chile pepper. In another study, watermelon peel waste was utilized with zinc oxide nanoparticles (ZnO-NPs) as hydrogel to protect the plant against *Fusarium* wilt (Abdelaziz et al., 2021).

This study is the first to report the use of watermelon rind extract against *Phytophthora* sp., Although the aqueous extract did not show a high significant effect against all *Phytophthora* sp., the inexpensive nanoparticle method reported by Abdelaziz et al., (2021) could have broad potential for use against *Phytophthora* and other soil borne pathogens.

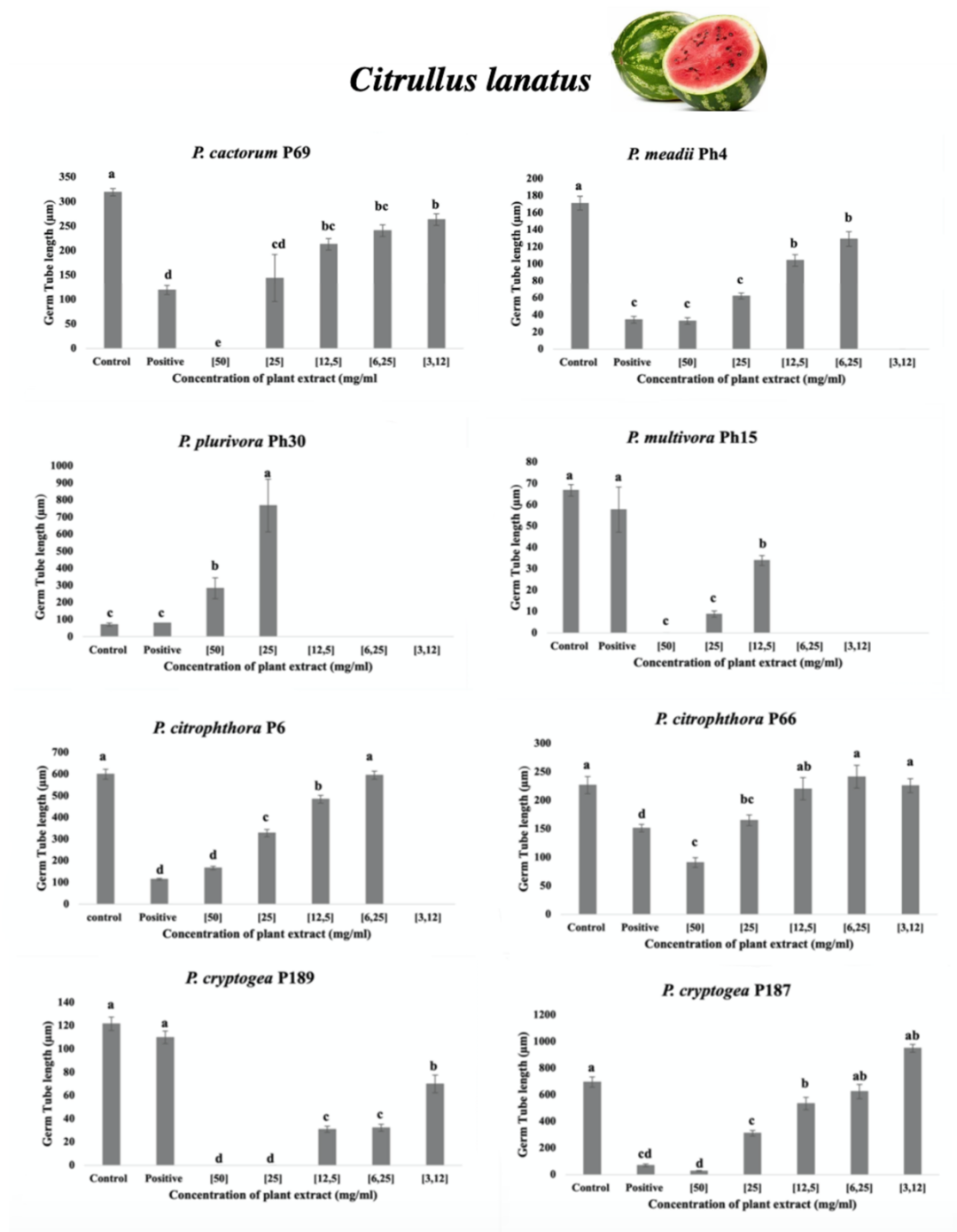
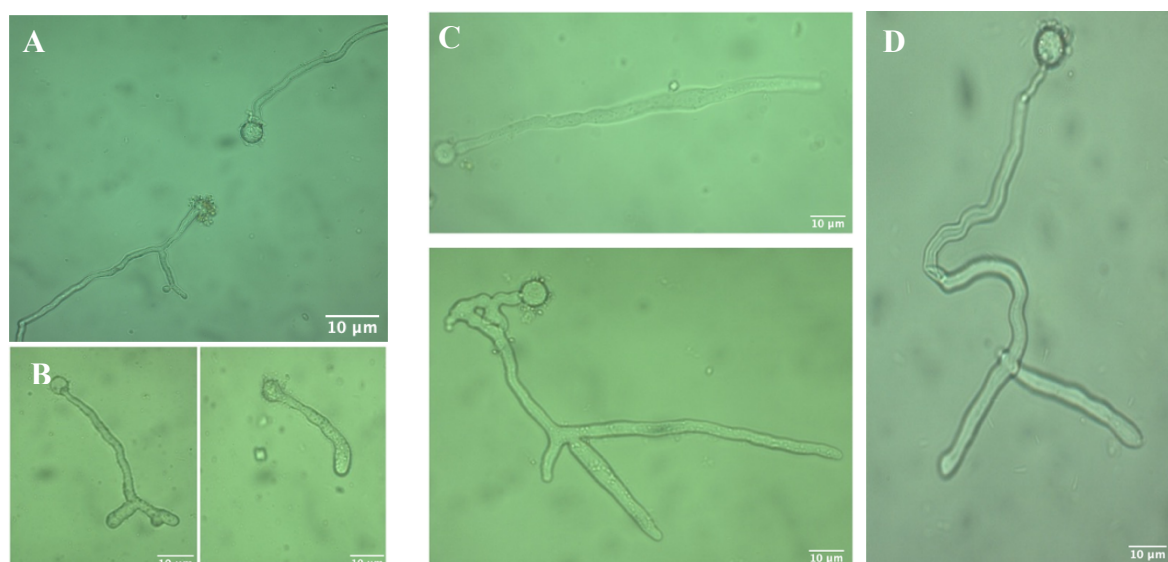


Figure 3. 2 Effect of watermelon rind extract on germ tube elongation of six different *Phytophthora* sp. at different concentration of plant extract, Positive = Volare® fungicide; Control = Water

Data presents mean $\pm$ standard error. Bars labeled with the same letter are not significantly different from each other according to Tukey's HSD at $p < 0,05$.

In addition, a microscopic investigation was conducted to determine whether the extract could affect mycelial morphology.

It is interesting to note that, even though watermelon extract did not show a high inhibitory effect on the mycelial growth of *P. citrophthora* at 50 mg.ml⁻¹, it did demonstrate damage to



the mycelial structure.

Figure 3. 3 Morphology of two isolates of *Phytophthora citrophthora* (P66, P6) germinated zoospore with and without aqueous watermelon rind extract treatment (A) Germinated zoospore without inoculation. (B) Unusual germinative tube zoospores treated with Volare[®] fungicide (C) Granulated mycelium and deformation with *P. citrophthora* (P6) zoospore treated with 50 mg.ml⁻¹ watermelon extract (D) *P. citrophthora* (P66) zoospore cells became thin near at the beginning of germination when treated watermelon extract. The photographs were taken under a bright-field microscope with objective (20x).

The comparison between the microscopic observations of *P. citrophthora* isolates with the untreated control revealed a clear difference between both treatments.

The morphology of the mycelium in the control (Figure 3.3; A) was normal, linear and with a constant diameter. However, after treatment with 50 mg.ml⁻¹ of aqueous extract, *P. citrophthora* mycelia were severely deformed and showed numerous twisting with loss of linearity. In some cases, it starts to be thinner after zoospores germination and then gets thicker, in addition to the presence of granulations (vesicles) and excessive branching (Figure 3.3; C, D).

These observations were consistent with previous studies where many changes may occur in the morphology of fungal hyphae after treatment with plant extract (Ali et al., 2015a; S. Zhang et al., 2022) and our results are in agreement with Wang et al., (2016) who showed that after treatment with cuminic acid, mycelia displaying excessive branching and were severely deformed.

Viburnum tinus

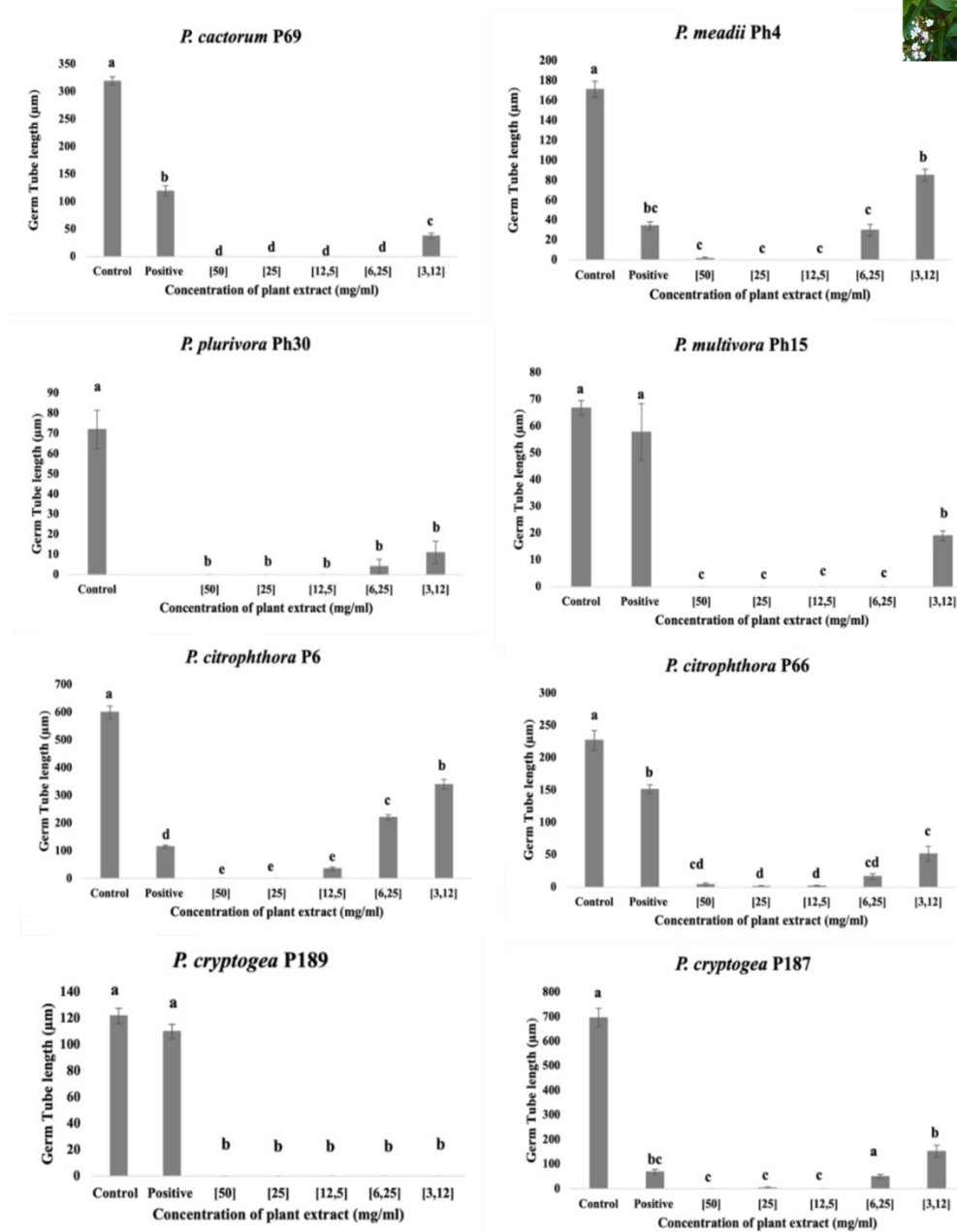


Figure 3. 4 Effect of *Viburnum* extract on germ tube elongation of six different *Phytophthora* sp. affected by different concentration, Positive = Volare® fungicide; Control = Water. Data presents mean $\pm$ standard error. Bars labeled with the same letter are not significantly different from each other according to Tukey's HSD at $p < 0,05$.

In contrast to previous plant extracts, a successful extract from this study has been recorded with aqueous *Viburnum tinus* extract that showed 100% inhibition of zoospores germination at 12,5 $\text{mg} \cdot \text{ml}^{-1}$ on all evaluated *Phytophthora* sp. and a high inhibitory effect for some *Phytophthora* sp. was observed even at 3,12 $\text{mg} \cdot \text{ml}^{-1}$, the lowest tested concentration.(Figure 3.4).

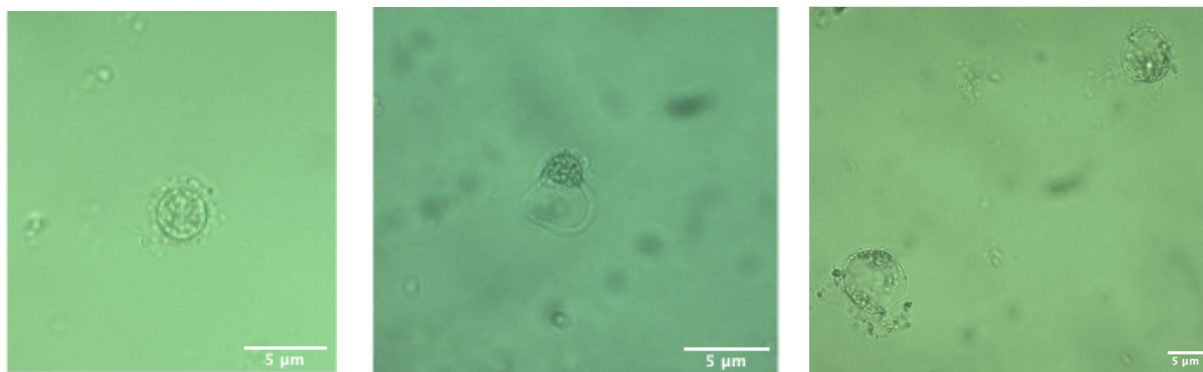


Figure 3. 5 Effect of aqueous *Viburnum tinus* plant extract on *Phytophthora* zoospores.

Even though, *Viburnum* aqueous extract completely inhibited zoospores germination, it also displayed a high fungicidal activity. At different concentrations, zoospores formation and cellular structure were seriously affected. These observations were verified through microscopic examination, where the extract showed zoospores lysis and blast at higher concentration. This resulted in cellular leakages of nucleic acids and soluble materials from outside the cell wall, and loss of the zoospores cytoplasm membrane. Additionally, there was an increase in zoospore granulation at lower concentration (Figure 3.5).

The effectiveness of different species of *Viburnum* botanical materials have been reported in previous studies. *Viburnum awabuki* and *Viburnum sargentii* showed moderate inhibitory effects against *Phytophthora* disease and other phytopathogenic fungal diseases including rice blast, tomato gray mold and tomato late blight in *in vitro* tests. Methanol extract of *V. awabuki* wasn't effecting in controlling late blight disease, however, a significant effect was obtained with *V. sargentii* that showed a disease control value of 31 % against *P. infestans* at a concentration of 3000 µg.ml⁻¹ (K.J. et al., 2006).

Recently, it has been reported the antifungal effect of acetone, ethanol or methanol extract of *Viburnum opulus* on different *Fusarium* strains by implementing machine learning methods (Zongur et al., 2022). In addition, it has been reported the effectiveness of *V. tinus* leaves on animals as a molluscicidal agent (Mohamed et al., 2005).

However, in our study, *V. tinus* showed active inhibitory effects on zoospore and mycelia growth of different *Phytophthora* sp. and this represent the first report of activity towards this genus.

Rose sp.

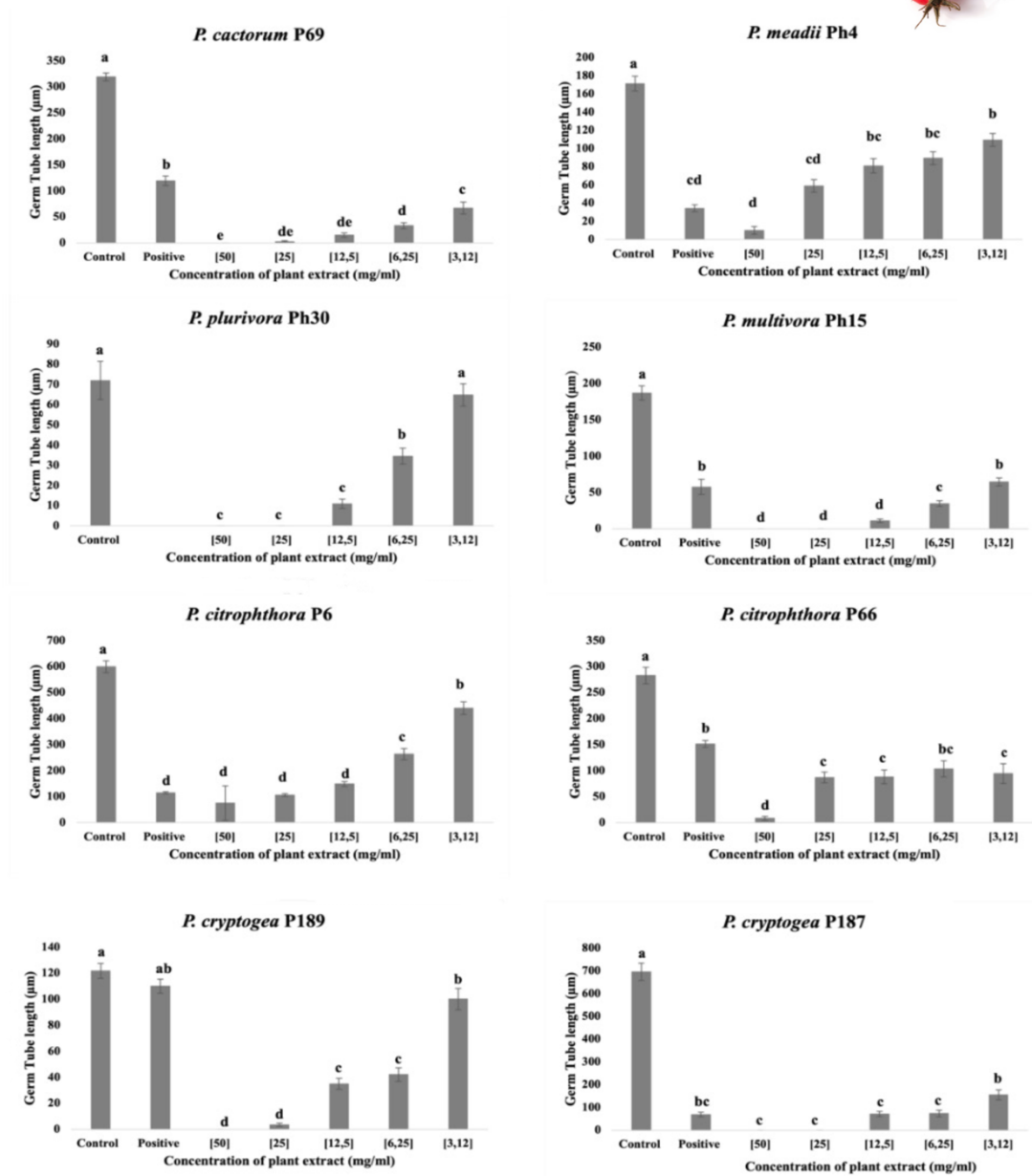


Figure 3. 6 Effect of rose hips extract on germ tube elongation of six different *Phytophthora* sp. affected by different concentration, Positive = Volare® fungicide; Control = Water. Data presents mean $\pm$ standard error. Bars labeled with the same letter are not significantly different from each other according to Tukey's HSD at $p < 0,05$.

Additionally, from this study, water rosehips extract represents the first report of activity against *Phytophthora* sp. It has antibacterial, antifungal and anti-inflammatory properties (Adamczak et al., 2012) and it is widely used in both pharmaceutical and food industries due to the presence of large range of important biologically active health-promoting compounds such as ascorbic acid, carotenoids, tocopherols, polyphenol flavonoids and triterpenoic acids (Bhave et al., 2017).

Previously, few study have been carried out regarding the effect of rosehip extract on *Phytophthora* sp. According to K.J. et al., (2006), methanol extract of *Rosa rugosa* and *Rosa multiflora* fruits, did not show an antifungal effect against late blight disease in *in vitro* test. Similarly, using a paper disc diffusion bioassay no inhibitory activity against *Phytophthora melonis* and *Pythium aphanidermatum* was observed by using one of the three different extracts: water, methanol and ethanol extract of *Rosa damascene* leaves. (Ghasemi et al., 2012). However, from this study, based on the results obtained from the *in vitro* zoospores bioassay, it was found that aqueous rose fruit extract was highly active against oomycetes with control efficacy of more than 50% for the six *Phytophthora* species at 12,5 mg.ml⁻¹. The efficacy of the treatment was positively correlated with extract dose and the germ tube elongation of *Phytophthora* sp. was significantly suppressed with all tested concentrations. The extract displayed an MIC value of 25 mg.ml⁻¹ for *P. cryptogea*, *P. multivora*, *P. pluriovra*, and *P. cactorum* (Figure 3.6).

Effect on mycelium

To further determine the extracts with the greatest activity, both the *Viburnum* and *Rose* extracts, that showed the most promising antimicrobial properties against all tested *Phytophthora* sp., were chosen for further testing on mycelial growth inhibition against *P. cryptogea* using an agar diffusion assay.

The results on mycelium antifungal activities did not correspond closely to the inhibition of zoospores by botanical extract observed in the previous experiment. Both *Viburnum* and *Rose* extract were highly inhibited even at lower concentration. The extract was not efficacious against *P. cryptogea* (P189, P187) mycelium growth at a concentration of 50 mg.ml⁻¹ and both *Viburnum* and *Rose* treatments did not show a significant effect comparing to the control (Figure 3.7).

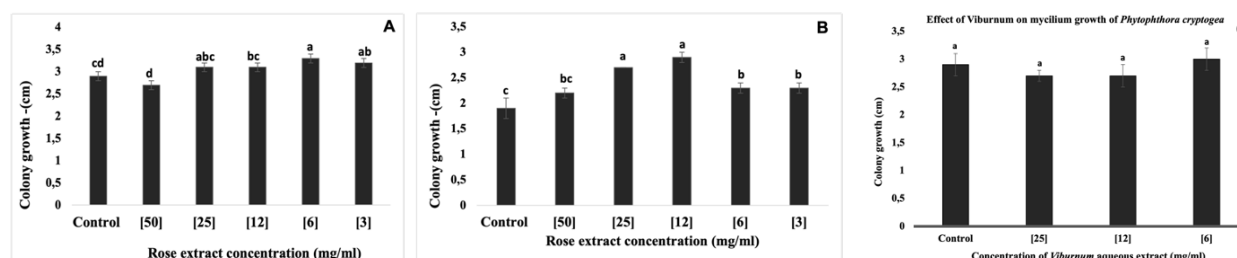


Figure 3. 7 Effect of Aqueous extract on mycelium growth of *Phytophthora cryptogea* after 7 days: (A) Aqueous rose extract on *Phytophthora cryptogea*, isolate (P 189) and (B) on *P. cryptogea*, isolate (P187). (C) Effect of *Viburnum* extract on *P. cryptogea* isolate (P 189). Average values are presented, and bars indicate the standard deviation (n=8). Bars labeled with the same letter are not significantly different from each other according to Tukey's HSD at p<0,05.

This in accordance with other studies that have demonstrated the same effect, Röhner et al., (2004) reported the effect of acetone extract on zoospore germination of *P. infestans* while it wasn't active on mycelial growth.

In addition, Li et al., (2020) reported a complete inhibition of sporangium production and zoospores release with much lower concentrations of *Psoralea corylifolia* and *Sophora flavescens* ethanol extracts than the rate used for *P. nicotianae* mycelial growth inhibition.

The reasons for this discrepancy are not known; however, it may be the result of the aggressiveness of *P. cryptogea* or a more thorough for the presence of other botanical

compounds that promoted *Phytophthora* growth and decrease the effect of inhibitory substances. Or it may be due that the extract contains few antifungal compounds which are ineffective for inhibiting mycelium growth, thus a higher level of extracts concentration could be applied to increase the rate of inhibition.

Generally, a successful infection of plants by *Phytophthora* sp. is dependent on zoospore germination (Ali et al., 2015b). Additionally, the fragility of zoospores is mainly due to the absence of a cell wall before encystment, where the outer surface is that of the plasma membrane (Banko et al., 2006; Hardham, 2007). Therefore, one possibility for the highest efficacy of plant extract treatments on this pathogenicity stage could be related to its lower activity on cellulosic *Phytophthora* mycelium cell walls.

On the other hand, for rosehips extract, other possibilities for controlling *Phytophthora* could be based on the application of not heating the water extract to reduce the effect of thermal degradation of natural compounds or by using the extract as a bio-stimulant for plant to enhance disease resistance through the presence of ascorbic acid, (Vitamin C), which is found in high concentrations in rosehips (Krzyzaniak et al., 2018; Singh et al., 2021).

Effect of aqueous extract on cress seedling

Extract that showed a remarkable inhibitory effect on the tested oomycetes was selected for further examination in a phytotoxicity bioassay on cress seedlings.

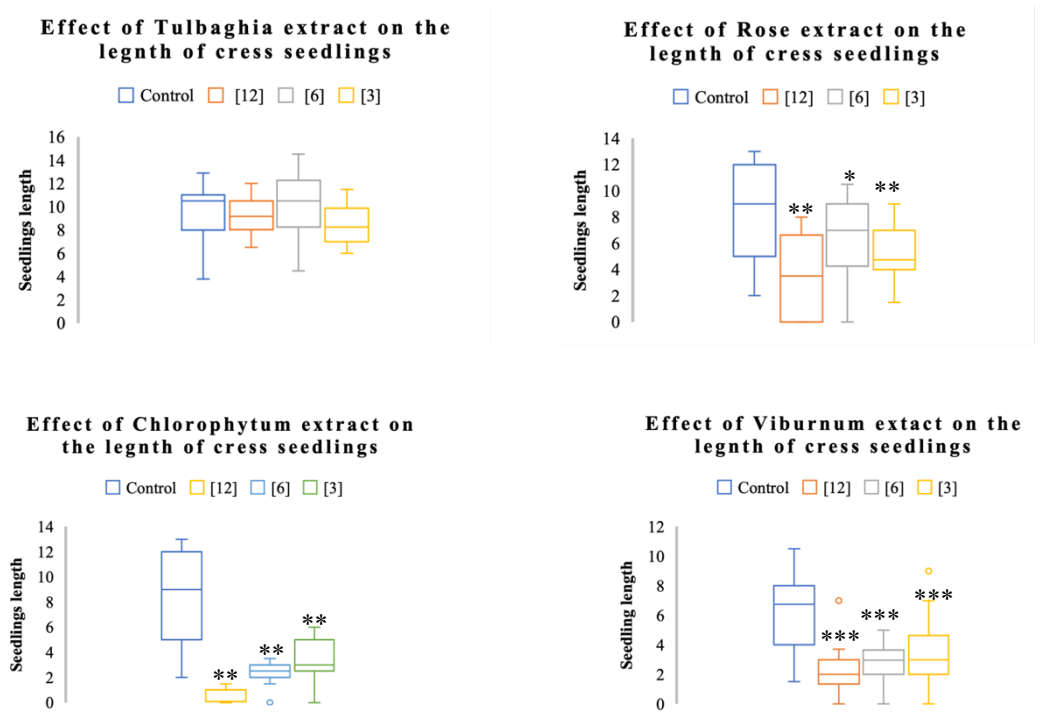


Figure 3. 8 Box plot representing the effect of three concentration (12, 6 and 3 mg.ml⁻¹) of plants extract on the length of cress seedlings (cm). Box plots represent mean (bold horizontal line) and 25% and 75% quartiles (box perimeters). The whiskers show 95% confidence interval, the small circles are outliers. The results of DunnettTest: (Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05).

Besides the previously tested extracts: *Chlorophytum* sp., *Rose* sp., and *Viburnum tinus*, *Tulbaghia violacea* was selected as an additional extract in this study due to its high inhibition rate on *Phytophthora* sp. and to other different pathogenic fungal diseases. The results of this experiment are discussed in detail in the following chapter (Chapter 4).

Table 3. 2 Concentration response of cress seedlings growth to water *Tulbaghia* leaves extract.

Extract concentration (mg.ml ⁻¹)	Percentage of Germination	Seedlings length (cm) ^y
Control	100	9.5 a
[12]	100	9.2 a
[6]	100	10 a
[3]	100	8.4 a

^y Means with aqueous extract followed by the same letter are not different by Dunnett honestly significant difference (P = 0.05) test.

Regarding the phytotoxicity test, it was found that *Tulbaghia* leaves extract was the only one that did not inhibit cress seedlings growth with the three tested concentration (12, 6 and 3 mg.ml⁻¹) (Figure 3.8), (Table 3. 2). However, with other extracts, at all concentrations, means were different as a result of an apparent botanical compound that inhibited seedlings growth and resulted in radicle length smaller than the control.

Furthermore, the extract derived from *Viburnum* leaves was found to reduce seedlings growth length by half compared to control under the concentration of 3 mg.ml⁻¹. However, at higher concentration, it completely suppressed seeds germination (Table 3.3).

This in accordance with other studies that found that extracts of *Artemisia annua* were phytotoxic to tomato plants even though it can completely inhibit *P. infestans* sporangia germination and significantly reduce foliar blight (Dorn et al., 2007).

Table 3. 3 Concentration response of cress seedlings growth to water *Viburnum* leaves extract.

Extract concentration (mg.ml ⁻¹)	Percentage of Germination	Seedlings length (cm) ^y
Control	100	6.14 a
[50]	0	0 c
[25]	59	0.5 c
[12]	85	2.1 b
[6]	89	2.6 b
[3]	90	3.2 b

^y Means with aqueous extract followed by the same letter are not different by Dunnett honestly significant difference (P = 0.05) test.

Similarly, Harrison et al., (2012) found that 70% methanol watermelon seed exudates of different genotypes can highly inhibit Proso millet radicle growth and *P. capsici* zoospore germination.

Viburnum tinus leaves extract are known to be rich in iridoid glycosides and other phytochemical constituents, such as coumarins, flavonoids oleanolic acid and bioflavonoids, which were also identified as major contents (Mohamed et al., 2005) and could be the phytochemicals responsible for the antimicrobial property and the phytotoxicity effect. Moreover, due to it is allelopathic effect on cress seedlings, *Viburnum* plant extract can be

considered as a potential herbicidal that could be used before planting as a biofumigant in an integrated management program in order to suppress weeds and reduce *Phytophthora* inoculum in an infected soil.

It should be noted that *Viburnum* leaves water extract was found to suppress zoospores growth of all tested *Phytophthora* sp., although the plant is highly susceptible to different *Phytophthora* infection (Henricot & Waghorn, 2014; Martini et al., 2004). This may indicate that the inhibitory substances extracted from *Viburnum tinus* are not involved in resistance to *Phytophthora* disease or they are not present in inhibitory concentrations for mycelium growth.

Garlic extract: case study

Garlic, which contains various organosulfur compounds, is already known for its antifungal potency and anti-*Phytophthora* activity (Hayat et al., 2016). It has been gradually becoming an alternative to synthetic chemicals and has been registered as a bio fungicide in more than 10 European countries.

Due to its biological properties, which may vary depending on the methods of extraction and between garlic cultivars, this experiment was performed to identify the bioactivity and anti-*Phytophthora* potency of different garlic extract on *P. nicotianae*.

Among the selected plants, twelve different local varieties of freshly prepared aqueous garlic (*Allium sativum* L.) extract, kindly provided by “DIBAF Lab”, were tested for the germination of zoospores on two isolates of *P. nicotianae* (P306, P 392).

From this study, our findings strongly confirm the antifungal potential of garlic extracts. The anti-*Phytophthora* activity was recorded on all tested cultivars with a similarity in *Phytophthora* inhibition between both *P. nicotianae* isolates (P392, P306).

Furthermore, the strongest potency was observed among nine out of the twelve garlic varieties (S0, C4, G1, P8, P3, C2, C7, BR and Sp) with a reduction of zoospores germination by more than 90%. (Figure 3.9).

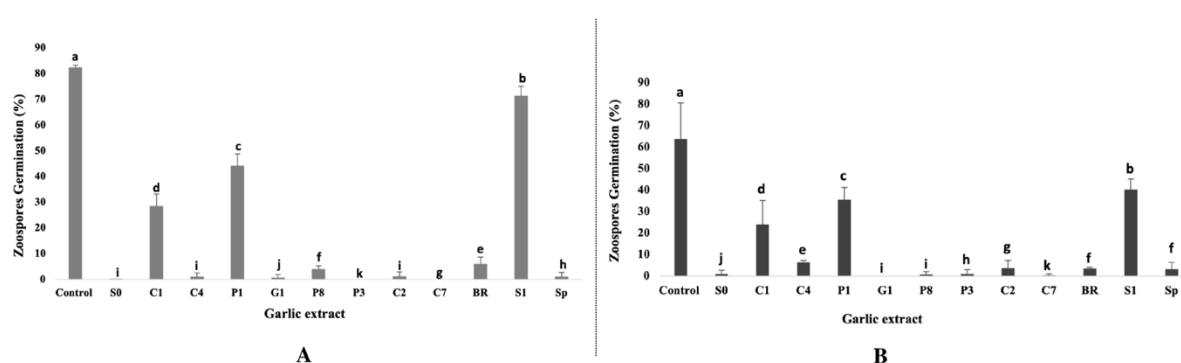


Figure 3. 9 Effect of different varieties of aqueous garlic extract on zoospores germination of two isolates of *P. nicotianae* A(P306), B(P392).

Data presents mean $\pm$ standard deviation, Bars labeled with the same letter are not significantly different from each other according to Tukey's HSD at $p < 0.05$.

The present study highlights the potential of garlic extracts as a natural alternative for controlling *Phytophthora nicotianae*. It confirms the antifungal potential of garlic extracts, and

demonstrates the variability in anti-*Phytophthora* activity among different Italian garlic cultivars.

These results suggest that the diversity in anti-*Phytophthora* activity among different garlic cultivars could be related to the diversity in allicin the primary antifungal substance found in garlic, and other sulfur-containing compounds among the selected cultivars.

This study also supports previous findings that have reported significant variability in antifungal activities of different garlic cultivars. Hayat et al., (2016) reported the antifungal potency of 28 Chinese garlic against four kinds of phytopathogenic fungi: *Fusarium oxysporum*, *Botrytis cinerea*, *Verticillium dahliae* and *Phytophthora capsici*. Similarly, Jang et al., (2019) found differences in thiosulfinate contents between four cultivars garlic extract that significantly inhibit *P. cactorum* growth and other different fungal pathogens.

Further research is needed to understand the mechanism of action of these compounds on *Phytophthora* growth as well as to determine the optimal garlic cultivars and extraction methods for use in disease management.

3.4 Conclusion

In summary, the present study, found that aqueous extracts of Watermelon, *Chlorophytum*, Rosehip and *Viburnum* were effective against different *Phytophthora* sp. in an *in vitro* setting. The extracts displayed a significant inhibition rate in a dose-dependent manner on zoospores of the six tested *Phytophthora* species, with the growth inhibition being highly variable among different plant extracts, and *Viburnum tinus* extract showing the most significant inhibitory properties. These findings suggest that the tested aqueous extracts contain valuable antimicrobial constituents for controlling *Phytophthora* disease. However, further research is needed to understand the mechanism of action of these extracts and their individual purified compounds on both the reproductive and vegetative life cycles of *Phytophthora*, as well as to determine their stability and safety for use in disease management.

3.5 References

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CHAPTER 4: USE OF AQUEOUS *TULBAGHIA VIOLACEA* EXTRACT TO SUPPRESS DIFFERENT *PHYTOPHTHORA* SPECIES AND FUNGAL PATHOGEN

Abstract

Phytophthora, a virulent soil-borne plant pathogen, is a significant global burden, causing extensive losses of ornamental plants in many nurseries worldwide. Along with other pathogenic fungi, it has become a growing concern in agriculture and the food industry, as it has been linked to many serious threats to forest, horticultural trees and crops, ornamental plants, and postharvest fruits. The regular use of chemicals to control these pathogens has negative impacts on the environment, poses health risks to non-target organisms, and may also lead to the development of resistance. Therefore, there is a need to find and establish more effective long-term strategies for sustainable agriculture and to improve crop growth and health through an integrated management approach that minimizes the use of chemicals fungicides. Several review studies suggest herbal medicine *Tulbaghia* sp. and its natural bioactive compounds have antibacterial, antiviral, and antifungal effects. Although the effects of this plant have been investigated as antifungal agents in several studies, to the best of our knowledge, its leaves aqueous extracts' antifungal potential against *Phytophthora* sp. and other pathogenic fungus are unknown. This study therefore considers the application of leaves aqueous extract of *Tulbaghia villoacea* to test the anti-*Phytophthora* activities against two isolates of each of the following species: *Phytophthora nicotianae*, *P. plurivora* and *P. cryptogea*. Additionally, the antifungal activity was carried out on three pathogenic fungus *Alternaria* sp., *Gnomoniopsis castanea*, *Fusarium oxysporum* and *F. brachygibbosum*. Furthermore, for a better understanding of its toxicity to biological control agents and safer application to the environment, an assessment of its application on *Trichoderma hamatum* has been conducted.

Results of these studies provided evidence that concentrations of 5 mg.ml⁻¹ and 0.5 mg.ml⁻¹ significantly ($p < 0.05$) inhibited the growth of mycelia and the germination of spores of most tested species, respectively compared to the control. It is apparent that the antifungal nature of *T. violacea* aqueous leaves extracts against *Phytophthora* sp. and other fungal pathogen shown here for the first time indicates a potential use of this extract as biofungicide.

4.1 Introduction

Phytophthora sp., a highly virulent soil-borne plant pathogen, is common in many plant nurseries. Other pathogenic fungi, such as *Fusarium brachygibbosum* and *Gnomoniopsis castanea* sp., have become a growing safety concern in agriculture and in food industries because they have been reported to be associated with many serious threats to forests, horticultural trees and crops, ornamental plants, and postharvest fruits.

Due to consumers' concerns about the food safety, there is a great need for alternative, safe antifungal preservatives added to cereal to control *Fusarium brachygibbosum*, the cause of head blight disease (EFSA Plant Health (PLH) et al., 2021), and *Gnomoniopsis castanea* sp., the causal agent for the outbreak of chestnut postharvest decay (Visentin et al., 2012). Additionally, there is a high demand for a biofungicide to control the activity of a three globally distributed, economically important *Phytophthora* pathogens: *Phytophthora nicotianae*, *P. plurivora* and *P. cryptogea* (Jung et al., 2017), as well as *Fusarium* wilt, one of the major

diseases caused by pathogenic *Fusarium oxysporum* strains (de Lamo & Takken, 2020) and *Alternaria* sp. a foliage plant pathogenic disease (Adhikari et al., 2017).

Plant extracts contain a wide range of secondary metabolites, which possess several biological activities and seem to be very promising as an alternative to synthetic chemicals (Nxumalo et al., 2021). In addition, many species belonging to the Alliaceae family contain active sulfur volatile compounds produced by enzymatic action that have an extensive history of antibacterial, antiviral and antifungal activity (Kubec et al., 2013; Lindsey & Van Staden, 2004).

Tulbaghia violacea is a perennial monocotyledonous bulbous plant indigenous to Southern Africa and belonging to the Alliaceae family. It is commonly known as society garlic or wild garlic and bears resemblance to *Allium* in odor (Taneja & Singh, 2020). The genus *Tulbaghia* includes 20-30 species, with at least 25 species having similar karyotypes (Báez et al., 2020; Samkeliso Takaidza et al., 2018; Balogun & Ashafa, 2019; Báez et al., 2020).

Cultivated all over the world, *T. violacea* presents an economic importance for its medicinal effects and potential as an ornamental plant for nursery growers (Sadhu et al., 2018). It also has been used to treat sewage in mesocosm subsurface constructed wetlands (Burgos et al., 2017). Additionally, it is considered as a great food source for bees and its characteristic alliaceous aroma attracts greater interest from the food industry (Aremu & Van Staden, 2013; Kubec et al., 2013). Furthermore, *in vitro* plant cultures of this plants are used extensively to produce bioactive metabolites (Eid & Metwally, 2017).

The effects of *Tulbaghia* sp. have been investigated extensively in traditional medicine (Aremu & Van Staden, 2013; Madike et al., 2020; Saibu et al., 2015) and have been found to have diverse applications in the treatment of numerous diseases including cancer (Aremu & Van Staden, 2013; Balogun & Ashafa, 2019; Bungu et al., 2006; S. L. Lyantagaye, 2013; Mackenzie et al., 2017; Motadi et al., 2020; Mthembu & Motadi, 2014; Olorunnisola et al., 2011; Saibu et al., 2015; S. Takaidza et al., 2018; Taneja & Singh, 2020), HIV/AIDS patients (Chinsembu, 2016; Klos et al., 2009; Maroyi, 2014; Ncube, Ngunge, et al., 2011). With different solvent extracts, *Tulbaghia* sp. demonstrated antioxidant, anti-hypertensive, anti-atherosclerosis, antithrombotic anticoagulant, anti-diabetic effect, and the ability to induce testosterone production (Bungu et al., 2008; Ebrahim & Pool, 2010; Murugesan et al., 2019; Olorunnisola et al., 2011, 2012). Additionally, *T. violacea* has the potential to treat headaches, cough, colds, intestinal worms, gastrointestinal ailments, asthma, fever, and pulmonary tuberculosis as well as having anti-inflammatory and pain relieving properties (Balogun & Ashafa, 2019; Ncube, Ngunge, et al., 2011) (Adebayo et al., 2015).

The effects of *Tulbaghia* extracts have been shown to have an antimicrobial activity against a variety of human bacterial diseases. They significantly inhibit the growth of *Escherichia coli*, *Staphylococcus aureus*, and *Mycobacterium aurum* A+, *Mycobacterium avium*, *Mycobacterium scrofulaceum*, *Mycobacterium microti* (Bamuamba et al., 2008), *Staphylococcus aureus*, *Enterococcus faecalis* and *Pseudomonas aeruginosa* (Netshiluvhi & Eloff, 2019). Additionally, they have been shown to possess anti-parasitic properties against *Trypanosoma brucei brucei*, *Leishmania tarentolae* (Krstin et al., 2018). *Tulbaghia* extracts have also been found to be a therapeutic or prophylactic anticoccidial agent against *Eimeria* sp., in poultry and livestock (Naidoo et al., 2008) and a tick repellent at a concentration of 10 w/v% against *Rhipicephalus appendiculatus* (Staffa et al., 2020), However, (Richter et al.,

2014) reported that *Tulbaghia* essential oils exhibit insecticidal properties and it can strongly repel the Russian wheat aphid (*Diuraphis noxia*).

Antifungal activities of *Tulbaghia* extracts have also been evaluated in several studies. These studies have tested the extracts on *Candida albicans* (Krstin et al., 2018; Motsei et al., 2003; Ncube, Ngunge, et al., 2011; Thamburan et al., 2006) a human pathogenic yeast. The extracts have also been found to have significant activity against some important plant pathogenic fungi. An extensive and systematic review of the literature was carried out, and relevant findings on the anti-fungal activities of *Tulbaghia* are presented in (Table 4.1). Most studies have been conducted on *Aspergillus sp.* for example, aqueous plant extract of *T. violacea* significantly decline β -glucan and chitin synthase production in *Aspergillus flavus* (Belewa et al., 2017) and it can affect ergosterol production by inhibiting oxidosqualene cyclase.(Somai et al., 2021).

Table 4. 1 Antifungal activities of *Tulbaghia violacea* plant extract

Plants characteristic			Experimental setup			References
Part used	Type	Solvent	Antifungal activities	bioassays	Tested concentration(mg.ml ⁻¹)	
Bulb	F	Water	<i>Aspergillus flavus</i>	<i>in vitro</i>	5 to 25	(Somai et al., 2021)
Bulb	D	Acetone	<i>Fusarium oxysporum</i>	<i>in vitro</i>	0,1; 0,3; 0,4; 0,8; 1,5	(Ncise et al., 2020)
Leaf	D	Acetone	<i>f. sp. glycines strain Aspergillus fumigatus</i>	<i>in vitro</i>	0.42 to 2.50	(Netshiluvhi & Eloff, 2019)
Bulb	F	Water	<i>Aspergillus flavus</i>	<i>in vitro</i>	0, 2.5, 5, 7.5, 10, 12.5	(Belewa et al., 2017)
Bulb	F	Water	<i>Aspergillus flavus</i> ; <i>Aspergillus parasiticus</i>	<i>in vitro</i>	0, 5, 10, 15, 20, or 25	(Somai & Vuyokazi, 2011)
Bulb	F	Water	<i>Aspergillus flavus</i>	<i>in vitro</i>	5 to 25	(Belewa et al., 2011)
Leaf	D	Methanol	<i>Puccinia triticina</i>	<i>in vitro</i> , glasshouse	1	(Cawood et al., 2010)
Leaf	D	Acetone	<i>Aspergillus fumigatus</i>	<i>in vitro</i>	10, (2-fold dill)	(Netshiluvhi & Eloff, 2016)
Stem +Leaf	D	Methanol	<i>Sclerotium rolfsii</i>	<i>in vitro</i>	1	(Van Den Heever et al., 2008)
Leaf+Bulb	D	Methanol	<i>Pythium ultimum</i> , <i>Botrytis cinerea</i> , <i>Fusarium oxysporum</i> , <i>Sclerotium rolfsii</i> <i>Sac</i> , <i>Rhizoctonia solani</i> , <i>Botryosphaeria dothidea</i> , <i>Mycosphaerella pinode</i>	<i>in vitro</i>	0.125, 0.25. 0.5, 0.8, 1.0, and 2.0	(Nteso & Pretorius, 2006a)
Bulb	F	Water	<i>Pythium ultimum</i> ,	<i>in vitro</i>	50; 10	(Lindsey & Van Staden, 2004)

			<i>Botrytis cinerea</i> , <i>Rhizoctonia solani</i> .			
Stem +Leaf	D	Methanol- crude dissolve with water	<i>Mycosphaerella pinodes</i>	Field, detached leaves	0.25, 0.5, 1.0, 2.0	(Nteso & Pretorius, 2006b)
leaf + Bulb	F	Methanol- crude dissolve with water	<i>Botrytis cinerea</i>	Postharvest assay	0.0, 62.5, 125.0, 250.0, 500.0; (0.0, 1.95, 3.91, 7.81, 15.63, 31.25 and 62.5	(Fielding et al., 2015)

F: fresh; D: dried

According to the information provided, it seems that there have been no previous studies on the effects of *Tulbaghia* aqueous leaf extract on *Phytophthora* diseases, *Gnomonopsis castanea*, *Alternaria sp.*, *F. brachygibbosum*. and *Trichoderma hamatum*. As a result, a research study was conducted to evaluate the effectiveness of the plant extracts in reducing populations of these major threats to agriculture and forest plants. In this study, we measured the impacts of *Tulbaghia* extract using *in vitro* bioassays on mycelium growth development and spores germination. To work in sustainability, we used water as a solvent and we chose to use leaves instead of underground organs to avoid affecting their abundance and possibly making them susceptible to extinction (Aremu & Van Staden, 2013).

4.2 Materials and methods

4.2.1 Fungal strains and growth conditions

The study evaluated the inhibitory activity of *Tulbaghia* plant extract against two *Phytophthora* isolates, including *P. cryptogea* (P189, P187), *P. Plurivora* (Ph30, P135) and *P. nicotianae* (P306, P392), as well as fungal pathogens such as *Gnomonopsis castanea* strain G7, *Alternaria sp.*, *Fusarium oxysporum* and *F. brachygibbosum*, which were isolated from infected castanea, tomato fruit, garlic, and wheat respectively, and one biological control agent, *Trichoderma hamatum*. The isolates were taken from the mycological collection at the DIBAF plant protection laboratory at Tuscia University in Viterbo, Italy.

The *Phytophthora* isolates were grown on carrot agar (CA) and the fungal pathogens on Potato dextrose agar (PDA), and both were maintained at 25°C. To produce zoospores, plates containing mature zoosporangia were flooded with fresh cold water and incubated at 4°. After one hour, zoospores were collected.

For all the fungi, when required, conidia were dislodged from the mycelium by flooding a plate with water and scraping the spores off the surface with a sterile glass rod, before being counted under a hemocytometer (Thoma Counting Chamber; BLAU BRAND, Germany).

4.2.2 Preparation of aqueous *Tulbaghia* leaves extract

With the aim of promoting the use of leaves as a conservation strategy, 200g of freshly gathered leaves of *T. violacea* were thoroughly washed under running water and immediately homogenized for 1 min at maximum speed blender (Johnson, Annabella, Italy) with 500 ml of 4°C cold distilled water, according to a method slightly modified from Somai & Vuyokazi, (2011).

The homogenate was then chilled for 15 min in a refrigerator and centrifuged at 10,000 x g for 10 min at cold temperature using (Beckman, J2-21M/E Centrifuge, UK) to separate the pulp from the liquid. The aqueous supernatant was filtered through Whatman filter paper to remove the remaining pulp and obtain a pure extract. The filtrate was then evaporated to dryness using a freeze-dried (VirTis, USA).

A stock solution was prepared by dissolving the dried extract powder in distilled water and storing it in the dark at -20 °C. Dilutions were made from this stock solution ranging (0.5, 1, 2.5, 5, 10, 25, to 50 mg.ml⁻¹).

The extraction yield (%) was calculated by dividing the weight of the extract by the weight of the sample multiplied by 100.

4.2.3 Anti-*Phytophthora* and anti-fungal activity: Mycelium inhibition

To investigate the minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) of *Tulbaghia* extract on the mycelial growth of *Phytophthora sp.* and other pathogenic fungi, an *in vitro* agar dilution method was used, following the method described by Nteso & Pretorius, (2006a).

The PDA medium (2 ml) was supplemented with *Tulbaghia* extract was poured into sterilized microscopic slide to create a final concentration of (2.5, 5, 10, 25, to 50 mg.ml⁻¹). PDA was used as negative control, while the systemic fungicide Arpel WDG was used as a positive control for *Phytophthora*, and Folpan 80 WDG for the pathogenic fungus.

A 6 mm diameter disc was cut from each two *Phytophthora* isolates and fungus species from the periphery of an actively growing culture and placed on the top of the slide.

Four slides were prepared for each treatment and isolates, representing four replicates, put inside sterilized glass petri dish, and incubated at 25 ± 1°C for seven days. The experiment was performed in duplicate. The colony radius was measured with a ruler. The mean growth values were converted into the inhibition percentage of mycelial growth relative to the control treatment according to the Pandey et al., (1982) formula:

Growth inhibition % = $\left[\frac{((\text{growth in the control} - \text{growth in the sample}) / \text{growth in the control})}{1} \right] \times 100$.

The lowest concentration that completely inhibited the growth of *Phytophthora* was considered (MIC) and the minimum fungicidal concentration (MFC) was considered as the lowest concentration that completely inhibited *Phytophthora* growth after re-culturing onto a fresh PDA plate for 1 week incubation at 25 ± 1°C, indicating more than 99.5% killing of the original inoculums, as stated by Tao et al., (2014)

4.2.4 Anti-*Phytophthora* and antifungal activity: zoospores and conidia germination

To evaluate the effect of *Tulbaghia* treatment and to determine the minimum effective dilution on zoospore and conidia germination, 100 µl of zoospores (1 × 10⁴ spores/ml) from each *Phytophthora* species collected in cold water suspension and 50 µl of conidia (1 × 10⁵ spores/ml) were added to test tubes containing 50 µl of PDB broth and distilled water. The tubes were then supplemented with a range of *Tulbaghia* extract concentrations ranging between (0.5, 1, 2.5, 5, 10, 25, to 50 mg.ml⁻¹) and the volume was brought up to 200 µl. Negative control tubes received identical concentrations of spores but no plant extract, while the positive control received the appropriate concentration of fungicide. Tubes were incubated at 25°C for 3 hours for *Phytophthora sp.* and more than 12 hr. for the other fungi. Triplicate assays were performed per experiment and each experiment was done two separate times for reproducibility. The percentage of zoospores and conidia germinated was determined by

observing random fields of view on one slide through a compound microscope (10x objective lens) and counting a total of 100 spores. A spore was considered germinated if the germ tube was longer than the conidia or zoospore cyst.

4.2.5 Light microscopic examination of hyphal morphology.

The fungal mycelia and spores of the pathogen were used as samples for microscopic studies. Fungal physiology was monitored by light microscopy after growth with various subinhibitory doses of the plant extract. In addition, once the conidia were fully germinated, they were mounted on a fresh microscope slide with a drop of sterile water and were examined for any changes in mycelial physiology.

4.2.6 *In planta* Experiments

In order to evaluate the direct effect of *Tulbaghia* extract on *P. plurivora* development, an *in-planta* bioassay was performed on *Pinus sylvestris* seedlings. The seeds were surface sterilized with 5% sodium hypochlorite for 5 min and subsequently rinsed three times in sterile distilled water (sdH₂O), then cultivated in 12 Cell Culture Multiwell Plates filled with sterilized peat in a growth chamber at temperatures of 23 °C, 60±2% relative humidity, and a 12-h photoperiod. After two weeks, seedlings were artificially inoculated with *P. plurivora* (Ph30) by adding colonized 0.6 cm agar pieces on the top of soil surface, and each well was treated with 1.5 ml of *T. violacea* aqueous extract suspended in distilled water at a rate of 5 mg. ml⁻¹. A standard synthetic fungicide, Arpel WDG, was applied in the same way at the rate of 2500 mg. l⁻¹. Seedlings not treated with the extract or synthetic fungicide served as a negative control. Twelve seedlings were used per treatment. After 5 days of infection, disease incidence was recorded and presented as percentage of infected plants.

4.2.7 Statistical analysis

All data were expressed as the mean ± SE by measuring three or four independent replicates. Analysis of variance (ANOVA) using one-way ANOVA followed by Tukey test LSD (least significant difference) was used to test the significance of differences between means obtained among the treatments at the 5% level of significance.

4.3 Results

The results of this study suggest that *Tulbaghia* plant extracts was effective in inhibiting the growth of *Phytophthora* sp. and the test pathogens as compared with the negative control. However, their efficacy against pathogens varied significantly with concentration. In all treatments, the minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) were the same.

Water was the solvent used for the extraction, and the percentages yield of *T. violacea* crude aqueous yields extracts after freezing dried was about 3.3%.

4.3.1 Anti *Phytophthora* activities

The effect of *T. violacea* extract on the mycelial growth and zoospores germination of three *Phytophthora* sp. *in vitro* is shown in (Figure 4.1).

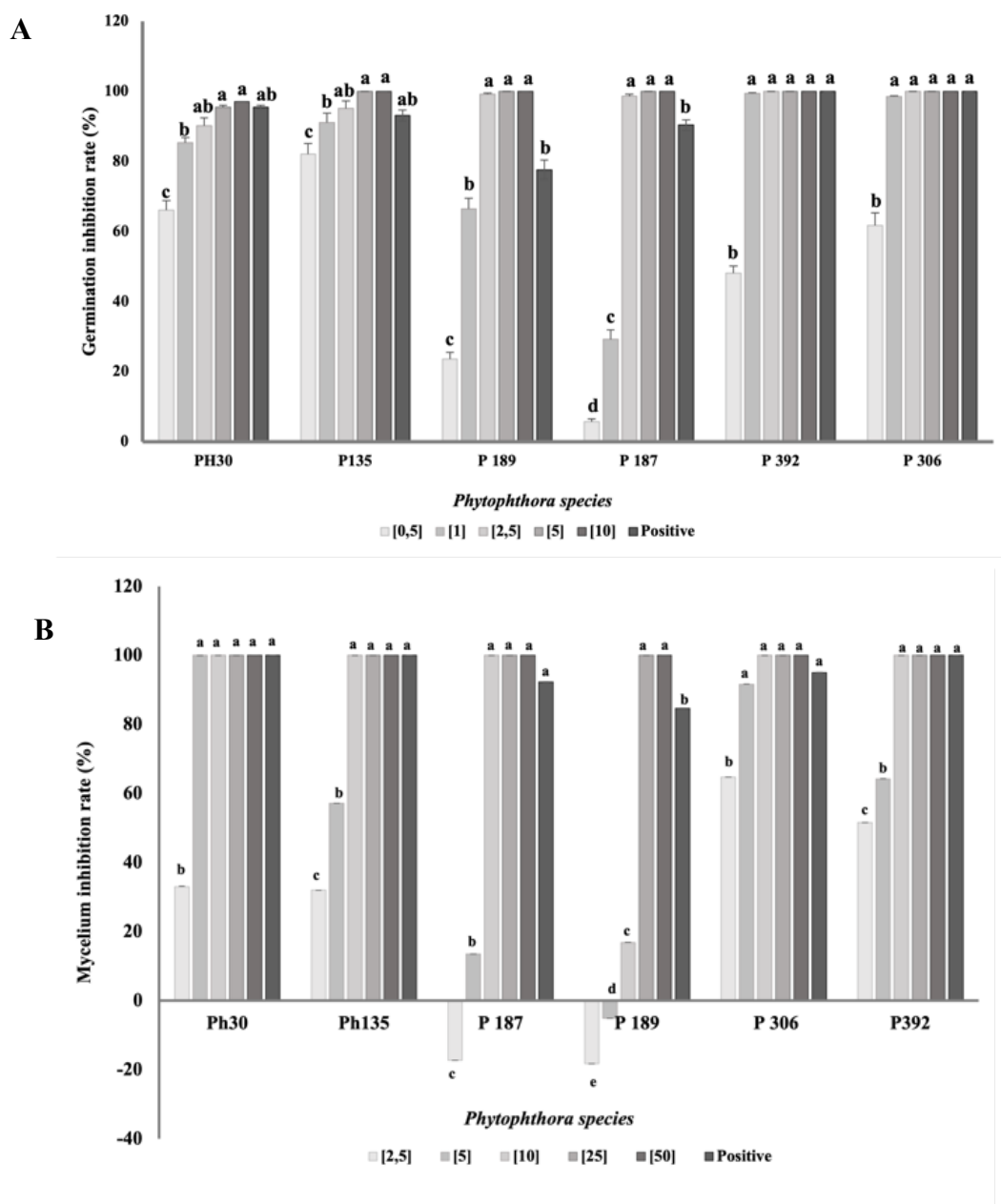


Figure 4. 1 Effect of aqueous *T. violacea* plant extract on three *Phytophthora* sp.: *P. plurivora* (Ph30, Ph135), *P. cryptogea* (P187, P189), *P. nicotianae* (P306, P392). A) Zoospores germination rate inhibition (%) as affected by different concentrations (wt/vol) of *Tulbagia* extract. Average values are presented, and bars indicate the standard error of the mean (n = 3). B) Percentage of mycelium growth inhibition of three *Phytophthora* sp. affected by different concentrations (wt/vol) of *T. violacea* extract. Average values are presented, and bars indicate the mean standard error of the mean (n = 4). Positive = Fungicide ARPEL WDG. The same letter for each isolates denotes no significant difference at P=0.05 according to Tukey test.

The mycelial growth inhibition varied significantly among different *Phytophthora* sp. in a dose-dependent manner as compared to the positive and negative controls within a period of 7 days.

Approximately 30% and around 50% of mycelial growth of *P. plurivora* and *P. nicotianae* respectively, were inhibited at a relatively low concentration of 2.5 mg.ml⁻¹. However, an incremental growth was recorded with both isolates of *Phytophthora cryptogea* (P187, P189).

The results of the study showed that the mycelial growth of *P. nicotianae* was completely inhibited at 10 mg.ml⁻¹, similarly for one isolates of *P. plurivora* (Ph135), however, 5 mg.ml⁻¹ was sufficient for inhibiting the second isolate (Ph30).

In contrast, the MIC for *P. cryptogea* ranges from 10 mg.ml⁻¹ for isolate (P187) to 25 mg.ml⁻¹ for (P189).

Thus, the (MIC) and (MFC) values of the *Tulbaghia* extract are between 5 mg.ml⁻¹ and 25 mg.ml⁻¹, with a concentration of 10 mg.ml⁻¹ being effective on most of the tested *Phytophthora* sp.

The MIC value for zoospores germination for both isolates was similar for the three tested *Phytophthora* species, ranging between 1 mg.ml⁻¹ and 5 mg.ml⁻¹.

The extract had an IC50 value of 0.5 mg.ml⁻¹ for *P. nicotianae* zoospores, with total inhibition at 1 mg.ml⁻¹ and the MIC value were 2.5 mg.ml⁻¹ and 5 mg.ml⁻¹ for *P. cryptogea* and *P. plurivora*, respectively.

The positive control with the Arpel WDG synthetic fungicide recorded 100% inhibition of mycelium and zoospores on both *P. nicotianae* and *P. plurivora*, however, on *P. cryptogea* it only showed a reduction of 80% compared to the control.

4.3.2 Anti-fungal activity

The results of the study showed that *T. violacea* extract was effective against all tested fungi. (Figure 4.2) indicates the minimum concentrations needed to completely inhibit conidia and mycelium growth of four common fungal pathogens found on plants compared with the control and represents the growth-inhibition effect on *T. hamatum*, a biological control agent.

The MIC values are the same as MFC ranged from 5 mg.ml⁻¹ and 25 mg.ml⁻¹ on mycelium. The *Tulbaghia* extract fully inhibited mycelium growth of *F. oxysporum* at 5 mg.ml⁻¹, while a full inhibition for *F. brachygibbosum*, *Alternaria* sp., and *T. hamatum* was observed at 25 mg.ml⁻¹

However, for conidia, the relative growth of each microorganism after 12 hours of incubation in the presence of different concentrations of *Tulbaghia* extract was compared to the control. There was no significant difference among the different pathogen species with an MIC value of 2.5 mg.ml⁻¹. *Tulbaghia* extract at 0.5 mg mL⁻¹ was able to reduce conidia germination of most fungal pathogen by 50%.

Moreover, *Gnomoniposis castanea* was found to be more sensitive to all concentration with an MIC of 0.5 mg ml⁻¹ on conidia and fungicidal activity at 2.5 mg ml⁻¹ on mycelium, which was very low in comparison with those of other pathogens.

Interestingly, *Trichoderma hamatum* was less affected by treatments with minor or no effects on suppressing conidia germination at 0.5 mg ml⁻¹ and 1 mg ml⁻¹ and around 20 % of conidia germination inhibition at 2.5 mg mL⁻¹.

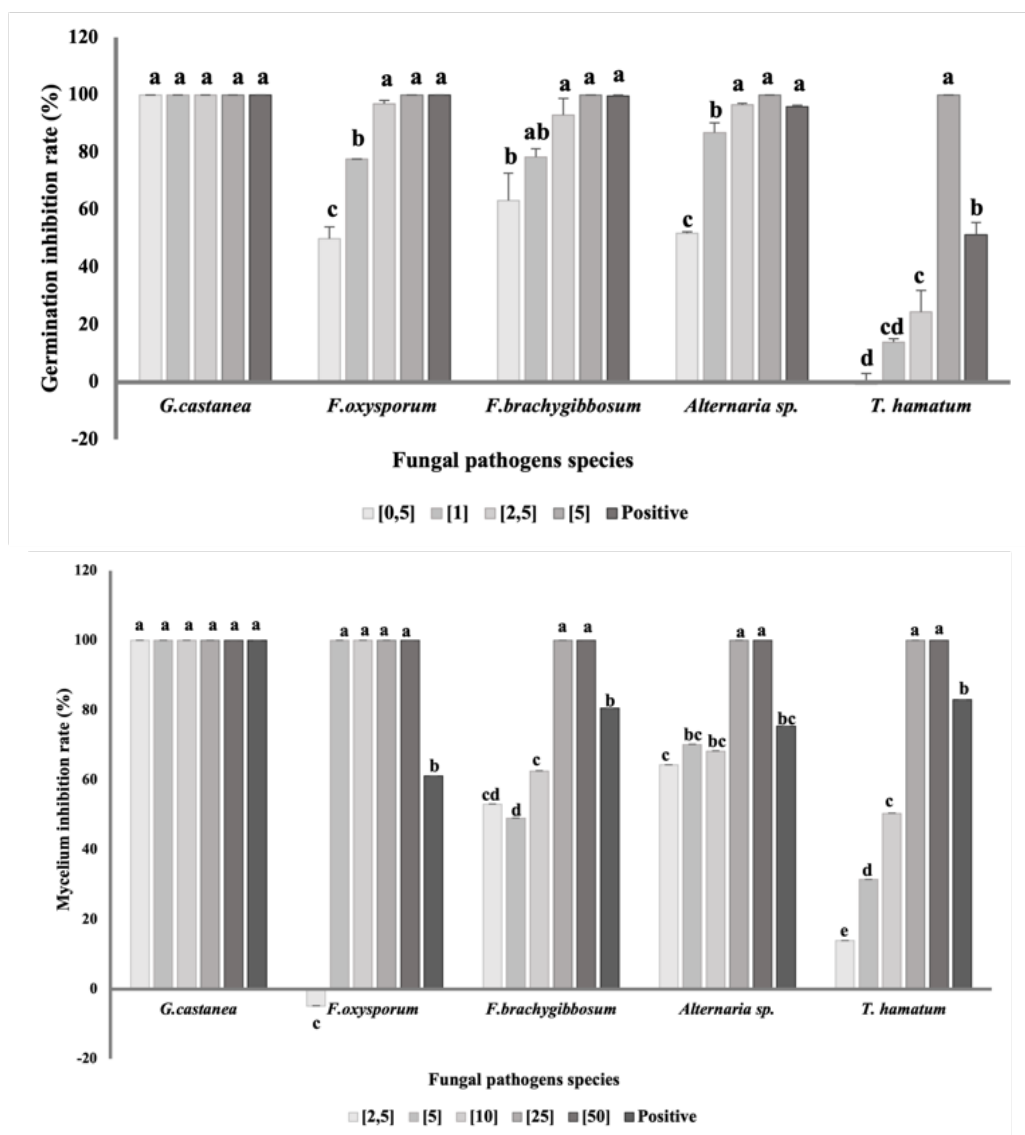


Figure 4. 2 Effect of aqueous *Tulbaghia* plant extract on mycelium and conidia germination of three fungal pathogens (*Fusarium oxysporum*, *F. brachygibbosum*, *Alternaria sp.*, *Gnomoniopsis castanea*) and on one biological control agent (*Trichoderma hamatum*). a) Percentage of growth inhibition of Conidia as affected by different concentrations (wt/vol) of the botanicals extract. Average values are presented, and bars indicate the mean standard error of the mean (n = 3). b) Mycelium growth inhibition (%) as affected by different concentrations (wt/vol) of *Tulbaghia* extract. Average values are presented, and bars indicate the standard error of the mean (n = 4). Positive = Folpan 80 WDG. The column with different lowercase letters between different concentrations indicates significant differences according to Tukey test ($P < 0.05$).

4.3.3 Light microscopic examination of hyphal and spore morphology

The effect of *T. violacea* extract on the morphology of pathogens was examined using a light microscope (Figure 4.3; 4.4; 4.5). After different concentrations of the extract ranging from 0.5 to 25 mg.ml⁻¹, zoospores were quickly immobilized, in contrast to the control in which zoospores remained mobile for a long time. A subsequent lysis and blast were observed at a concentration of 10 mg.ml⁻¹ and above, resulting in the loss of the zoospore cytoplasmic

membrane and the extrusion of abundant materials from the outside of the cell wall (Figure 4.3c, d, f).

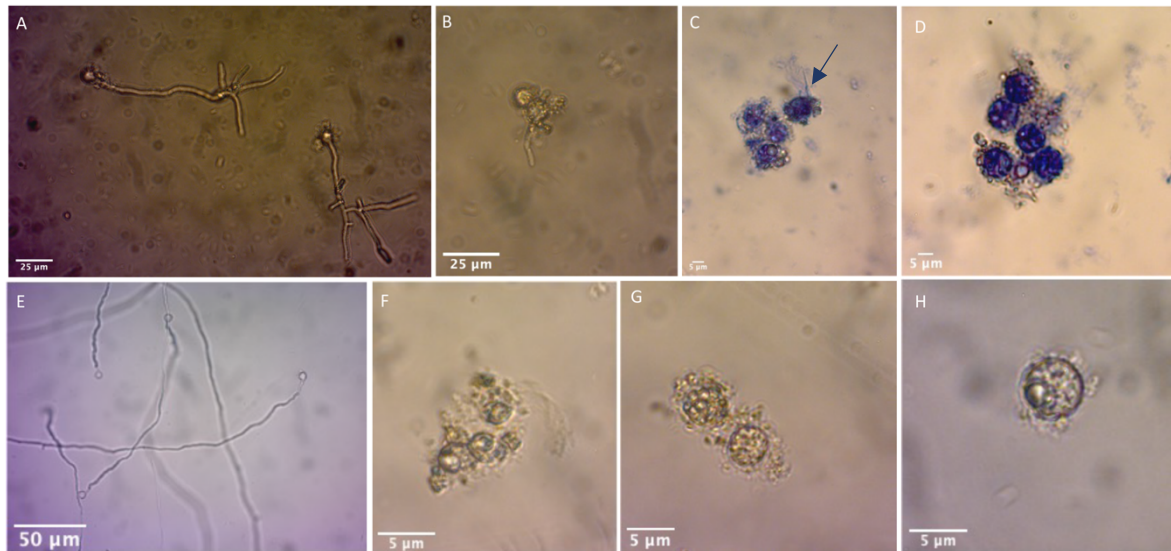


Figure 4. 3 Representative light microscopic images of zoospore morphology of three *Phytophthora* sp.: (A-B: *P. plurivora* (P135); C-D: *P. nicotianae* (P306); E- H: *P. cryptogea* (P189)) with and without *T. violacea* aqueous extract after 4 h incubation. (A), (E) Germinated zoospore without treatment. (B) Unusual germinative tube at 0,5 mg.ml⁻¹ of *Tulbaghia* extract and (C, D, F, G, H) Disruption of membrane and granulation of cell organelle and lysis (arrow) of zoospores by *Tulbaghia* extract after treatment with 5 mg.ml⁻¹.

It was also found that the extract caused a delay in zoospore germination (Figure 4. 3 B) and conidia germination (Figure 4.4), in addition to an increase in granulation for both zoospore (Figure 4.3 G) and *Phytophthora*'s mycelium (Figure 4.4; C, D). The control fungus and *Phytophthora* sp. grown on (PDA) had regular, normal, and homogenous hyphae (Figure 4.4; A, B, E, I). However, *Phytophthora* sp. mycelia treated with *T. violacea* extract for 7 day caused significant morphological changes. In addition to vesicle formation and granulation detected with *P. cryptogea*, an altered phenotypic change in hyphal morphology of *P. nicotianae* was detected, including hyphal swelling at the tips, crumpling and a warty surface of mycelia (Figure 4.4; E, F, G, H). However, hyphal ramification decreases in number and in length with the fungal pathogen *F. oxysporum* (Figure 4.4; I, J, K).

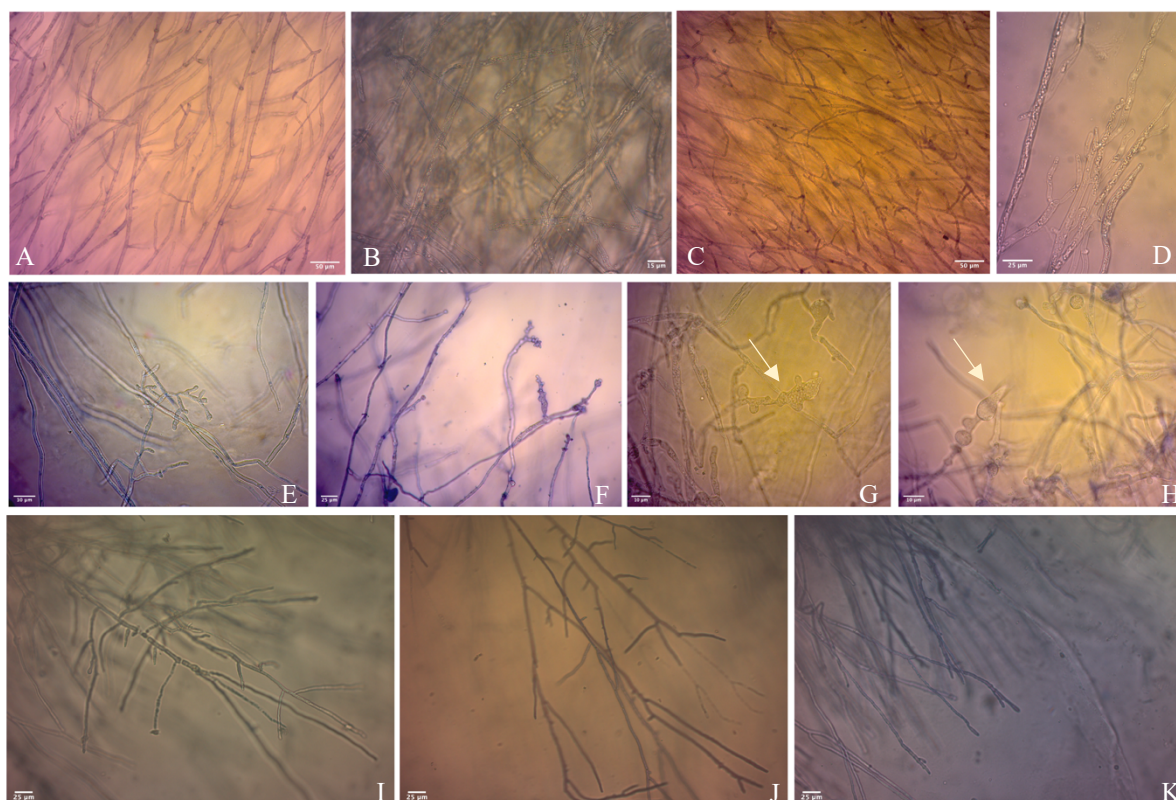


Figure 4. 4 Representative light microscopic images of morphological changes in *Phytophthora sp.* and *Fusarium oxysporum* with and without treatment with *Tulbaghia* extract. In each row are image for different pathogen species. First row: *P. cryptogea* (P189) increase in granulation (C-D). Second row: *P. nicotianae* (P306) Arrows refer to the morphologic changes in the hyphae, such as hyphal swelling (G) and the disruption of sporangia formation (H). Third row: *Fusarium oxysporum* hyphae at the edge of the colonies after incubation with *Tulbaghia* were shorter and denser with less branches (J-K) compared to the control (I) hyphae.

The figures (A, B, E, I) represents untreated hyphae for each of *Phytophthora* and fungi.

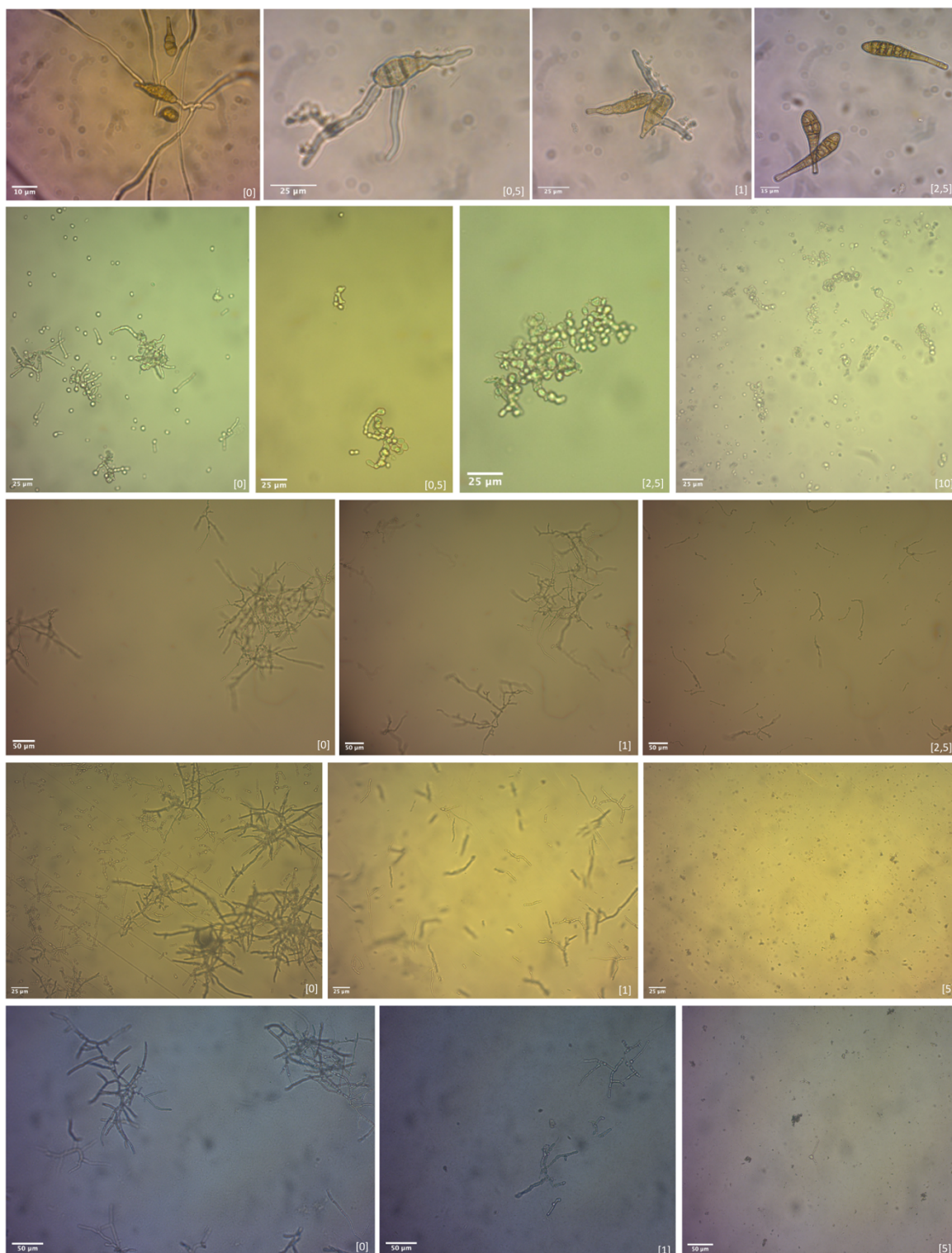


Figure 4. 5 Representative images of In vitro assessment of direct effect of *T. violacea* extract applied at different concentrations on fungal conidial germination. In each row are image for different fungal species: *Alternaria* sp (Top row), *Gnomonopsis castanea*, *Trichoderma hamatum*, *F. oxysporum*, *F. brachygibbosum* (bottom row). First image of each row represents the control with distilled water.

4.3.4 in planta test

The results of disease incidence after 5 days on treated *Pinus sylvestris* seedling with *T. violacea* extract indicate 11% incidence in seedlings treated with a fungicide alone or with *T. violacea* extract, and 8% with *T. violacea* extract alone. In contrast, in plants treated with *P. plurivora* alone, the incidence was 28%.

4.4 Discussion

During this study, it was proved that *T. violacea* aqueous extract is a potential bio-fungicide against economically important *Phytophthora* sp. such as *P. nicotianae*, *P. cryptogea*, *P. plurivora* and other pathogenic fungal disease associated with serious and safety concern diseases in food, ornamental, and forest plants.

T. violacea belongs to the genus *Allium*, and crude extracts of plants belonging to the family Alliaceae have been reported to possess antimicrobial and antifungal activities (Cawood et al., 2010). Several studies have demonstrated that sulfur compounds, such as those present in glucosinolates in certain *Brassicaceae* family (Rodríguez-Romero et al., 2021), or thiosulfinate marasmin, the main abundant compound in *Tulbaghia* extracts have antimicrobial activities (Ranglová et al., 2015; Krstin et al., 2018; Lyantagaye, 2011).

As a first experimental trial, we attempted to investigate the effect of dried leaves (60°C) aqueous extract of *T. violacea* on *P. nicotianae*. However, no significant difference was found between the treatment and control groups. Additionally, the storage temperature at (4 °C) significantly influenced the stability of the *Tulbaghia* extract's activities after long-term storage (Data not shown). To preserve their antimicrobial activities, we tried using fresh leaves aqueous extract stored at (-20°C) during this experiment. The stability and degradation of phytochemicals have been demonstrated by other studies, which have found that storage conditions or exposing the plant material to drying can dramatically affect the stability of marasmin in the *Tulbaghia* plant and subsequently its antimicrobial activities (Jäger & Stafford, 2012; Ranglová et al., 2015; Ghasemzadeh et al., 2016).

Even though the aqueous *T. violacea* extract contained different concentrations of other phytochemical compounds such as tannins, phenolics, terpenoids, glycosides, steroids, and saponins (Belewa et al., 2017; Samkeliso Takaidza et al., 2018; Pattoo et al., 2019), our study suggests that sulfur, the major volatile compound from the aerial parts of *T. violacea* (Pino et al., 2008) and more specifically marasmin, could be responsible for its fungicidal properties and anti-*Phytophthora* activities. Furthermore, the complexity of the antipathogenic phytochemical compounds in *T. violacea* (Pattoo et al., 2019) presents an advantage in limiting the pathogens' potential for developing resistance (Rodríguez-Romero et al., 2021).

This study demonstrates that extracts of *T. violacea* are effective in inhibiting the growth of *Phytophthora* sp. and different fungal pathogens by inhibiting both mycelia growth and spore germination. The study also shows that *T. violacea* extract has antifungal activity comparable to that of a broad-spectrum synthetic fungicide (Arpel WDG / Folpan 80 WDG) at a concentration of 10 mg. ml⁻¹ on most of the tested microorganisms.

In the literature, there are few studies on the interactive effects of *Tulbaghia* extract with oomycetes. Nteso and Pretorius (2006) reported the effect of methanol extracts of both aerial and below-ground parts of *Tulbaghia* on *Pythium ultimum*, with an MIC of 1 mg.ml⁻¹ or less. Moreover, Lindsey and Van Staden, (2004) reported that aqueous fresh bulb of *T. violacea*

extract exhibits low levels of activity against *P. ultimum* and other fungal pathogens such as *B. cinerea*, and *R. solani*, compared to other commercial fungicides.

This study shows for the first time the effectiveness of *Tulbaghia* extract in inhibiting mycelial growth of three different *Phytophthora* sp.. The results show an MIC value between 10 mg.ml⁻¹ and 25 mg.ml⁻¹ is required to inhibit the growth of *P. nicotianae*, *P. cryptogea* and *P. plurivora*, which is lower compared to the results reported by Lindsey and Van Staden, (2004) who reported that percentage inhibition of *P. ultimum* was about only 10% and 41% with 10 mg.ml⁻¹ and 50 mg.ml⁻¹, respectively of *T. violacea* aqueous extract. While some authors have demonstrated that leaf and bulb extracts exhibited comparable anticandidal activity (Ncube, Finnie, et al., 2011), this, suggests that the inhibition effect may be due to the fact that the quantity of active compound present in 26 g of *Tulbaghia* bulb extract was lower compared to 200 g of fresh leaves.

Directly after treatment, *Tulbaghia* extracts induce changes in the morphology of the microorganisms tested. The cellular materials in halted zoospores rapidly became granulated and dispersed into the surrounding water upon burst and lysis of their cell membranes. Motility inhibition and subsequent lysis of zoospores by different kinds of natural products have been reported previously by Islam et al., (2016). The increase in granulation observed in the present study is most likely due to enhanced reactivity between *T. violacea* extracts and fungal intracellular components, resulting in the formation of less soluble products as a kind of defensive response by the fungus to isolate toxic reactive compounds either from the plant extract or that formed after cellular reaction with the plant extract (Somai & Vuyokazi, 2011). Zoospores are one of the critical steps in the asexual disease life cycle of oomycetes, and encystment of the released zoospores seems to be a good indicator of disease reduction. Furthermore, further studies should be conducted to investigate the effect of *T. violacea* on the inhibition of sporangia production and on the weakening of the pathogen cell wall.

Tulbaghia extracts have been previously reported to have antifungal properties against economically important plant pathogenic fungi. This study found that all tested fungi were sensitive to the extract.

These results are in agreement with the findings of several authors who reported that rhizome water extracts suppressed conidia germination of *Aspergillus flavus* and *Aspergillus parasiticus* entirely at MIC value between 15 mg ml⁻¹ and 25 mg ml⁻¹ (Belewa et al., 2011; Somai et al., 2021; Somai & Vuyokazi, 2011). However, this study also found that concentrations lower than these values can also inhibit spore germination or germ tube elongation. 2.5 mg.ml⁻¹ of extract was able to suppress 100% conidia germination on all the tested fungal pathogen species. With 25 mg.ml⁻¹, *Tulbaghia* extract showed the highest mycelium growth inhibition against both *F. brachygibbosum* and *Alternaria* sp. These findings strongly support the effect of storage condition and different plant part extract on antifungal activities.

In another study using a different type of solvent, Netshiluvhi and Eloff (2019) demonstrated that acetone leaf extracts of *Tulbaghia* plants were able to suppress *Aspergillus fumigatus* growth with an MIC value of 1.67 ± 0.3 mg ml⁻¹. Similarly, Netshiluvhi and Eloff (2016) reported that crude methanol leaves and stem extracts were also effective between 0.8 mg ml⁻¹ and 2.1 mg ml⁻¹. The present study found that the antifungal activity of aqueous *Tulbaghia* extract was capable of significantly inhibiting conidia germination of *F. oxysporum*, *F. brachygibbosum*, *Alternaria* sp. and *Gnomoniopsis castanea* at 0.5 mg ml⁻¹. These results were consistent with previous studies that described the antifungal activity using organic solvent. This is similar also to the findings of Ncise et al., (2020) who showed that acetone extracts of plant roots on conidia of *F. oxysporum* f. sp. glycines strain had MIC values ranging from 0.1 mg ml⁻¹ to 1.5 mg ml⁻¹, depending on the watering regime and light intensity. Consequently,

for this current study, aqueous leaf extracts can be considered to have the same effect as acetone bulbous *Tulbaghia* roots.

Even though water is considered to have low efficacy in extracting some active compounds from plant materials (Mugao et al., 2020a), and organic solvents like ethanol are more effective in extracting Sulphur compared to water (Jäger & Stafford, 2012), this study shows that water can be used as a solvent to extract antimicrobial phytochemicals with low MIC and MFC values for some pathogens. This is consistent with other studies that have reported similar MIC value for water and ethanol extracts on both micropropagated and outdoor- grown plants, with a range of 3.125-12.5 mg/ml. (Ncube, Ngunge, et al., 2011).

Additionally, water is considered to be less toxic, Madike et al., (2020) reported that crude water extracts from leaves were less cytotoxic in comparison to crude ethanol extracts, which can inhibit the proliferation of the macrophages.

While many studies have investigated the antifungal effects of *Tulbaghia* extract on various fungal pathogens, there is limited knowledge of its potential as a biological fungicide. In comparison to commercial fungicides like carbendazim and difenoconazole, Nteso and Pretorius (2006) reported that 1mg.ml⁻¹ of below-soil *T. violacea* crude methanol extract produced the highest level of inhibition for *F. oxysporum* and *Rhizoctonia solani* and showed 100% inhibition of mycelial growth of most of the tested plant pathogens like *Botrytis cinerea*, *Sclerotium rolfsii*, *Mycosphaerella pinodes* and *Botryosphaeria dothidea*. However, Ncube et al., (2011) demonstrated that water extract at 12.5 mg ml⁻¹ had antifungicidal activity against *Candida albicans*. Furthermore, Belewa et al., (2011) investigated the potential of aqueous bulb extracts of *Tulbaghia violacea* to reduce *Aspergillus flavus* contamination in food spoilage and observed that the Minimum Fungicidal Concentration (MFC) was 20 mg.ml⁻¹. This study also shows that the fungicidal activity has been shown at 10 mg ml⁻¹ for most *Phytophthora* sp. and at 25 mg.ml⁻¹ on *P. cryptogea* and other fungal pathogens.

From the study, it was evident that different fungal species responded differently to the plant extract. *T. violacea* was found to be more effective against *G. castanea*, however *Trichoderma hamatum* was less affected by the treatments. Similarly, a variability in the response of different *Phytophthora* isolates to the extract at different concentrations was observed, even though the fungicidal action was present in all tested isolates at concentration 25 mg.ml⁻¹.

This observation can be assumed to indicate that the efficacy of the extract may depend on the sensitivity of the target pathogens to the extracts. This is in accordance with findings reported by Belewa et al., (2011) who observed a difference in the response of *A. parasiticus* and *A. flavus* conidia germination, with *A. parasiticus* showing resistant to the antifungal effects of *T. violacea* compared to *A. flavus*.

This study also aimed to evaluate the effect of certain concentrations of *T. violacea* on other surrounding microorganisms involved in biological control activities in the environment. The extracts exhibited weak antifungal activity against *T. hamatum*, suggesting that it could potentially be used in combination with *Tulbaghia* extract as a new approach to increase biocontrol efficacy against the tested microorganisms through a synergistic effect.

Due to their natural origin, *Tulbaghia* extract can be considered less toxic to human health as it has been used in traditional medicine for the treatment of various ailments. At certain concentration, it does not present any phytotoxicity to plants. This has been proven by a study conducted by Lindsey & Van Staden, (2004) who reported that a soil drench of the extract was effective at increasing plantlet survival.

However, like any other extract, care should be taken with extensive application of *T. violacea* extract to prevent any undesirable effects at higher concentrations.

Phytophthora plurivora has a very broad host range and *Pinus sylvestris* is a most common host of this species where it has been found infecting 24 stands in five European nurseries (Jung et al., 2016). thus, seedlings of *Pinus sylvestris* have been chosen for the *in-planta* test.

The results of this experiment indicated direct effects of *Tulbaghia* extract on *P. plurivora*. However, these effects decrease after 12 days. *Tulbaghia* extract has the ability to reduce the disease incidence in the seedling assays compared with the controls for a short period of time, one reason could be that extract might lose their efficacy after irrigation or it degraded quickly in soil.

A reduction of the population of *P. plurivora* in the rhizosphere soil is of key importance for forest nurseries, therefore, future studies with *Tulbaghia* treatment must be tested *in planta* in a formulated form under both controlled environments and field conditions.

4.5 Conclusion

In summary, this study suggests that *T. violacea* leaf aqueous extract has the potential to be a promising broad-spectrum biological fungicide against important soil borne, field, and storage pathogens and could be used as an efficient control method on seed borne pathogenic fungi to avoid pesticide human toxicity by synthetic fungicides.

However, further research is needed to isolate and quantify the bioactive compounds in the plant responsible for its antifungal properties, as well as to understand the mechanism of its fungicidal activity and the optimal conditions for its use.

Additionally, other species within the *Allium* genus and other plants with similar properties should also be explored for their potential as biological fungicides.

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CHAPTER 5: Lignin nanoparticles containing essential oils for controlling *Phytophthora cactorum* diseases

Summary

A review article have been published in the Forest pathology journal entitled “Lignin nanoparticles containing essential oils for controlling *Phytophthora cactorum* diseases”.

In this study, we investigated the use of thyme essential oils (EOs) both on their own and encapsulated in lignin nanoparticles as potential treatments for controlling *Phytophthora cactorum* in black poplar (*P. nigra*) seedlings. It is possible that the antimicrobial effects observed may be related to the high concentration of carvacrol.

The encapsulation process slows the evaporation of volatile essential oils, allowing for a slow and sustained release of the compounds that may inhibit the growth of *P. cactorum*.

To our knowledge, this is the first study to propose a strategy for controlling *Phytophthora* disease in the context of the European Union's bioeconomy strategy from 2012, which promotes the use of biomass residues to create environmentally friendly antimicrobial substances that operate within safe ecological limits.

Lignin nanoparticles containing essential oils for controlling *Phytophthora cactorum* diseases

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Abstract

Phytophthora cactorum is a plant pathogen affecting a wide range of hosts, causing economically damaging diseases, such as damping off and root rot, in fields and nurseries. Current plant protection strategies are often inadequate to control *Phytophthora* diseases. Hence, an attempt was made to evaluate the potential of a novel control method using lignin nanoparticles loaded with essential oil of *Thymus serpyllum* (EO-LNPs) for controlling *P. cactorum* infections on *Pinus nigra* in vitro and greenhouse conditions. Nanoparticles were characterized by Py-GCMS and the drug efficiency, and drug loading capacity were determined using HPLC. *T. serpyllum* essential oils (EOs) were characterized by gas chromatography (GC-FID and GC-MS). Under in vitro conditions, the median effective concentration (EC_{50}) values were 20.453 and 88.711 $\mu\text{g}/\text{ml}$, for EOs and EO-LNPs, respectively. Furthermore, in vivo tests revealed that thyme EOs and EO-LNPs were very effective in reducing the mortality of inoculated pine seedlings, with an inhibition rate of 93% and 100%, respectively. Results reported in this study open the possibility of using EO-LNPs for improving plant health in greenhouse settings. The design of new protection strategies relying on lignin nanoparticles conforms to the principles of the circular economy.

KEYWORDS

lignin, nanoparticles, *Phytophthora cactorum*, *Pinus nigra*, *Thymus serpyllum*; essential oils

1 | INTRODUCTION

Phytophthora is a genus containing more than 150 species (Yang et al., 2017) that are mainly plant pathogenic organisms. *Phytophthora* spp. are frequently associated with ornamental plants, especially in nurseries where *Phytophthora cactorum* is one of the most common species isolated (Jung et al., 2016). *P. cactorum* (Lebert & Cohn) (Erwin & Ribeiro, 1996) can infect an extremely large number of hosts, causing root rot, wilting and mortality as well as foliar and fruit (Erwin & Ribeiro, 1996; Jung et al., 2016).

There is a very high risk related to the introduction of *Phytophthora* species into new habitats via the movement of plants (Frankel et al., 2020; Santini et al., 2013). For instance, *P. tentaculata* was inadvertently introduced by restoration planting into new areas in California (Rooney-Latham & Blomquist, 2014; Rooney-Latham et al., 2015) while *P. ramorum* entered Europe through the trade of infested ornamental plants (Ivors et al., 2006; Mascheretti et al., 2008). Once established in a new country or region, these pathogens are very difficult to eradicate or manage. They can spread easily causing wilting and death of hosts, with further consequences of economic losses and damage to ecosystems and landscapes.

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For instance, the presence of *Pinus nigra* (European black pine) in Europe could be compromised by *Phytophthora* diseases. Black pine is a fast-growing conifer with a wide but fragmented distribution across Europe and Asia Minor, mainly found in mountain areas. It is characterized by slow regeneration after forest fires and therefore gets replaced by faster growing species. This process results in reduction of the black pine-dominated habitat in southern Europe. Thus, production of healthy seedlings in nurseries is key to preserving forest biodiversity. Unfortunately, nursery production of black pine is seriously affected by *P. cactorum* (Peterson, 1962).

The use of conventional synthetic fungicides for controlling diseases caused by *Phytophthora* spp. leads to development of resistant pathogens and has negative impacts on the environment and human health. Therefore, it is desirable to identify more eco-friendly control systems against *Phytophthora* spp.

Bio-based agrochemicals are becoming a "hot topic" regarding pest control in agriculture. However, currently the utilization of bio-based products in this field is still negligible compared to synthetic products. From this point of view, there are some substances which are considered promising, especially if delivered in the form of nanoparticles. Among the bioactive compounds which could be used as natural biocides are essential oils (EOs), which are "products obtained from a natural raw material of plant origin, by steam distillation, by mechanical processes from the epicarp of citrus fruits, or by dry distillation, after separation of the aqueous phase if any by physical processes" (ISO 9235:2013). The antimicrobial activity of these natural products has been widely studied and several biological effects (e.g., cytotoxicity, phototoxicity, mitochondrial damage) are well known (Bakkali et al., 2008; Kejløva et al., 2010). Due to the great number of constituents, EOs seem to have no specific cellular targets and could be considered as biocides with broad-spectrum activities (Antonelli et al., 2020; Bakkali et al., 2008; Isman, 2000).

In the literature, a small number of reports highlight the biocontrol activities of EOs on *Phytophthora* spp. such as *P. colocaliae* (Sameza et al., 2014), *P. capsici* (Liu et al., 2017) and *P. cactorum* (Lee et al., 2008), to name a few. EOs of *Eucalyptus globulus* inhibited growth of *P. colocaliae* (Sameza et al., 2014). Similarly, oils extracted from foliage of *Eupatorium adenophorum* significantly controlled *P. capsici* blight of pepper, through multiple mode of actions including disruption of the cell membrane system (Liu et al., 2017). A concentration of 28×10^{-3} mg/ml of several EOs, including citronellol, neral and geranial, led to approximately 100% inhibition of *P. cactorum* (Lee et al., 2008). One of the major concerns with the use of EOs in the field is the duration of action. Apart from EOs themselves, the delivery system has a crucial role as it can enhance the biocidal properties of an active principle while also regulating its release time. In recent years, lignin applied in a nanoparticle form has gained increasing interest as a drug delivery system (Alqahtani et al., 2019; Zikeli & Vinciguerra, 2020; Zikeli et al., 2018). Lignin nanoparticles are known to stabilize EOs and promote growth inhibition activity against microbes, but their efficacy has been demonstrated mainly against different bacteria (Chen et al., 2020; Freitas et al., 2020). Lignin possesses many interesting properties for industrial applications because of its biodegradability, along with antioxidant, antimicrobial

and stabilizer properties (Glasser, 2019; Hodášová et al., 2015; Zikeli et al., 2019; Zikeli & Vinciguerra, 2020). It is, therefore, a material with great potential for use as a carrier matrix in biocide activities. The consequences of using lignin in a drug delivery system are variable due to the role of hydrogen bond interactions, which depends on the quantity of aromatic and aliphatic hydroxyl groups in the specific type of lignin (Glasser, 2019). Indeed, results in this type of work are strictly correlated to the source of the lignin (hardwood, softwood, grass) and the process of extraction which determines the overall physicochemical properties of the lignin, and its cross-linking characteristics, which is necessary in biopolymer production (Glasser, 2019). Using lignin nanoparticles as a delivery system is also of great benefit in that the residues are abundant in agricultural and forest harvesting wastes. Furthermore, finding applications for lignin wastes is green chemistry, and also applies directly to circular economy principles avoiding single use in bioenergy (Colantoni et al., 2013; Delfanti et al., 2014; Paletto et al., 2019; Pieratti et al., 2019). Certainly, the potential for use of lignin wastes for high-value-added products is great and can be much more favourable than solely thermal use via combustion in terms of lower environmental impact (Lettner et al., 2020; Wang et al., 2018).

In the present study, lignin nanoparticles combined with EOs of wild thyme were evaluated for their ability to control mortality caused by *P. cactorum* in black pine (*P. nigra* J.F. Arnold, 1785) seedlings.

2 | MATERIAL AND METHODS

2.1 | Culture media and organisms

Strains of *P. cactorum* were isolated in 2019, from bleeding lesions on the collar of beech trees in Monti Cimini (Viterbo, Italy), as described by Vettraino et al. (2008), with some modifications. Briefly, tissues collected from the margin of necrotic areas were sterilized (ethanol 70%, 3 s, rinsed 3 times in distilled water, dried in a laminar flow hood) and transferred to PARBhy agar (Vettraino et al., 2005). *Phytophthora* isolates were identified initially based on colony growth patterns and morphological traits of sporangia, oogonia and antheridia. For two representative *P. cactorum* isolates, PF10 and PF11, identification was confirmed by sequencing the ITS rDNA and cox I regions, followed by a BLAST search at GenBank (Kroon et al., 2004; Vettraino et al., 2011).

2.2 | Essential oils from *Thymus serpyllum*

EOs of *T. serpyllum* were kindly provided by Flora srl, Florence, Italy. Flowering plants of *T. serpyllum*, common name wild thyme, were collected by hand on an organic farm in Turkey. EOs were extracted by hydrodistillation using a Clevenger-type apparatus and analysed by GC-MS (Shimadzu QP 2010 Plus) fitted with FID and Japan capillary column (0.32 mm i.d., length: 30 m, film thickness 0.25 μ m). The injector temperature was maintained at 280°C while the ion source

temperature was set at 230°C. A total of 0.2 µl of oil sample was injected into the column with a split ratio of 80:1. The temperature program was 60°C for 2 min, 250°C for 5 min at 10°C/min and 280°C for 15 min at 10°C/min. The peak normalization method was used to estimate the oil composition (%). Peaks were identified by comparing individual mass spectra with the NIST (National Institute of Standards and Technology) database and Wiley 229 mass spectrometry libraries. The analysis was carried out by Flora srl.

2.3 | Preparation and characterization of nanoparticle dispersion

Lignin nanoparticles loaded with essential oil (EO-LNPs) were prepared using a modified protocol from Zikeli and Vinciguerra (2020). Acidolysis lignin (AL) from beech wood was dissolved in DMSO together with EOs from wild thyme and subjected to dialysis against water. The ratio of AL to EOs in this work was highly in favour of EOs (AL:EOs = 1:3), in order to prepare a biocide delivery system (BDS) of high efficacy in the subsequent in vitro and in vivo experiments. Thus, 100 mg of AL and 300 µl of EOs were dissolved in 10 ml DMSO and, after centrifuging, filled into a SpectraPor® dialysis tube (SpectraPor 1 Dialysis Membrane Standard RC Tubing, 6–8 kDa, Spectrum Labs, USA) which was immersed in an excess of distilled water (4 L) for 1 h. The resulting EO-LNPs dispersions were stored at 4°C until further use for analysis and biocide experiments. Aliquots of the samples were freeze-dried for further analysis.

EO-LNPs were characterized by Py-GCMS after freeze-drying as described in Zikeli and Vinciguerra (2020). Drug loading efficiency and drug loading capacity were determined using HPLC and external calibration with pure EOs following formulas (1) and (2) below. Drug release was investigated using in vitro release experiments via a dialysis method as described in Zikeli and Vinciguerra (2020).

$$\text{DLE (\%)} = (\text{weight of EOs in EO-LNPs} / \text{weight of EO added initially}) \times 100 \quad (1)$$

$$\text{DLC (wt \%)} = (\text{weight of EOs in EO-LNPs} / \text{weight of EO-LNPs}) \times 100 \quad (2)$$

2.4 | Determining antifungal activity in vitro

Wild thyme EOs were evaluated for the ability to control *P. cactorum* PF10 and PF11 growth using the technique described by Tian et al. (2011). PDA (10 ml/Petri dish) was amended with various concentrations of EOs (24, 48, 96, 144, 192 and 240 µg/ml) via loaded nanoparticles (EO-LNPs) or unformulated EOs, dissolved in 0.1% DMSO. PDA supplemented with the same amount of DMSO or blank formulation (i.e., NPs without oils) used in treated cultures was used as control. Cultural media and DMSO were purchased from Merck (Milan-Italy). As control, a fungicide (Infinito®, Bayer) containing fluopicolide (62.5 g/L) + propamocarb hydrochloride (625 g/L) was used to a final concentration of 1.2 µl/ml. Five replicate cultures per treatment were used. The experiment was conducted twice. Petri dishes were inoculated with an agar plug (8 mm diameter) of each

isolate obtained from active margins of colonies grown on PDA. Cultures were incubated at 25 ± 2°C, in the dark.

After 7 days, the colony diameter was measured, and the mycelial growth inhibition (MGI) was calculated as follows:

$$\text{MGI} = [(dc - dt) / dc \times 100] :$$

where "dc" is mean colony diameter of control cultures and "dt" is mean colony diameter of treatment (Mohammadi et al., 2015).

Subsequently, median effective inhibitory concentration (EC₅₀) values for EOs and the fungicide "Infinito®" (expressed as ppm) against *Phytophthora* isolates were calculated. To test whether treatments were fungistatic or fungicidal, a disc plus pathogens was subcultured from treated cultures to fresh PDA. The effect was considered fungistatic if pathogen growth occurred at 25 ± 2°C after 10 days. The minimum inhibitory concentration values of NPs, oil and fungicide were determined as the lowest concentration of compound that inhibited *P. cactorum* growth 7 days after subculture and were expressed in µg/ml.

2.5 | Effect of the thyme essential oils on *Phytophthora cactorum* diseases in greenhouse conditions

Seeds of black pine (*P. nigra*) were disinfected (10% sodium hypochlorite for 15 min, rinsed three times in sterile distilled water and air-dried in a laminar flow cabinet) and sown in pots (2 × 2 × 3 cm), containing steam-sterilized soil (2:1 perlite/clay soil). One week after germination, *Pinus* seedlings were transplanted to new pots (15 cm diameter and 16 cm height) with different treatments. EOs (135 µg/ml) and EO-LNPs (135 µg/ml) were included in the test as well as the reference fungicide (1.2 µl/ml). A total of 12 treatments were prepared (A = untreated, B = *P. cactorum* PF10; C = *P. cactorum* PF11, D = LNPs; E = EOs; F = EO-LNPs; G = *P. cactorum* PF10 + EO-LNPs; H = *P. cactorum* PF11 + EO-LNPs; I = *P. cactorum* PF11 + EOs; L = *P. cactorum* PF10 + EOs; M = *P. cactorum* PF10 + Fungicide; N = *P. cactorum* PF11 + Fungicide). *Phytophthora* inoculum was prepared by inoculating sterilized millet seeds (Vettraino et al., 2001). Infested seeds were added to potting soil at a rate of 3% (wt/vol). All pots were maintained in a greenhouse at 23–27°C, 60%–70% relative humidity, 16 h light and 8 h darkness. Plants were flooded after 1 day for 48 h to promote sporulation.

Mortality of plants was recorded weekly throughout the duration of the experiment (3 weeks). The pathogen was reisolated from soils by baiting and confirmed morphologically.

2.6 | 6 Statistical analyses

Experiments were carried out in a completely randomized design. Measurements of mycelial inhibition were subjected to one-way ANOVA, and means comparisons were made using Duncan's multiple range tests. The effective dose for 50% (ED₅₀) inhibition was calculated using probit analysis. Mortality data were compared using

chi-square tests. Differences at $p \leq 0.05$ were considered to be significant. All statistical analysis was made using R software (R Core team, 2021).

3 | RESULTS

3.1 | Chemical composition of the essential oils

Based on the GC-MS analysis, 19 primary compounds were identified in the EOs of wild thyme. Most compounds, except for carvacrol, thymol, linalool, γ -terpinene and *p*-cymene, were present at concentrations below 1.5%. Carvacrol was the most abundant component (68.49%, Figure 1).

3.2 | Lignin nanoparticles dispersions loaded with essential oils

3.2.1 | Qualitative analysis of freeze-dried EO-LNPs using Py-GCMS

The main components of EOs from wild thyme were detected by Py-GCMS of the freeze-dried EO-LNPs dispersion. They included carvacrol and lignin pyrolysis products such as syringol, guaiacol, coniferyl alcohol or 4-vinylsyringol (Figure 2). The relative sizes of the peaks changed compared to the composition of pure EOs determined by GC-MS. In EO-LNPs, carvacrol was detected in higher amounts (74.8%) together with thymol (4.1%), while *p*-cymene, γ -terpinene and linalool were detected only in trace quantities (Figure 2, Table 1).

3.2.2 | Quantification of EO-LNPs dispersion using HPLC

EO-LNPs (6.9 mg/ml of lignin) loaded with 15.8 mg/ml of EOs from wild thyme were prepared via simultaneous entrapment that

resulted in highly loaded LNPs dispersions. The DLE and DLC values for EO-LNPs were approximately 61.9% and 69.6%, respectively.

3.2.3 | Release behaviour in vitro of EO-LNPs in vitro using dialysis and HPLC

Under the experimental conditions used here, release behaviour in vitro showed a strong retaining effect against EOs release from the EO-LNPs dispersions. In the first 24 h, much lower quantities of wild thyme EOs were released from EO-LNPs compared to the pure EOs dispersion. After 24 h, whereas almost 100% of EOs were released from the pure EOs dispersion, EO-LNPs dispersion still contained approximately 20% of EOs initially present (Figure 3, Table 2).

3.2.4 | Scanning electron microscopy (SEM)

SEM photos showed distinct particles at the nano-scale, but also chain-like agglomerates with a length of up to several micrometres (Figure 4a). At high magnification (Figure 4b–c), EO-LNPs with diameters of approximately 100 nm can be identified. However, these particles were present in irregular rounded shapes and a number of patches may be detected.

3.3 | Anti-oomycete Assay

No significant differences in virulence were detected between the *P. cactorum* PF10 and PF11 (ANOVA, $p < 0.05$). Thus, data for the two isolates were combined. The antifungal activity of thyme EOs was tested against *P. cactorum* in vitro. Both *P. cactorum* PF10 and PF11 were sensitive to the fungicide Infinito®, since neither isolate grew on PDA amended with the compounds at 1.2 $\mu\text{g/ml}$. Thyme EOs caused either partial or complete inhibition of *P. cactorum* growth. A dose-dependent increase in inhibition rate was observed

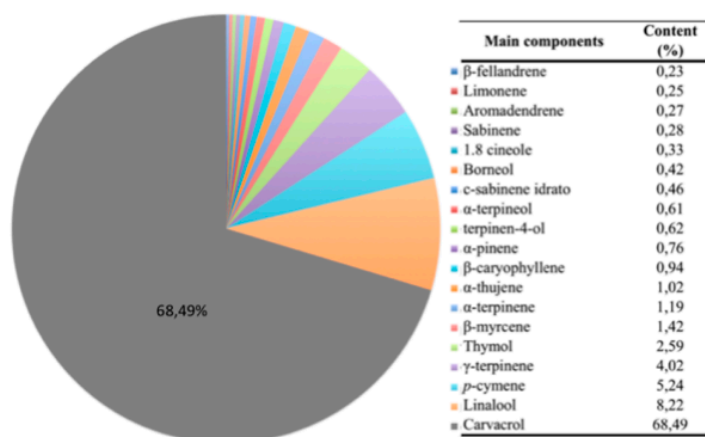
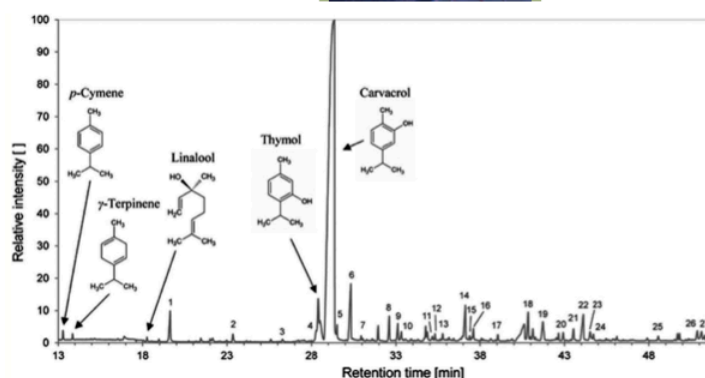


FIGURE 1 Chemical composition of *T. serpyllum* EOs obtained from gas chromatography-mass spectrometry (GC-MS) analysis

FIGURE 2 Py-GCMS chromatogram of freeze-dried lignin nanoparticles loaded with *T. serpyllum* EOs



with *P. cactorum* strains treated with EOs or EO-LNPs, with EC_{50} values of 120.453 and 88.711 $\mu\text{g}/\text{mL}$, respectively (Figure 5).

EO-LNPs inhibited pathogen growth at a minimum concentration of 48 $\mu\text{g}/\text{mL}$, lower than for EOs (120 $\mu\text{g}/\text{mL}$). There was a total (100%) inhibitory effect at 240 $\mu\text{g}/\text{mL}$ for EO-LNPs with double value for EOs (480 $\mu\text{g}/\text{mL}$). Thyme EOs caused the death of *Phytophthora* isolates in 7 days, when used at concentrations over 480 $\mu\text{g}/\text{mL}$.

The fungicides and plant EOs applied as a soil drench on *Pinus* seedlings significantly (Chi-square; $p < 0.05$) reduced mortality of the plants, compared to the nontreated plants in greenhouse assays. The fungicide Infinito® had the most significant effect against the pathogen with 100% healthy seedlings despite inoculation. In contrast, approximately 27% of inoculated plants died. Mortality of treated pine seedlings was first observed by 10 days after inoculation. In the presence of EOs, 7% of seedlings died, within 3 weeks of inoculation. In contrast, no mortality was observed in the EO-LNP treatments.

4 | DISCUSSION

In this work, we explored thyme EOs, free and encapsulated in lignin nanoparticles, as new agents for the treatment and control of *P. cactorum* in *P. nigra* seedlings. According to our understanding, and the information obtained from the reviewed literature, this is the first study to elaborate a strategy for controlling *Phytophthora* growth within the framework of EU bioeconomy strategy, 2012. The use of biomass residues to develop eco-friendly antimicrobial substances, in fact, promotes good practices to operate the bioeconomy within safe ecological limits.

Thyme EOs have proved to have high efficacy in constraining several microorganisms including *Chaetomium*, *Fusarium*, *Aspergillus* and *Stachybotrys* species (Antonelli et al., 2020) and *Phytophthora* spp. (Lu et al., 2013; Mohammadi et al., 2015; Quintanilla et al., 2002). However, to date, *T. serpyllum* has not yet been studied in relation to its biological control effect against *Phytophthora* species.

EOs of *T. serpyllum* contain several molecules, including borneol, isobutyl acetate, caryophyllene, 1,8-cineole, citral, citronellal,

citronellol, *p*-cymene, geraniol, linalool, α -pinene, γ -terpinene, α -terpineol, terpinyl acetate and thymol in relatively high concentrations, but carvacrol is the major active compound (PDR for Herbal Medicines).

Carvacrol is already known to have bactericidal and fungicidal activities against Gram-positive and Gram-negative bacteria, fungi and oomycetes (Antonelli et al., 2020; Ayres Cacciatore et al., 2020; Hyldgaard et al., 2012; Martín et al., 2010; Szczepanski & Lipski, 2014).

It is a volatile secondary metabolite insoluble in water with a single hydroxyl group ($-\text{OH}$) adjacent to the methyl group in the aromatic ring. The hydrophilic nature of the $-\text{OH}$ group and the ability of carvacrol to exchange its protons have important roles in its antimicrobial activities. It alters the structure and function of cell membranes causing an increase in permeability. Its antifungal activity, together with that of thymol, is linked to the formation of lesions in the cell membranes and a reduction in ergosterol content in fungi. Carvacrol affects physiological processes inside the cell, influences virulence factors, affects gene expression and thus reduces virulence of pathogens (Liu et al., 2019). It can alter hyphal morphology of fungi and *Phytophthora* spp., increasing cytoplasmic coagulation, vacuolations, hyphal shrivelling and protoplasm leakage (Soylu et al., 2006). Another mechanism that could be involved in the interaction between carvacrol and plant resistance is associated with phenols, known to act as activators of plant defences as highlighted on elms (Martín, Solla, Witzell, et al., 2010; 2010; Martín et al., 2012).

Data reported here confirmed that EOs of *T. serpyllum* were effective in management of *P. cactorum* diseases of *P. nigra* seedlings. We might hypothesize that the antimicrobial activities could be linked to high concentrations of carvacrol. However, a synergism with other compounds characterizing the plant extract cannot be excluded (Heghes et al., 2019). For instance, *T. serpyllum* EOs analysed in this study contained considerable amounts of *p*-cymene (5.4%) and linalool (8.22%), which cause increases in intracellular cAMP (Antonelli et al., 2020), and γ -terpinene, which is, together with *p*-cymene, a biochemical precursor of thymol and carvacrol in the phenol biosynthetic pathway (Mahboubi & Ghazian Bidgoli, 2010; Saei-Dehkordi et al., 2010).

TABLE 1 Relative abundances of pyrolysis products from freeze-dried lignin nanoparticles dispersions loaded with essential oil from wild thyme (EO-LNPs)

#	Pyrolysis product	RT [min]	EO-LNPs [%]
-	<i>p</i> -Cymene	13.23	0.3
-	γ -Terpinene	13.78	0.1
-	Linalool	18.32	0.2
1	Guaiacol	19.59	1.3
2	4-Methylguaiacol	23.32	0.4
3	4-Ethylguaiacol	26.25	0.1
4	4-Vinylguaiacol	28.24	0.6
-	Thymol	28.41	4.1
-	Carvacrol	29.15	74.8
5	Eugenol	29.29	0.6
6	Syringol	30.14	3.5
7	Isoeugenol (cis)	30.82	0.2
8	Isoeugenol (trans)	32.51	1.0
9	4-Methylsyringol	33.02	0.7
10	Vanillin	33.22	0.4
11	Homovanillin	35.11	0.2
12	4-Ethylsyringol	35.27	0.3
13	Acetoguaiacone	35.72	0.3
14	4-Vinylsyringol	37.07	1.9
15	Guaiacylacetone	37.34	0.2
16	4-Allylsyringol	37.55	0.4
17	4-Propenylsyringol (cis)	39.01	0.3
18	4-Propenylsyringol (trans)	40.76	1.4
19	Syringaldehyde	41.63	1.3
20	Homosyringaldehyde	42.87	0.4
21	Acetosyringone	43.47	0.7
22	Coniferyl alcohol (trans)	44.06	1.9
23	Coniferaldehyde	44.47	0.7
24	Syringylacetone	44.68	0.3
25	Sinapylalcohol (cis)	48.52	0.2
26	Sinapylalcohol (trans)	50.86	0.7

Note: The symbol "-" refers to compounds present as traces.

The data presented here confirmed analytical pyrolysis as potent technique for the analysis of polymeric constituents in addition to volatile compounds (Ferraz et al., 1997; Romagnoli et al., 2018; Vinciguerra et al., 2011). Data from Py-GCMS showed that, when considering the ratio of EOs to lignin, a much higher value (79.5%) in favour of EOs was evident compared to the 54% reported by Zikeli and Vinciguerra (2020). However, Zikeli and Vinciguerra (2020) already indicated that a proportion of the EOs might not be fully entrapped but present as loosely bound or free pure oil micelles around the EO-LNPs in the dispersion. Pure EO micelles might still be retained and hindered from evaporation by the EO-LNPs present in the suspension.

Based on the DLE and DLC values, EOs from *T. serpyllum* were considered suitable to be entrapped into lignin nanoparticles. The DLE values for the EO-LNPs prepared in this work were over 60% and comparable with values reported in other publications (Dai et al., 2017; Zikeli & Vinciguerra, 2020). Conversely, the DLC attained higher values than data reported in the literature (Dai et al., 2017; Zikeli & Vinciguerra, 2020), probably for an overestimated quantification due to the presence of pure EO micelles in the EO-LNPs dispersion.

EO-LNPs delayed the release of EOs into the surrounding medium within the first 48 h of the experiment. The ratio of lignin to EOs was highly in favour of EOs in the present work, but even a low quantity of lignin matrix material compared to the loaded EOs was sufficient to successfully retard the release of EOs from the aqueous dispersion of EO-LNPs. It is worth noting that the efficiency of the process is led not only by the amount but also the structure of lignin, which is a variable to be considered in further investigations (Ferraz et al., 1997). The higher concentration of EOs, strongly in favour of EOs in the presented work, resulted in unified drop-like spots of EOs visible under SEM, but probably caused by the heating effect of the SEM electron beam. Consequently, the prepared EO-LNPs with diameters around

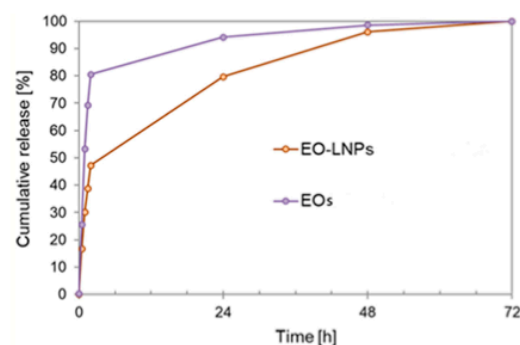


FIGURE 3 Release profile of *T. serpyllum* EOs incorporated into EO-LNPs compared with the release profile of pure *T. serpyllum* EOs dispersed in water

TABLE 2 Essential oil cumulative release of the lignin nanoparticles essential oil dispersions (EO-LNPs) and the pure essential oil dispersions (EOs) over time

Essential oil cumulative release [%]		
Time [h]	EO-LNPs	EOs
0.5	16.6	25.5
1	30.1	53.2
1.5	38.6	69.1
2	47.2	80.5
24	79.7	96.8
48	96.0	98.5
72	98.7	100.0

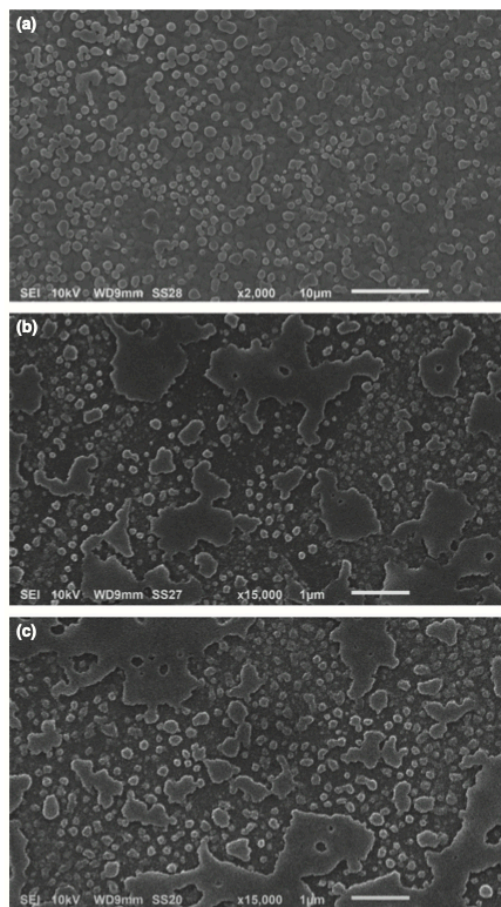


FIGURE 4 Scanning electron microscopy photos of EO-LNPs. (a): $\times 2,000$ magnification; (b) and (c): $\times 15,000$ magnification

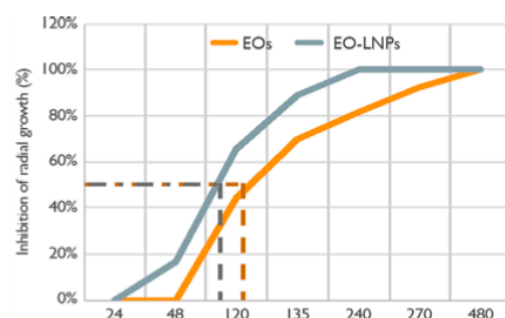


FIGURE 5 Percentage of inhibition of the radial growth of *P. cactorum* on PDA amended with EOs and EO-LNPs

100 nm, which can be clearly seen in Figure 4 at high magnification (Figure 4b–c), show more irregular shapes compared to those presented in Zikeli and Vinciguerra (2020). This irregularity is probably derived from the high concentration of EOs in the structure of the nanoparticles, particularly the high load of carvacrol molecules. Thus, the mass ratio of EOs and lignin was probably the main driving force influencing the form and shapes of EOs loaded lignin nanoparticles, when comparing the present work with that of Zikeli and Vinciguerra (2020).

This study showed that encapsulated EOs of wild thyme were more effective than pure EOs in controlling *P. cactorum* both in vitro. The encapsulation slows evaporation of volatile EOs, allowing a delayed and continuous release of the compounds that could affect *P. cactorum* growth. Treatments with EOs and EO-LNPs had no impact on growth of pine seedlings in our study. Nevertheless, phytotoxicity can be of concern when certain EOs are applied to plants or soil. Bi et al. (2012), for example, reported that oregano EOs inhibited seedling emergence of common bean (*Phaseolus vulgaris*), beet (*Beta vulgaris*), carrot (*Daucus carota*) and pepper (*Capsicum annuum*). Therefore, specific EOs should be selected for different hosts to avoid phytotoxic consequences.

In conclusion, our results indicated that the application of *T. serpyllum* EOs might be an alternative method to synthetic chemicals for integrated management strategies against diseases caused by *P. cactorum*. The formulation of biologically derived active compounds as nanoparticles increased the efficacy of the control strategy.

In addition to their biocompatibility and excellent stability, innovative lignin nanoparticles exhibited a strong capacity to carry thyme EOs with sustained release. The use of lignin in agriculture provides an example of the circular economy concept and will increase the added value chain towards sustainability instead of a simpler use for lignins in the bioenergy sector (Vinardell & Mitjans, 2017; Wang et al., 2018).

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DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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CHAPTER 6: *PHYTOPHTHORA* BIOCONTROL BY USING BACTERIAL BIOLOGICAL CONTROL AGENTS: CASE STUDY *GLUCONOBACTER KONDONII* AND *MEYEROZYMA GUILLIERMONDII*

Abstract

The bacterial antagonist *Gluconobacter kondonii* (G7) was isolated from kiwi fruit and screened against *Phytophthora cactorum*, *P. cryptogea*, and *P. cinnamomi* using dual culture on Potato Dextrose agar and Yeast Peptone Mannitol agar medium. The inhibitory effects of (G7) were evaluated in dual culture plate assays alone and in combination with the yeast *Meyerozyma guilliermondii* (Y1). The protective effect of (G7) against *P. cactorum* was also examined in *in planta* assays and the enzymatic traits and plant growth promoting effects were assessed *in vitro* and on *Pinus* seedlings.

Our results showed strong activity against *Phytophthora* pathogens, with the highest inhibition rate around 80 % with *P. cactorum* and more effective than individual applications in controlling *P. cinnamomi* with an inhibitory effect around 60%. In culture, (G7) produced indolic acetic acid (IAA) siderophore, biofilm and protease enzymes and could solubilize phosphate. (G7) also reduced disease incidence on *Pinus nigra* seedlings and promoted lateral root growth significantly ($P=0.039$) comparing to control.

However, *Gluconobacter kondonii* may cause potential on kiwi fruit surfaces after wounds caused by agricultural tools, insects' bites, and environmental factors.

6.1 Introduction

Phytopathogenic *Phytophthora* are the causal agents of some of the most destructive plant diseases (Erwin & Ribeiro, 1996). Different *Phytophthora* species present worldwide have been shown to cause high loss in forest tree production area and in conifer nurseries, among them *P. cinnamomi*, *P. cactorum* and *P. cryptogea* are considered as a serious pathogens of herbaceous and woody hosts and proved to be highly aggressive to the bark and root systems of both mature and young forests tree in temperate and mediterranean regions of Europe (T Jung et al., 2018; Pérez-Sierra et al., 2013; Vettraiño et al., 2005).

Phytophthora cactorum, for example, was found to be common in the rhizosphere of 85% of sampled trees collected from riparian poplar plantations in Serbia (Milenković et al., 2018) and was found causing Ink Disease in declining chestnut stands in Italy and Greece (Vettraiño et al., 2005). With other various *Phytophthora* species such as *P. cinnamomi*, *P. cambivora*, *P. cactorum*, *P. citricola* and *P. cryptogea*, it has shown declining and rapid mortality to walnut trees (Vettraiño et al., 2003). Furthermore, it was frequently encountered in natural oak forests in Italy with *P. cryptogea*, *P. cinnamomi* and other eight species (Vettraiño et al., 2002).

In a recent literature survey, Jung et al., (2016) demonstrated that *P. cinnamomi*, *P. cactorum*, along with *P. plurivora* and *P. cambivora* are the most common species found in fields and container stands of forest European nurseries. In Portugal and Mediterranean regions of France and Spain, *P. cinnamomi* and *P. cryptogea* with *P. cambivora* were found to prevail on oak species, whereas in nursery stands of different *Pinus* spp. the most frequent were *P. cinnamomi*, *P. cactorum*, *P. cryptogea* and *P. plurivora* (Jung et al., 2016).

Similarly, McKeever & Chastagner, (2016) found *P. cinnamomi* and *P. cambivora* to be the prominent species during a survey conducted in major U.S Christmas tree production regions. More recently, a general survey in Pennsylvania revealed 231 *Phytophthora* sp. with *P.*

cactorum, *P. cryptogea*, and *P. cinnamomi* among the most frequent isolated species from diseased forest plants (Bily et al., 2022).

Controlling *Phytophthora* plant pathogens in nurseries is usually managed intensively with regular use of fungicides and fungistatic chemicals, which pose a significant challenge in terms of durable disease control methods (Jung et al., 2016).

As a result, due to increasing fungicide resistance in many nurseries (Olson et al., 2013), and the risks these chemicals pose to human health and the environment (Capieau et al., 2004; Fang & Tsao, 1995), there is an emphasis on the need to develop alternative disease control strategies such as biological control. Recently, managing *Phytophthora* in nurseries through antagonistic organisms has proved to be a promising solution (Anith et al., 2003; M. Elliott et al., 2009) and as a result, this area has received growing interest. However, bacterial biological control from *Acetobacteraceae* family have not been well evaluated.

The genus *Gluconobacter* belongs to the family *Acetobacteraceae*, which is an aerobic acetic acid bacterium (AAB), that is phylogenetically affiliated with the major genera of *Acetobacter*, *Acidomonas*, *Ameyamaea*, *Asaia*, *Bombella*, *Commensalibacter*, *Endobacter*, *Gluconacetobacter*, *Gluconobacter*, *Granulibacter*, *Komagataeibacter*, *Kozakia*, *Neoasaia*, *Neokomagataea*, *Nguyenibacter*, *Saccharibacter*, *Swaminathania*, *Swingsia* and *Tanticharoenia* (Gomes et al., 2018). Strains of the genus *Gluconobacter* are responsible for the production of vinegar (Gomes et al., 2018), but they are different from strains of the genus *Acetobacter* in that they cannot oxidize acetic acid to carbon dioxide and water (Kadere et al., 2010).

Currently, the genus *Gluconobacter* comprises five safe species (Gomes et al., 2018; Malimas et al., 2007; Neffe-Skocińska et al., 2022) that can grow at lower pH values, with some species recognized as thermotolerant (Mamlouk & Gullo, 2013; Saichana et al., 2009). *Gluconobacter* is one of the earliest food-associated microorganisms, occurring in flowers and on intact and injured fruits, with a wide range of applications in alcoholic fermented beverages such as vinegar, beer, and apple cider vinegar (Cousin et al., 2017; Neffe-Skocińska et al., 2022; Valero et al., 2005). Additionally, it is frequently used in the industrial biotechnology, pharmaceutical, and chemical industries, where it has been used for the production of dihydroxyacetone (Liu et al., 2021), D-Gluconic acid, keto-D-gluconic acids and L-Sorbose, an intermediate in the industrial production of vitamin C (De Muynck et al., 2007; Leverentz et al., 2006; Rosenberg et al., 2014; Zhao et al., 2015). Studies of newly isolated enzymes from *Gluconobacter* are expanding the range of potential applications of this bacterium, including bioconversions and biosensors (Švitel et al., 2006).

Although, there have been few studies on the control of fungal diseases in different crops through the use of strains of the genus *Gluconobacter* (Olivera et al., 2021), their biocontrol effects on *Phytophthora* sp. have not been investigated.

This study aimed to obtain fundamental knowledge for efficiently screening and effectively using *Gluconobacter* sp. as potential biological control agents (BCAs) against common *Phytophthora* diseases in forest nurseries.

The study included the investigations of *in vitro* anti-*Phytophthora* activities against three forest pathogens (*P. cactorum*, *P. cryptogea*, and *P. cinnamomi*), an examination of the inhibitory effect against *P. cactorum* pathogens on *Pinus* sp. seedlings, characterization of their plant growth promotion abilities, and their phytotoxicity effects on plant and fruits.

6.2 Materials and Methods

6.2.1 Bacteria isolation

Gluconobacter sp. and *Meyerozyma guilliermondii* with potential anti-*Phytophthora* capacities were screened from yellow kiwi fruits variety “Soreli” in an agricultural field in Cisterna di Latina (LT), Italy (41.5889°N, 12.8296°E). Pieces of 3–5 mm in diameter, showing softening and tissue collapse, were aseptically excised from five spoiled kiwi fruits, and placed into Potato Dextrose Agar (PDA) medium. The sample were incubated at 24°C ±1°C for 48 to 72 hours. Three bacterial isolates (G2, G6, G7) and one yeast isolate (Y1) were chosen for molecular testing.

The Microbial isolates were preserved in Yeast Peptone Mannitol (YPM) with 40% glycerol at -20°C.

6.2.2 Test for bacteria optimum culture growth medium

To optimize and examine cell growth, 0.1 ml of *Gluconobacter* bacterial culture was inoculated in 5 ml of each of the following nutritional liquid media: Potato Dextrose Broth (PDB), Nutrient Broth (NB), Tryptic Soya Broth (TSB), Glucose, Yeast and Malt (GYM), Yeast Peptone Mannitol (YPM), Lysogeny Broth (LB), Yeast Extract Sucrose (SYES), and Man Rogosa and Sharpe (MRS). The experiment was carried out in a mobile, sterile condition of growth in a rotary shaker incubator (149 RPM). After 24 hours of incubation at 28°C, the microbial growth was determined by measuring the optical density at 600 nm (OD₆₀₀).

6.2.3 Molecular identification and characterization of Bacteria and Yeast strains

Genomic DNA was isolated from the *Gluconobacter* strains, and the 16S ribosomal subunit DNA (rDNA) gene extraction was performed using the genomic DNA extraction kit (NucleoSpin® Plant II, Macherey-Nagel, Germany) according to the manufacturer's instructions. PCR reactions were conducted using universal bacterial primers 8F, (5'-AGAGTTTGGATCCTGGCTCAG-3'), and 1492R, (5'-GGTTACCTTGTTACGACTT-3') (TURNER et al., 1999) and the amplification was carried out on Bio Rad (C1000 Thermal Cycler, UK) in a total reaction mixture volume of 25 µl using the Taq Master 2X Mix tubes (VWR, Milano, Italy). The PCR reaction was performed under the following conditions; an initial denaturation at 94°C for 2 min, 34 cycles of denaturation for 30 sec at 94°C, annealing for 45 sec at 58°C, extension for 30 sec at 72°C, and a final extension step at 72°C for 10 min. The presence of the predicted PCR products was confirmed by 1.5% agarose gel electrophoresis and the PCR bands were analyzed under UV light. The PCR products were purified using the Gel/PCR DNA Fragments Extraction Kit (Genaid), and purity was confirmed using a Qubit fluorometer. A volume corresponding to 12 µL of 11.2 ng/ul of PCR product was added to a 20 µL reaction mixture, containing 10 pM of 8F primer and water, and then sent for sequencing. Nearly complete rDNA sequences were obtained, and homology blast searches were performed against GenBank databases. Additionally, a whole genome sequence of *Gluconobacter* sp. was carried out using 340ng /ul of extracted DNA.

Similarly, yeast PCR amplification was carried out using: 1 µl of 10 µM universal primers each for forward (ITS1: 5'-TCC GTA GGT GAA CCT GCG G-3') and reverse (ITS4: 5'-TCC TCC GCT TAT TGA TAT GC-3') (Fujita et al., 2001), 12,5 µl of Taq Master 2X Mix tubes (VWR, Milano, Italy), approximately 1 µl of DNA template and sterile Milli-Q water to 25 µl. The PCR reaction was used to amplify ITS1-5.8SrDNA-ITS2 region using the following conditions: an initial denaturation at 95°C for 2 min, followed by 35 cycles of 1 min at 95°C, 1 min at 55°C, and 1:30 min at 72°C with a final extension of 5 min at 72°C. Amplicons (400

bp) were purified, sequenced and then searched against yeast ITS deposits within GenBank databases.

6.2.4. Mechanisms of *Phytophthora* inhibition

The dual-plate confrontation assay was used to test antagonistic effect of the isolated bacteria and yeast against plant pathogens (*Phytophthora* sp.) by measuring their ability to produce extracellular antifungal metabolites and volatile organic compounds (VOCs).

6.2.4.1. Dual culture methods with *Gluconobacter* sp.

The *Gluconobacter* sp. mycelial inhibition *in vitro* assay was quantitatively assessed by measuring *Phytophthora* radial growth on plates according to the method described by Syed-Ab-Rahman et al., (2018a), with some modifications. The dual-culture test was carried out by using a standardized bacterial suspensions 10^8 CFU·mL⁻¹ (OD_{600nm}=1.62) prepared from overnight cultures against two isolates of each of the following *Phytophthora* species: *P. cactorum*, *P. cryptogea*, and *P. cinnamomi* on two different medium, PDA, (VWR Chemicals) and Yeast peptone mannitol (YPM) consisting of: Mannitol (25 g), Peptone (3 g), Yeast extract (5 g), Agar (12 g) and distilled water (1000 ml).

The medium was inoculated with 9 droplets of 3 µL of bacterial suspensions along the boundaries equidistant from each other. A plug with a diameter of 0,6 cm of one of the six different *Phytophthora* species was placed in the center of PDA or YPM plates. Petri dish plates inoculated only with *Phytophthora* agar plug in the center served as controls. The measurements for Mycelial Growth Inhibition rate (% MGI) were calculated after incubation at 28°C for 7 days using the formula $(C-P/C \times 100)$, where C is the mycelial growth (in cm) of the pathogen in the control plate and P is the mycelial growth of the pathogen in the dual-culture. The trials were repeated twice with three replications of plates. The results of the measurements were analyzed statistically using one-way ANOVA, and further analyzed with Tukey's HSD Test using R software.

6.2.4.2. Dual culture method by mixing *Gluconobacter* sp. and Yeast

The antagonistic activity of a combination of two microbial isolates, *Gluconobacter* sp. (G7) and the yeast *Meyerozyma guilliermondii* (Y1), were tested against three phytopathogenic *Phytophthora* sp. using a dual culture assay on PDA medium. Overnight microbial cultures were grown separately at 24°C for 48 h on Yeast Peptone Mannitol Broth (YPMB) liquid medium. The cultures were then centrifuged at 7,500 rpm for 10 minutes, and the supernatant was removed. The microbial pellets were resuspended in sterile distilled water to a concentration of 10^8 CFU·mL⁻¹. An Equal proportion of the microbial suspensions were mixed and spread in two straight lines containing twelve droplets of (3 µL) at a distance of 30 mm from the center of a 90 mm Petri dish. After 1 day of incubation at 24°C, an agar plug of 6 mm diameter with growing mycelium of one of the six *Phytophthora* spp. was placed in the center. After incubation periods of 7 and 12 days at 24°C, the percentage of mycelium growth inhibition was calculated. Dual culture tests were performed in triplicate and all data collected were analyzed by ANOVA and Tukey's test (P=0.05).

6.2.4.3. *Phytophthora* inhibition assays by Bacterial Volatile Compounds (BVCs)

The ability of *Gluconobacter* sp. to produce extracellular volatile organic compounds (VOCs) that inhibit *Phytophthora* growth was tested using the double plate technique. The interaction experiments were performed using YPM medium. One plugs of mycelium 6 mm diameter was taken from the margin of the growing colonies and placed upside down in the center of a YPM plate. One hundred microliters of an overnight *Gluconobacter* bacterial suspensions of 10^7 CFU ml⁻¹ concentration were spread over the surface of another YPM Petri dish. The plates containing fungal mycelium plugs were then placed upside-down over the YPM plates inoculated with the bacteria. Each pair of plates was sealed with Parafilm® to prevent the leak of bacterial volatiles compounds and incubated at 24°C in darkness for 7 days until the fungi had grown over the control plates without bacteria. Antifungal activity was recorded as the width of the zone of growth inhibition between the control plate and the fungi and the bacteria tested. The assays were performed once with three replicates.

6.2.5. *Gluconobacter* sp. fungistatic or fungicidal effect

The specific antifungal activity of *Gluconobacter* sp. (fungistatic or fungicidal) was assessed according to a method described by De Corato et al., (2014). To determine the nature of the antifungal activity, completely inhibited *Phytophthora* mycelium discs were re-plated on the upper surface of PDA medium and qualitative observations were recorded from the 5th–10th day after incubation in the dark at 24°C. The presence of fungal growth indicated fungistatic action, while the lack of growth indicated a fungicidal effect.

6.2.6 *In vitro* screening for *Gluconobacter* sp. plant beneficial traits

6.2.6.1 Qualitative test

1-Siderophore production

The strain was assayed for the production of siderophore on Chrome Azurol S agar (CAS) using the overlay technique according to a method described by Pérez-Miranda et al., (2007) with some modifications. The medium (per liter) for the overlay was as follows: Chrome Azurol S (CAS) 60.5 mg, hexadecyltrimethyl ammonium bromide (HDTMA) 72.9 mg, Piperazine-1,4-bis (2-ethanesulfonic acid) (PIPES) 30.24 g, and 1 mM FeCl₃·6H₂O in 10 mM HCl 10 mL. Agar (1.5%, w/v) was used as the gelling agent. The CAS agar medium was applied over YPM and PDA agar plates inoculated 3 days before with the bacterial strains and incubated at 25°C for 10 days. The colonies producing yellow to orange halos were considered positive for siderophore production. *Pseudomonas fluorescens* (L126) kindly provided by Marco Minetti, DAFNE, University of Tuscia (Viterbo), Italy) was used as the positive control.

2-Biofilm production

A qualitative method for biofilm detection was done as described by Christensen et al., (1982). 100 µL of fresh culture of *Gluconobacter* sp. was inoculated in 900 µL of (YPM) medium in a 2 ml Eppendorf tube. The tubes were incubated at 24°C for 48 hours. After incubation, the tubes were decanted and washed with phosphate buffer saline (pH 7.3) and dried. The tubes were then stained with crystal violet (0.1%). Excess stain was washed with deionized water and the tubes were dried in an inverted position. The scoring for the tube method was done according to the results of distilled water as negative control and the positive strains of *Pseudomonas fluorescens*. Biofilm formation was considered positive when a visible film lined the wall and the bottom of the tube.

3-Protease production

Protease activity was determined by using 10% skimmed milk agar plate method (Brown & Foster, 1970). The assessment was done on a modified milk agar (pH 7.0) containing (per liter): 100 mL of Ultra-high temperature processing (UHT) milk, 5.0 g of Tryptone, 2.5g of Yeast extract, 1.0 g of Dextrose, and 15.0 g of agar. Inoculation was performed by spotting 4 spots of 10 µL of overnight bacterial cultures with an optical density (OD) of 1.53 on agar plates and subsequent Petri dish incubation at 28°C for 2 days. Clear zones surrounding the bacterial colonies indicated a positive reaction for protease activity.

4-Cellulase production

For a rapid and qualitative screening of cellulolytic activity, 10 µl of a *Gluconobacter* bacterial suspension, with an optical density (OD) of 0.3 were inoculated into 1L of carboxy methyl cellulose (CMC) agar media plates containing 0.5 g KH₂PO₄, 0.25 g MgSO₄, 0.25 g Carboxymethyl cellulose and 2 g gelatine for bacterial growth activity (F. Islam & Roy, 2018). The plates were then incubated at 25°C for 48 hours. The cellulolytic activity was visualized by flooding the plates with Lugol's iodine solution for 20 min. A cellulase-positive producing bacteria were identified by the appearance of a light orange to clear zone against red (CMC) agar background. The diameter of the clear zone was measured.

5-Pectinase production

The detection of pectinase-producing bacteria was carried out by inoculating 10 µl of a water-diluted bacterial suspension by spot puncture on Pectinase Screening Agar Medium (PSAM) medium according to a method described by KC et al., (2020) with some modifications. The medium was prepared with the following composition (g/L); (NH₄)₂HPO₄, 3.0; KH₂PO₄, 2.0; K₂HPO₄, 3.0; MgSO₄, 0.1, pectin, 10.0; and agar, 25.0. To enhance the growth of both *Gluconobacter* and the positive control *Pseudomonas fluorescens*, 25.0 g of Mannitol; 5.0 g of yeast extract, and 5.0 g of Peptone were added, and the pH of the media was adjusted to 4.5. The presence of characteristic clear zones upon flooding with iodine-potassium iodide solution around colonies after 3–4 days of incubation at 25°C was considered to indicate pectolytic activity.

6-Phosphate solubilization

Quantitative analysis of Phosphate-solubilizing bacteria was performed on Pikovskaya's (PKV) agar medium (Pikovskaya, 1948), consisted of g/L: yeast extract, (0.50); Dextrose, (10.00); Calcium Phosphate, (5.00); Ammonium Sulfate, (0.50); Potassium Chloride, (0.20); Magnesium Sulfate, (0.10); Manganese Sulfate, (0.0001); Ferrous Sulfate, (0.0001); and Agar, (15) dissolved in 1000 mL distilled water. Four spots of bacterial suspensions (10 µL with OD= 0.3) were pipetted onto the agar plates. After 24 hours of incubation at 25°C, a visible halo zone around the colonies was observed. The solubilization efficiency unit of the strain was calculated as the diameter of the entire visible halo zone divided by the diameter of the zone with bacteria colonies.

6.2.6.2. Quantitative test

1- Auxin production (IAA)

The production of indole 3-acetic acid (IAA) by *Gluconobacter* strains was measured using the method characterized by Bano & Musarrat, (2003). Bacteria were first cultured in 5 mL (YPM) broth medium for 2 days at 27°C, then 200 µL of the culture with an optical density (OD₆₀₀) of 2,08 (10⁹ CFU/ml) was transferred into six subsamples of 10 mL of medium each, either without or with L-tryptophan (0.1% wt/vol). The cultures were incubated under sterile conditions in a rotary shaker incubator (149 RPM) at 27 °C for 4 days. After incubation, the cultures were centrifuged at 15000 × g for 15 min. 100 µL of culture supernatant was diluted with 900 µL of water and mixed with 2 mL of Salkowski's reagent (50 mL of 35% perchloric acid and 1 mL of 0.5 M FeCl₃). The mixture was incubated in the dark for 30 min and then examined for the development of pink color, which indicates indole acetic acid production. *Pantoea* sp. was used as the positive control and was kindly provided by Prof. Maurizio Ruzzi, (DIBAF, University of Tuscia (Viterbo), Italy). The IAA concentration in culture was calculated using a calibration curve of pure curve of pure IAA (Sigma–Aldrich, Italy), as a standard, ranging from 0 to 30 µg/ml. The absorbance of the mixture was measured at 535 nm by a spectrophotometer.

2-Cellulase Production

The cellulase production ability of (G7) bacteria was assessed by a Filter-paper-degrading activity (FPA) assay. First, the bacteria were inoculated into 5 mL of preculture (YPM) broth medium. After 2 days of incubation at 27°C, 400 µL of the culture with an optical density (OD₆₀₀) of 2,08 (10⁹ CFU/ml) was transferred into 5 sub-samples containing each 20 mL of the cellulase production medium with the following composition (g/l): NaNO₃, 3; KCl, 0.5; KH₂PO₄, 1; MgSO₄ 7H₂O, 0.5; FeSO₄·7H₂O, 0.01; CaCl₂, 0.1; Yeast extract, 5; Avicel PH-101, 5; pH= 7. The flasks were incubated in a rotator shaker (180 rpm) at 27°C.

Extra cellular enzymes solution was isolated at one day intervals. *Gluconobacter* sp. culture was centrifuged twice at 15000 × g for 15 min and the supernatant liquid was stored at 20°C for future use.

Filter-paper-degrading activity (FPA) was determined by measuring the amount of reducing sugar liberated from Whatman filter paper n°1 (1 × 6 cm strip, 50 mg), using the methods described by Ghose, (1987). The reaction mixture was prepared by mixing 0.5 ml of crude enzyme solution as described before with 1ml of 0.05M Na-citrate buffer, pH 4.8 and 1 x 6 cm filter paper was added to the tested tubes. The mixtures were incubated at 50°C for 60 min. The reaction was stopped by adding 40 µL of DNS reagent. The experimental tubes were heated in boiling water at 100°C for 5min. The tubes were then cooled in an ice box and the solution was diluted with 2,5 ml of distilled water. The optical density was measured at 540 nm.

Cellulase assay were performed in three replicates. The enzyme activities were expressed in international units (IU), defined as the amount of enzyme activity releasing 1 µmol of glucose reducing-sugar equivalents per ml of the sample per min under standard conditions. (Luziatelli et al., 2014).

6.2.7. Test for Acid production

A pH meter (HI223-Hanna instruments) was used to measure of the pH value of PDB and YPM broth of *Gluconobacter* (G7) culture media. Additionally, the pH was checked on inoculated

agar plates amended with the pH indicator Bromocresol purple, which changes color to yellow when the pH is lower.

6.2.8. In planta experiment

6.2.8.1. Inoculum preparation

Phytophthora inoculum was prepared using millet grains. First, 75 ml of millet grains were autoclaved twice consecutively with 30 ml of carrot juice in a 250 ml conical flask. Carrot Agar (CA) agar plugs of one isolate of *Phytophthora sp.* were mixed with the medium. After 4 weeks, *Phytophthora* -infested millet grains were used as inoculum.

6.2.8.2. Plant Material and Experimental Conditions

Two experiments were conducted between May 2019 and March 2020 to test the biocontrol activity of *Gluconobacter* strain on plant species. The first experiment included four treatments on *Pinus nigra*, while the second experiment was conducted on two plant species: *Pinus nigra* and *Pinus sylvestris*, using two *Phytophthora* isolates.

The biocontrol activity of *Gluconobacter* strain was tested on 2–3-week-old black pine (*Pinus nigra*) seeds obtained from the National center of Biodiversity of Peri, Verona, Italy, and on scots pine (*Pinus sylvestris*) from Skogsplantor, Sweden. Seeds were stored at 4°C in a cool refrigerator. Before use, seeds were surface sterilized in 10 % sodium hypochlorite for 15 min. After two washes with sterile distilled water, seeds were immersed in a solution of 10% hydrogen peroxide for 10 min, then rinsed three times with sterile distilled water. They were then air-dried in a laminar flow cabinet and were sown at 1 cm depth of growth media containing sterilized soil prepared with a mix of 2 parts perlite and one part soil by volume in each container (volume 80 mL) (Soil Nutrient content: N 250 mg/L; P₂O₅ 300 mg/L; K₂O 900 mg/L; Mg 150 mg/L; S 450 mg/L; pH 6.4 (CaCl₂); 28% Organic matter; Grandiol GmbH, Germany). Seedlings emerged approximately after one week.

The first experiment was conducted at room temperature (20–25°C) in a plastic pot under natural light conditions. The following four treatments were used: Control (in sterile distilled water), 400 µL of *Gluconobacter sp.* with bacterial suspensions corresponding to a cell density of (10⁸ cells/ml) prepared from overnight cultures, bacterial suspension with *P. cactorum* (P69) inoculated at the same time, and *P. cactorum* alone. *Phytophthora* was prepared by adding infected millet seeds at the rate of 0.5% (v/v) in the potting mix, as described by Robin et al., (1998). Each treatment involved 20 seedlings (1 seedling for each replication), and a total of 80 container seedlings were set-up in a randomized design. After 2 weeks, the plants were removed from the soil, washed, and examined for foot and root rot symptoms, as indicated by browning. Roots without any disease symptoms were classified as healthy.

The second experiment was conducted in a growth chamber with a temperature range of 25–23°C, Humidity 70%±5, and a photoperiod of 12 hours of light and 12 hours of darkness.

Seventeen single-plant replications per treatment were arranged in a round carton pot in a completely randomized design. The treatments in the pot bioassay included: 1) Uninoculated control; 2) Volare[®] chemical fungicide as a positive control; 3) Two *Phytophthora* isolates inoculated each one with *Gluconobacter* strain; 4) Bacteria alone.

Plants were inoculated with 800 µL of bacterial suspensions (OD= 1,6) and one week later it was inoculated with *Phytophthora* pathogens. The inoculation was repeated after one month.

In both experiment, seedling growth parameters were measured including shoot and root lengths (cm), and seedling biomass (shoot and root dry mass) (mg). The shoots and roots were separated at the boundary of the uppermost part of the root. The biomass was measured after drying the separated shoots and roots at 40°C for one week.

6.2.9. Pathogenicity and Hypersensitivity test

6.2.9.1. Pathogenicity test on kiwi fruits

To check the pectinolytic activities of the isolated bacteria on postharvest fruits, pathogenicity tests were performed using green kiwi fruits.

The surface of the working area was disinfected with 70% ethanol and a bunsen burner was set up to maintain an aseptic environment. Fresh kiwi fruits were surface sterilized with 70% ethanol and left to dry under a hood chamber.

For inoculation, bacteria were first incubated on (YPM) medium at 25°C for 2 days. Then artificial wounds approximately 2 mm deep were aseptically made using a pipette, and ten microliters of bacterial water suspension containing 10^9 CFU/ml was inoculated to healthy fruits. An equal volume of sterile distilled water was used onto the control samples. The inoculated fruits were placed in sterile beakers, then wrapped using aluminum foil and incubated at 27°C for 48–96 hours. This test was repeated three times, The symptom development was observed daily.

6.2.9.2. Hypersensitivity Response (HR) on tobacco leaves

The *Gluconobacter* isolate was tested for its ability to induce a hypersensitive reaction (HR) on tobacco leaves (*Nicotiana tabacum*). Plants were grown for 8 weeks in a growth chamber with a controlled temperature at $25^\circ\text{C} \pm 2$ and relative humidity of $70\% \pm 5$.

An aqueous bacterial suspension with an OD= 0.3 from a 72-hr. prepared culture was infiltrated into the mesophyll using a hypodermic syringe against the bottom of tobacco leaf. Water was used as a negative control. The development of HR was monitored within 24 and 48 hours post- infiltration (hpi).

6.3 Results

6.3.1 Morphological and Molecular Identification of *Gluconobacter* Bacteria

After purification of growing colonies isolated from rotted kiwi fruits and many subcultures on PDA medium supplemented with or without streptomycin, phenotypic identification using stereo binocular microscope and optical microscope revealed two different morphotypes.

One microbial isolate with medium-size white colonies was identified as yeast (Y1) and the other as bacterial strain (G7).

The isolate strain (G7) cells were rod-shaped and it was a Gram-negative bacteria with the appearance of a viscous nature for KOH string test (Kandi, 2015). After 2-3 days incubation subculture on PDA medium at 25°C it revealed an entire colony with beige-creamy color on the border and pinkish in the center, approximately 1-2 mm round size with raised elevation over the medium as shown in (Figure 6.1).

However, on YPM agar medium the growth was noted after one days of incubation and entire pink color colonies with bigger size were common in all plates.

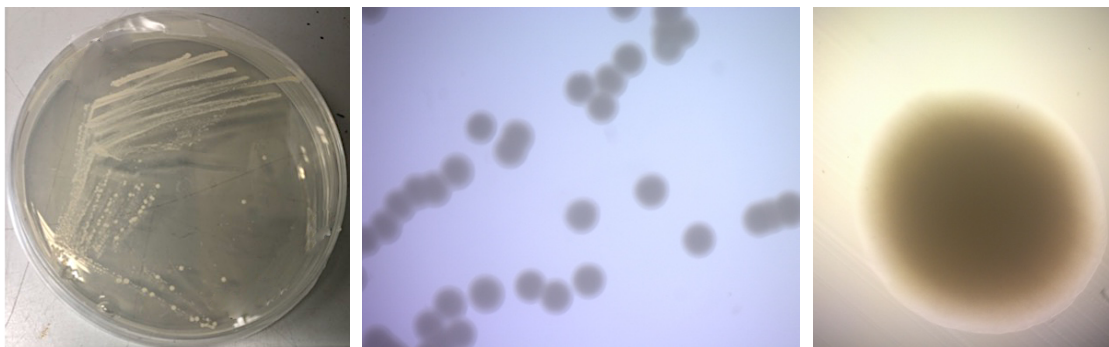


Figure 6. 1: Colony patterns of *Gluconobacter* sp. grown on PDA agar medium. Colony and cell morphology of the isolates were studied using stereo binocular microscope (Olympus, Japan).

After 24 hr. incubation, the result of (G7) growth on different medium measured by optical density (OD_{600}) was as follows:

Potato Dextrose Broth (PDB): 0,68; Nutrient Broth (NB): 0,054; Tryptic Soya Broth (TSB):0,654; Glucose, Yeast and Malt (GYM): 0,121; Yeast Peptone Mannitol (YPM): 1,25; Lysogeny Broth (LB): 0,045; Yeast Extract Sucrose (SYES): 0,0115; Man Rogosa and Sharpe (MRS): 0,59.

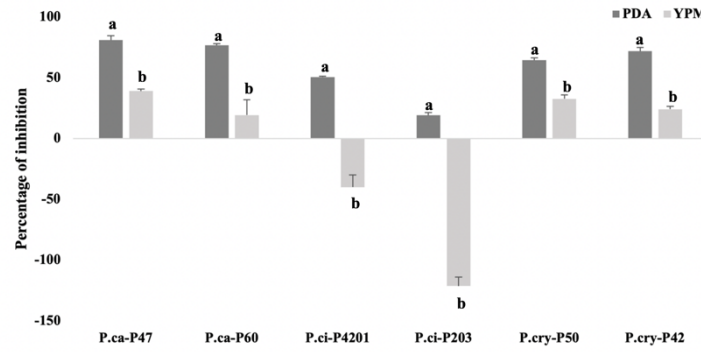
The maximum bacterial growth was noted with YPM broth followed by PDB and TSB as these mediums contain carbon and nitrogen sources that enhance bacterial growth.

Moreover, the amplification of the 16S rRNA and ITS gene sequencing confirmed that the antagonistic bacteria and yeast belonged to *Gluconobacter* sp. (G7) and *Meyerozyma guilliermondii* (Y1) respectively. However, whole genome sequencing facilitates species identification and revealed that the bacterial isolate was most closely related to *Gluconobacter kondonii* strain.

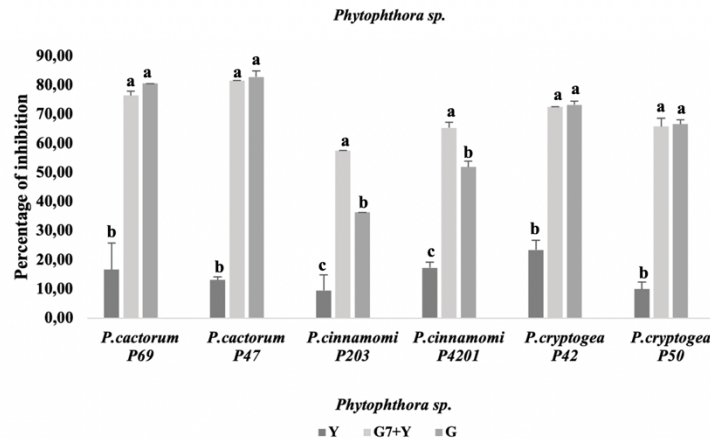
6.3.2. Anti-*Phytophthora* activities of *Gluconobacter* and *Meyerozyma* isolates

In our study, the mycelial growth of all *Phytophthora* sp. was inhibited by the diffusible compounds produced by *Gluconobacter* (G7) on PDA medium (Figure 6.2, A) and not by the production of volatile antifungal compounds. The inhibition rate was more significant against *P. cactorum* and *P. cryptogea*. The highest percentage of inhibition was presented in the trial with *P. cactorum*, with an average of 80.8% and 76.6% for both isolates.

A



B



C

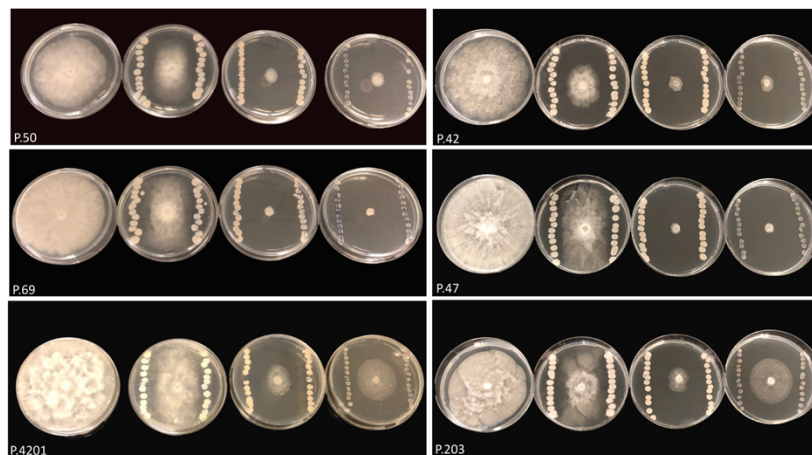


Figure 6. 2 Growth inhibition in *in vitro* dual cultures of *Gluconobacter kondonii* (G7) and *Meyerozyma guilliermondii* (Y1) against three *Phytophthora* pathogens (*P. cryptogea*, (P 50, P42); *P. cactorum*, (P. 69, P47); *P. cinnamomi*, (P. 4201, P 203)) shown in figure (A, B) and pictures (C): A) Percentage of *Phytophthora* mycelium growth inhibition on two different medium PDA and YPM; B) Percentage of *Phytophthora* mycelium growth inhibition with the combination of antagonisms and individual yeast and bacterial isolate applications C) A dual culture figure representing the inhibition of microbial antagonisms (strait dot line) on three *Phytophthora* sp. with the following treatments; From left to right: Control with *Phytophthora* alone, with yeast *Meyerozyma guilliermondii* alone, with a combination of *Meyerozyma guilliermondii* and *Gluconobacter kondonii* and bacterial isolate *Gluconobacter kondoni* alone. Calculated mean values are from three replicates. Vertical bars represent standard deviation and different letters indicate significant differences according to Tukey's test ($p < 0.05$).

In the case of *P. cinnamomi*, the average inhibition percentage was 50.5% and 19% on PDA. However, on YPM medium a different growth result was observed with an increase in mycelium growth for both isolates.

In addition, a fungicidal effect has been noticed with *P. cactorum* with completely inhibition of *Phytophthora* growth after re-culturing onto a fresh PDA plate (data unpublished).

Moreover, in an *in vitro* test after 12 days of observation, the combinations of *Gluconobacter* (G7) with the yeast isolate *Meyerozyma guilliermondii* (Y1) showed a strong anti-*Phytophthora* activity against the three *Phytophthora* sp. than with each microbial antagonism alone. The level of antagonism exhibited on dual plate assay against three *Phytophthora* pathogens is summarized in (Figure 6.2; B, C).

Yeast alone inhibited the growth of all *Phytophthora* species with a range of 10 to 25%. A similar inhibition rate was observed with a combination of yeast and bacteria or with bacteria alone on both pathogens isolates of *P. cactorum* and *P. cryptogea*. However, the combination of two microorganisms showed even greater inhibitory effects, particularly against *P. cinnamomi*, with an inhibitory effect of around 60% for both isolates.

Additional tests have been conducted on dual agar plates by inoculating *Gluconobacter* (G7) with two *Trichoderma* species that are considered as fungal biological control agents (Santos et al., 2020).

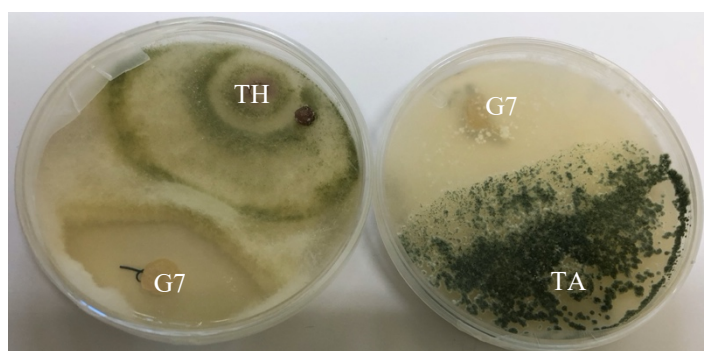


Figure 6. 3 Dual plate assay of bacterial isolate (G7) *Gluconobacter kondonii* tested on two *Trichoderma* sp.; (TA): *Trichoderma hamatum* and (TH): *Trichoderma harzianum* after 15 days.

Interestingly, *Gluconobacter* did not fully inhibit *Trichoderma* growth and a complete growth for both species was observed on all plates (Figure 6.3), indicating that *Gluconobacter* compounds are not highly relevant for fungal inhibition.

6.3.3. Plant Growth Promoting Traits

Screening for direct and indirect traits commonly associated with plant growth promoting bacteria (PGPB) including siderophore production, biofilm formation and auxin biosynthesis, as well as enzymes production were assessed according to (Syed-Ab-Rahman et al., 2018a) with some modifications. The tests revealed that *Gluconobacter* strain (G7) produces IAA, siderophore, protease, and biofilm and can solubilize phosphorus, but was negative for pectinase and cellulase production in both quantitative and qualitative analyses.

1-Siderophore Production

The qualitative methodology for siderophore was examined by using the CAS medium. Since the growth of *Gluconobacter* sp. is inhibited with the traditional CAS method due to the lack of carbohydrate source, the “Overlay O CAS” method was the most appropriate.

The overlay is spread after the microorganisms have been grown for 3 days on YPM and PDA plates, positive siderophores production were confirmed by the development of yellow/orange halos surrounding the colonies. With the positive strain *Pseudomonas fluorescence*, a change in color was observed after a few hours, indicating a strong secretion of a siderophores that can remove iron from CAS dye. However, the *Gluconobacter* isolate showed the production of siderophores 4 days after inoculation on CAS agar medium overlayed on YPM inoculated plates with the appearance of a reddish zone surrounding the inoculum on PDA plates, as observed in the CAS assay (Figure 6.4).

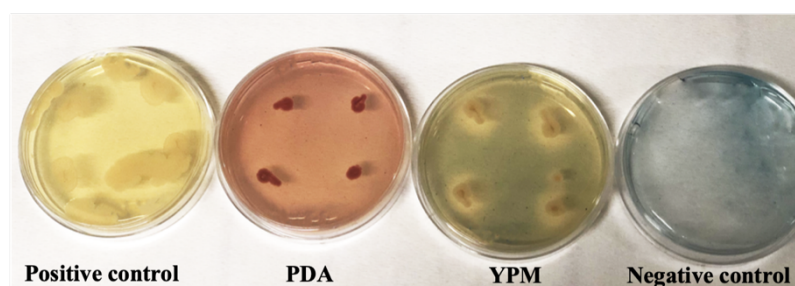


Figure 6. 4 Siderophore production by *Gluconobacter* isolate on two different media: PDA and YPM and by *Pseudomonas fluorescence* the positive control.

2-IAA Production

In *in vitro* test, a progression of pink color in the presence or absence of tryptophan in the YPM culture broth showed the production of IAA. The maximum production by *Gluconobacter* sp. was recorded one day after culture in a range of 81 $\mu\text{g/mL}$ to 15 $\mu\text{g/mL}$ after four days of inoculation in a media supplemented with l-tryptophan. Half of the quantity of IAA was also produced even without additional IAA precursor tryptophan, indicating the ability of

Gluconobacter sp. as strong IAA producer. The presence of tryptophan in the medium substantially enhanced the IAA production, as shown in (Figure 6.5).

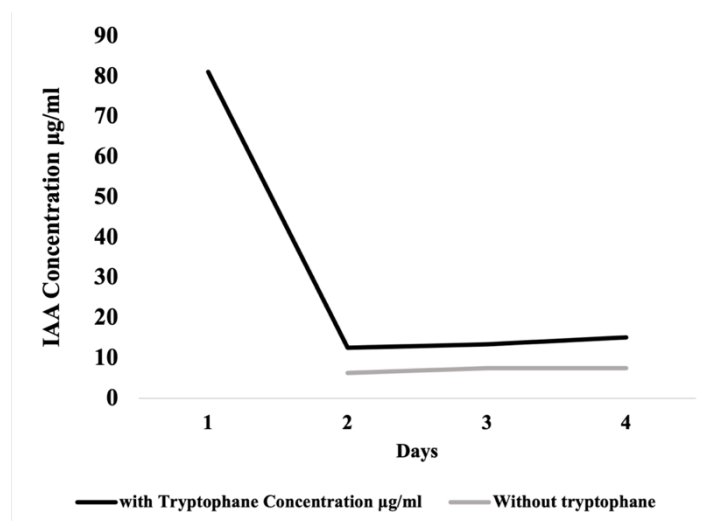


Figure 6. 5 Indole acetic acid (IAA) produced by *Gluconobacter* isolate (G7) in presence and without of L-tryptophan in the growth medium.

3-Biofilm Production

Biofilm production results showed that *Gluconobacter* (G7) inoculated in a static YPM broth medium have the ability to form biofilm at the air–liquid (A–L) interface. In contrast the positive control *Pseudomonas fluorescence* (L126) produce biofilms in abundance on the inner surface of plastic tubes compared to the negative control (Figure 6.6).

4-Phosphate Solubilization.

Gluconobacter (G7) was found to solubilize phosphate, producing distinct halos zones (clear zone) with a diameter of 1.73 cm around the colony on the plate (Figure 6.7).

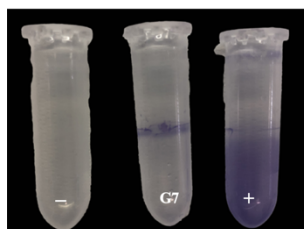


Figure 6. 6 Biofilm production assay of *Gluconobacter* isolate (G7) using 2 ml tubes after 24 hr. growth.
(+) Represent positive control with *Pseudomonas fluorescence* (L126).
(-) Represent negative control with water.

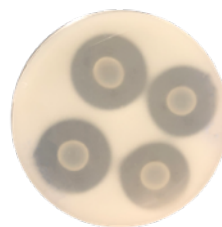
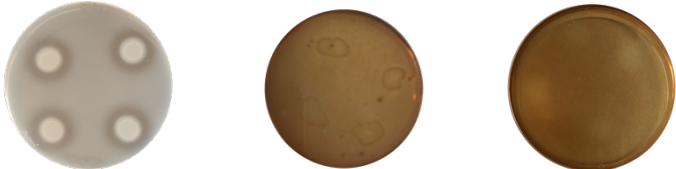


Figure 6. 7 Phosphate solubilization of *Gluconobacter* (G7).
Clear zone; A (cm)= 1.73; Colony zone; B (cm)= 0.7; Ratio; A/B= 2.47

5-Cell Wall-Degrading Enzyme Activities

Gluconobacter (G7) has been tested for its ability to produce cell wall-degrading enzymes. A positive reaction is indicated by a clear halo surrounding the bacterial colonies grown on protease medium. However, no clear halo zones were observed on pectinase and cellulase medium, indicating that the (G7) bacterial strain synthesized only protease enzyme and does not produce extracellular cellulases or pectinase enzymes.



Test	Protease	Pectinase	Cellulase
Clear zone; A (cm)	2,12	0,88	0,71
Colony; B (cm)	1,27	0,88	0,61
Ratio; A/B	1,67	1	1,16

Table 6. 1 Cell wall degrading enzyme of *Gluconobacter* (G7) in plate assays.

The colony diameter, halo zone diameter, and solubilization ratio of the isolates are presented in (Table 6.1).

6.3.4 Test for acid production

The pH of the agar medium was monitored after 7 days of inoculation with *Gluconobacter* (G7) bacteria. The pH value on PDB medium decreased from 5.37 in control to 3.63 in the inoculated medium, while on YPM broth there was a slight reduction from 6.71 to 5.52. Additionally, a clear yellow color surrounding the bacteria inoculation on the agar plate (Figure 5.8) indicates acid production by *Gluconobacter* bacteria.

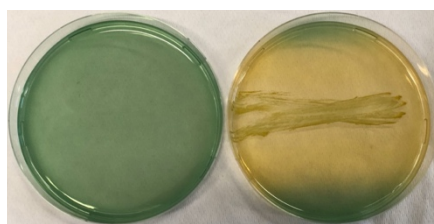


Figure 5. 8 *Gluconobacter* (G7) producing acid after 2 days inoculation on WL Nutrient Agar (Medium) containing Bromocresol green as a pH indicator. The yellow color indicating a pH below 3.8.

5.3.5 *in planta* assay of *Gluconobacter* strain on *Pinus* seedlings

The efficiency of the *Gluconobacter* strain in protecting and enhancing *Pinus* seedlings growth was evaluated with two different *in planta* experiments; the first trial was conducted on *Pinus nigra* seedlings.

After 2 weeks inoculation with *P. cactorum* (P69), the seedlings showed variable growth. Compared to the control, the highest root and shoot length of *Pinus nigra* seedlings were recorded with *Gluconobacter* bacterial treatment, 6,2 cm, and 7,8 cm respectively. However, no increases in leaf length were observed among all treatments, and the only significant difference was recorded between *Gluconobacter* treatments and *P. cactorum* inoculation (Figure 6.9, A).

Additionally, the maximum in-plant dry weight of 23 mg was recorded only with bacterial inoculation (Figure 6.9, B).

Interestingly, as compared to water-treated controls, a significant increase in the number of lateral roots formation per plant was recorded with bacterial inoculation. 85% of seedlings inoculated with *Gluconobacter sp.* formed lateral roots, with an average of 2,5 of roots per plants (Figure 6.9, D), while 45% of non-inoculated seedlings were able to do so. The combined treatment of the oomycete and bacterium allowed 45% of seedlings to produce lateral roots.

Moreover, the treatment significantly protected the seedlings from *P. cactorum* root rot in black pine. Seedlings inoculated with *P. cactorum* displayed 65% mortality, while un-inoculated controls showed 5%. However, when seedlings were treated with *Gluconobacter*, there were no seedling deaths. When *Gluconobacter* was used as a treatment against *Phytophthora* root rot, the mortality dropped from 65% to 35%.

Furthermore, the inoculation of plants with *Gluconobacter* and *P. cactorum* significantly reduced root rot by 41 % compared with the non-inoculated control in plants infected with *P. cactorum*. (Figure 6.9, c).

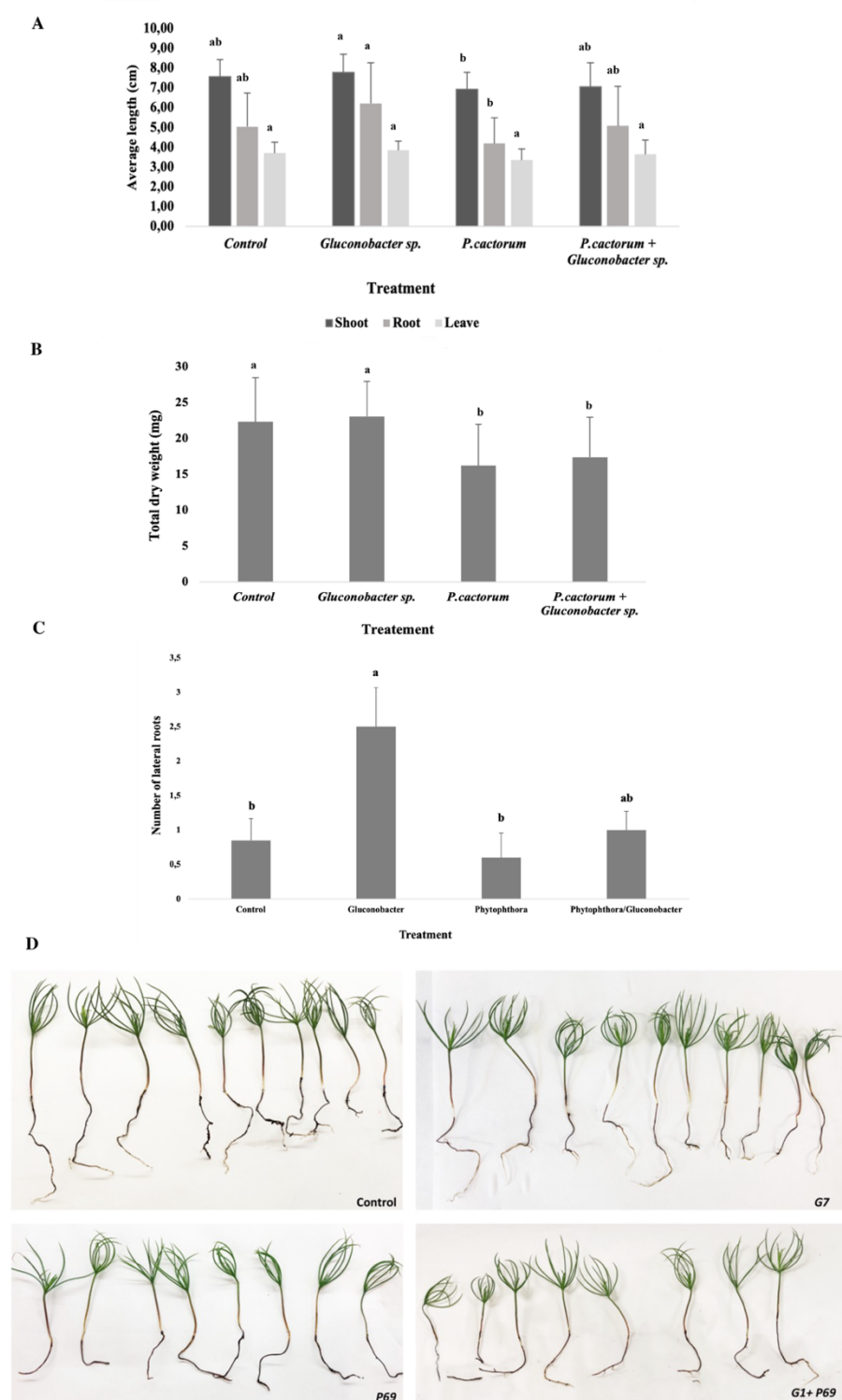


Figure 6. 9 Effect of bacterial *Gluconobacter* (G7) inoculation on *Pinus nigra* shoot, root and leaves length (A), plant dry weight (B) and mean number of lateral roots formation per seedlings (D) and a figure for representative samples of treated *Pinus nigra* seedlings after 14 days of growth (C). Values are mean $\pm$ SE (n = 20). Different letters indicate significant differences at $P < 0.05$ (ANOVA and Tukey's test for length and dry weight and a Kruskal test for the lateral root number $P = 0.039$).

Furthermore, concerning the second *in planta* test, the outbreak of the global Covid-19 pandemic had a significant impact on completing the test of the biological control agent *Gluconobacter* (G7) on the two different *Pinus* species inoculated with two isolates of *P. cactorum*. The results of this experiment did not show any significant difference between treatments, as a high mortality of plants was observed due to the lack of irrigation caused by the lockdowns.

6.3.6 Test for Phytopathogenic and Hypersensitivity Response (HR) of *Gluconobacter*

Pathogenicity tests of *Gluconobacter* sp. on fresh green kiwi fruit were conducted to complete Koch's postulates and to determine if the isolated bacteria are the causal agent of fruit rot.

The results showed a water-soaked lesions, brown discoloration, and decay symptoms with a fermentation scent appeared on inoculated fruit in 4-7 days after incubation with 10^9 CFU/ml bacterial suspension at 28°C (Figure 6.10; A2), whereas the control fruit remained asymptomatic (Figure 6.10; A1).

However, the spraying method failed to incite symptoms, indicating that this isolate needs wounds to infect kiwi fruit.



Figure 6. 10 Hypersensitivity reaction on kiwi fruit (A2) and tobacco leaves (B) after *Gluconobacter* (G7) inoculation. Control inoculated with water didn't show any symptoms (A1).

Moreover, the potential antagonist *Gluconobacter* bacteria induced HR by producing necrosis on tobacco old leaves after 1 month inoculation (Figure 6.10; B), however, on the negative control, it did not show any virulence symptoms. These tests confirm that *Gluconobacter* could present a risk for postharvest kiwi fruits and plants.

6.4 Discussion

In the present study, *Gluconobacter kondonii* has been studied as a potential antagonist against common forest *Phytophthora* species.

The isolates showed the best growth on Yeast peptone mannitol agar, followed by Potato Dextrose Broth and Tryptic Soya Broth. As this genus of *Gluconobacter* is part of the acetic acid bacteria (AAB) group, which is known for its high oxidative ability against sugars, mainly glucose, but also arabinose, fructose, galactose, mannose, ribose, sorbose and xylose (Kerstens et al., 2006), and the species *G. kondonii* is well known for its growth on mannitol agar (Malimas et al., 2007).

The *Gluconobacter* (G7) strain exhibited potent inhibitory effects towards three *Phytophthora* sp.: *P. cinnamomi*, *P. cactorum* and *P. cryptogea*. The inhibitory effects in the *in vitro* dual

culture assay test on *Phytophthora* mycelial growth tested on PDA showed a strong inhibition rather than on the YPM plate. The response observed suggested that anti-*Phytophthora* activities of bacterial antagonism might be linked to the decreased of medium pH due to acidification (Guzzon et al., 2014) or it may involve the production of secondary metabolites from the PDA medium that are different from those of the YPM. Several previous studies, have reported the influence of the type of culture medium on the activity and the efficacy of BCAs (Colombo et al., 2019; Peigham-Ashnaei et al., 2008). Moreover, the low pH has been confirmed on agar and Potato Dextrose broth medium, according to Kaur et al., (2006), gluconic acid is considered as significant antifungal agent produced by *Pseudomonas species* in biocontrol of take-all on wheat roots and it will be considered as a major antagonistic mechanism involved in the biocontrol activity of *Gluconobacter* bacteria.

In fact, low pH is considered not suitable for *P. cinnamomi* growth and it is rare for this species to be detected in sites characterized by soil pH below 5.4 (Vettraino et al., 2005). However, in this study, the acidity of the PDA agar medium caused by *Gluconobacter* was not an inhibiting factor for the tested *P. cinnamomi* isolates.

Moreover, since it is common to find some AAB coexisting with yeasts (Drysdale & Fleet, 1988; Jakob et al., 2019), a mixture culture of *Gluconobacter* bacteria (G7) with the yeast *Meyerozyma guilliermondii* (Y1), both isolated from kiwi fruit, was tested on PDA dual plate assay. Data showed a strong *Phytophthora* inhibition growth, especially with *P. cinnamomi* with an increase of 10 to 20% inhibition rate compared to bacterial antagonism alone. This is consistent with the finding of a previous study which reported the synergetic effect of both biocontrol agents, *Hanseniaspora osmophila* yeast and *Gluconobacter cerinus*, where separately and together they inhibited *in vitro* mycelial growth of *Botrytis cinerea* and *Penicillium expansum*, the main responsible of Gray and summer bunch rot diseases of table grapes (Olivera et al., 2021).

Additionally, several authors have evaluated the effect of *Meyerozyma guilliermondii* as a potential biocontrol agent. Huang et al., (2021) reported the biocontrol efficiency on Apple ring rot, caused by *Botryosphaeria dothidea* *in vitro* and *in planta*. Similarly, *Meyerozyma* biocontrol yeast was able to reduce the decay incidence and lesion diameter against postharvest gray mold in apple fruit (Sun et al., 2021) and to control blue mold decay in pears (Han et al., 2021) when combined with melatonin. As previously reported, yeasts are promising BCAs (Olivera et al., 2021). However, from this study *Meyerozyma guilliermondii* alone did not show an efficacy inhibition rate in comparing with its combination with *Gluconobacter* bacteria, thus a deeper understanding of the action mechanism is required to develop the appropriate biological control methods.

During this study, it was found that *G. kondonii* can be considered a beneficial bacterium with antifungal activities against several *Phytophthora sp.* and with various plant growth promoting (PGP) traits. One possible explanation for this could be related to *Gluconobacter*'s close association with plants. It has been reported as a phyllosphere inhabitants (Miura et al., 2019) and a predominant bacterial endophyte colonizing plants (Kandel et al., 2017; Karthikeyan & Rajeswari, 2019). This mechanism allows bacteria are be considered as a good inoculant candidates and can establish a successful stimulation of plant growth (Ansari et al., 2016; de Souza et al., 2015).

The responses observed in the *in vitro* tests suggested that the mode of action of *Gluconobacter* BCA against *Phytophthora* pathogens also involves the production of a complex of hydrolytic

enzymes and other antimicrobial metabolites that have various plant growth promoting (PGP) traits.

For example, the change in Chromeazurool agar color indicates that *Gluconobacter* was positive in siderophore production. The presence of siderophores, which have high specificity for chelating or binding the insoluble (Fe^{3+}) from the environment to the cell, can enhance plant growth by improving plant nutrition. Additionally, from a competition point of view, it contributes to plant protection by making iron unavailable to the phytopathogens through the sequestration of Fe from the environment (de Souza et al., 2015). One possible explanation for the presence of red color on PDA medium is that these organisms could produce more than one type of siderophore (both hydroxamate and catechol) (Pérez-Miranda et al., 2007). Furthermore, the type of bacteria inoculated medium and the position of CAS overlay layer medium (The first layer is a CAS medium or a bacterial growth medium) could change in aerobic and anaerobic conditions of bacterial growth and subsequently affect the type and the timing of siderophore production (unpublish data).

Another important growth-promoting trait of *Gluconobacter* bacteria is their ability to solubilize phosphate, which might be due to the direct oxidation of glucose to gluconic acid by these bacteria (Velizarov & Beschkov, 1994). This process has been reported to enhance the solubilization of mineral phosphate by the potential to lower pH in the environment (Alori et al., 2017; Babu-Khan et al., 1995). The ability of *Gluconobacter* strain to solubilize phosphate could promote plant growth by improving agricultural productivity by increasing the availability of nutrients in the soil in the form of soluble phosphates that can be absorbed directly by plants (Syed-Ab-Rahman et al., 2018b).

The detection of IAA production in *G. kondonii* suggests its potential as a plant growth-promoting bacteria. The detection of IAA production in several plant growth-promoting rhizobacterium such as *Bacillus* strains has shown they produce between 59 and 86 $\mu\text{g ml}^{-1}$ of IAA.

B. subtilis strain (330-2) has been reported to produce a significant amount of 24.13 $\mu\text{g ml}^{-1}$ of IAA (Ahmad et al., 2017; Ahmed & Hasnain, 2010).

However, from this study, the amount produced by *G. kondonii* seems to be higher than other *Gluconobacter* species and our result is in agreement with a previous study where *Gluconobacter* sp. had shown to produce 22 μM (3.9 $\mu\text{g/mL}$) of IAA (Barazani & Friedman, 1999). In *in planta* test, *Gluconobacter* increased the number of adventitious roots, which might be related to its production of exogenous auxin that has been reported to inhibit primary root elongation and promote the formation of lateral roots (Alarcón et al., 2019). This benefits the plant by increasing the ability to absorb a large volume of nutrients and water after root loses caused by *Phytophthora* diseases (El-Tarabily, 2008).

Plant growth-promoting bacteria are thought to form biofilms on roots as a beneficial feature for effective bacterial attachment and root colonization (Timmusk et al., 2005). Biofilm can provide various advantages that help increased resistance to certain environmental stresses (Danhorn & Fuqua, 2007) and it can affords protection from various pathogens causing diseases (Beauregard et al., 2013; Karthikeyan & Rajeswari, 2019). The present *Gluconobacter* strain slightly formed biofilm at the air-liquid (A-L) interface when tested in an Eppendorf tube after 24 h of incubation on YPM broth. Our results are consistent with a previous study where Jakob et al., (2019) demonstrate the formation of biofilm by *Gluconobacter* sp. by metabolizing sucrose via polyfructose building levansucrases (LS).

Protease is considered as a principal antifungal agent by hydrolyzing the peptide bonds in the cell wall of phytopathogenic fungi, causing cell death. Several studies have demonstrated the biocontrol potential of antifungal protease produced by a plant growth-promoting bacterium, such as *Bacillus amyloliquefaciens*, *B. licheniformis*, and other *Bacillus* species (Harman et al., 2004; Ji et al., 2020; Tseng et al., 2008). Notably, *Gluconobacter* strain secreted a protease hydrolytic enzyme, it was detected preliminarily as a formation of a clear zone around the colony on skim milk agar medium after 48 hr. incubation, possibly is responsible for degrading the contents of *Phytophthora* cell wall.

The *Gluconobacter* strain was unable to produce pectinase, which is consistent with the findings of Huber Cristina, (2016) who stated that acetic acid bacteria do not produce pectinolytic enzymes. Additionally, the strain was unable to produce cellulase enzyme as determined by qualitative and quantitative standard filter paper assay using Whatman N° 1 filter paper as the sole carbon source. There is limited research on cellulase production by *Gluconobacter* sp. and others acetic acid bacteria. Further studies are needed to confirm this result, as Liang et al., (2014) found that different carbon and nitrogen sources can affect cellulase production in microorganisms.

Additionally, in *in planta* test, the application of *Gluconobacter* demonstrated its potential as a biocontrol agent by reducing the severity of *Phytophthora* disease and suppressing *P. cactorum* root rot symptoms on inoculated *P. nigra* seedlings. However, further research is needed to determine its effectiveness against other *Phytophthora* pathogens.

The results of *Gluconobacter*'s biocontrol activities are in line with previous studies, which have shown that bacterial isolates can exhibit both plant-growth promotion (Singh et al., 2020) and pathogens suppression. For example, Guzzon et al., (2014) demonstrated the *in vitro* antagonistic activity of *Gluconobacter oxydans* and *Gluconobacter cerinus* against *Botritis cinerea* on WL nutrient agar (Wallerstein laboratory Medium). Additionally, *G. oxydans* has also been shown to be effective in inhibiting mould growth caused by *Penicillium expansum* and preventing patulin and mycotoxin accumulation (Bevardi et al., 2012; Markov et al., 2019). Additionally, Asante et al., (2008) reported that EtOAc-soluble metabolites produced by *Gluconobacter* sp. can inhibit *Fusarium oxysporum* mycelia growth. Furthermore, *Gluconobacter* sp. play an important role as bioprotectants against food-borne human pathogens, the effectiveness of *Gluconobacter asaii* has been tested in combination with a bacteriophage on *Listeria monocytogenes* on fresh-cut honeydew melon pieces (Hong, 2015) and it has been reported to have an inhibitory effect on fresh-cut apples fruits (Leverentz et al., 2006; Siroli et al., 2015).

However, according to the pathogenicity test, *G. kondonii* bacteria was associated with kiwi fruit rot and, due to acid production, it caused necrosis on tobacco old leaves indicating its potential to cause disease in plants and storage green kiwi fruits. This species of *Gluconobacter* has not been previously reported as a specific plant pathogens and it does not elicit hypersensitive response (HR) on tobacco plants (Cha et al., 1997). To the best of our knowledge, there have been no descriptions of a bacterial soft rot disease on kiwi. This is the first report of *Gluconobacter* on kiwi fruit as a potential plant disease.

The fermentation processes in the diseased tissue were not unexpected, as the bacteria *Gluconobacter* is known for its ability to ferment sugars and sugar derivatives (Saichana et al., 2015). Other studies have suggested that *Gluconobacter* strains associated with others bacteria have been causing rot and postharvest losses of fruit. For example, Van Keer et al., (1981) reported *Gluconobacter* to cause soft rot on pear and apple. Moreover, under storage environments, onion bulbs were affected by rot due to numerous different species, and

Gluconobacter was a major bacterial group detected (Yurgel et al., 2018), while in a survey of fresh-market tomato fruit in Oahu, Hawaii, *Gluconobacter* was associated with decay on tomatoes, and the pathogenicity test confirmed the results on wounded cherry and grape tomatoes (Firas et al., 2017). It has been reported as well that *Gluconobacter* sp. caused “sour rot” on grapes by a combination of yeasts and insect (Hall et al., 2018). A similar result was also observed when *Gluconobacter cerinus* build a symbiotic relationship with *Bactrocera dorsalis* by fermenting and damaging fruit, the fermenting substrate that are used as food resource and mating site for insects (Hamby & Becher, 2016; He et al., 2017). Recently, there has been some research done where it shows the presence of an enzyme levansucrases T1-LS in the genera *Gluconobacter* as well as secreted by proteobacteria known to be plant-pathogenic (Jakob et al., 2019).

It is interesting to note that *Pantoea citrea* was identified as the causal agent of pineapple pink disease and that *Gluconobacter oxydans* with several distinct bacteria was suspected to be one of the causative agent (Kado, 2003).

Therefore, to identify *Gluconobacter* as a potential pathogenic microorganism and develop new strategies for plant disease management kiwi fruit storage, it is an important to understand the factors that contribute to infection. This includes studying how the pathogen can survive in long periods of inactivity without apparent disease symptoms, the host’s physiological tissue changes, and the epiphytic and endophytic microorganisms associated with the fruit. Understanding the microbiomes of both healthy and diseased fruit is crucial, as plant diseases are often caused by multispecies synergistic interactions. (Lamichhane & Venturi, 2015). Previous studies have shown the role of yeast, acetic acid bacteria, and insects in the development of sour rot (Hall et al., 2018). Insects have contributed to pathogenic soft rot *Enterobacteriaceae* transmission for decades (Rossmann et al., 2018), It can act as vectors for many plants pathogenic bacteria and play an important role in pest management strategies.

It is important to note that while many BCAs have shown promising bio control activity, their use may not be practical due to their potential pathogenicity on plants (Appendix F, Table 1). Therefore, screening methods are a critical step in the development of novel commercial BCA-based products to ensure that only non-pathogenic BCAs are selected for use.

6.5 Conclusion

The present study, focused on the isolation of *Gluconobacter kondonii* strain which showed potential as a biocontrol agent against *Phytophthora* sp. and had also a plant growth promoting trait.

However, further research is needed to investigate the potential anti-*Phytophthora* activity of *Gluconobacter* secondary metabolites, the effect of acidity produced by the bacteria, and the potential for a bacteria-yeast mixture to improve biocontrol effectiveness, especially in *in planta* considering the microbial bioformulation for a non-spore forming bacteria.

Additionally, the present study aimed to investigate the potential pathogenicity of *Gluconobacter* on kiwi fruit, which could be related to complex plant-associated multispecies interactions, and this study highlight the pathogenicity of some epiphytic and /or endophytic bacteria present on fruit surface.

To the best of our knowledge, this is the first report to demonstrate a potential pathogenicity of *Gluconobacter* sp. against kiwi, however, this study was conducted under *in vitro* conditions and further studies are required to investigate whether these results hold true under postharvest and storage conditions.

Even though *Gluconobacter* is known to thrive in natural environments (Huber Cristina, 2016) and is not pathogenicity to humans (Samuel et al., 2016), its use as a biocontrol agent may not

be practical due to potential spoilage activity. However, it could be used as a novel approach for mass trapping for fruit flies.

The evaluation of biocontrol agents using a phenotype-based screening method such as a dual culture assay is a valuable but less representative method with some limitations for the exploration of the full biocontrol potential of the appropriate candidates and the high probability of having a side effect on other host plants. Therefore, in addition to the *in vitro* studies and *in planta* test, the screening programs should be extended by including other parameters such as the whole genome sequence and the integration of omics data to have a more comprehensive screening.

6.6 References

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CHAPTER 7: ISOLATION AND CHARACTERIZATION OF BACTERIAL BIOLOGICAL CONTROL AGENTS WITH ANTI-*PHYTOPHTHORA* ACTIVITY AGAINST *PHYTOPHTHORA NICOTIANAE*.

Abstract

Five bacterial isolates obtained from environmental and food sources were screened for anti-oomycete activity and evaluated *in vitro* and *in planta* for their potential as biological control agents against *Phytophthora nicotianae* disease.

The isolates that exhibited more than 20% inhibition against *P. nicotianae* in dual plate *in vitro* tests were selected for further study, based on their ability to solubilize phosphate, produce hydrogen cyanide (HCN), siderophores, and cell wall degrading enzymes.

One strain, closely related to *Pseudomonas fluorescence* (G1), and unidentified bacterial species isolated from Castanea fruits (Cas) were chosen for evaluation of their biocontrol effect on ornamental plants.

The volatile organic compounds and anti-*Phytophthora* metabolites produced by the (Cas) isolate significantly reduced the radial growth of both *Phytophthora* isolates (P. 306 and P. 392) and decreased mortality from 50% to 17% on one isolate of *P. nicotianae* (P392) when used as a treatment on *viola* plants.

(G1) was also chosen for further studies to investigate its persistence in soil after multiple irrigations in plastic and cellulose biodegradable carton pots. Results showed that different pot types had a significant effect on bacterial persistence in soil, with (G1) able to increase in number after one week of inoculation on carton pots, while numbers remained constant on plastic pots. These findings could represent a first step towards the use of bacterial biological control agents formulated in carton pots for an eco-friendly control and sustainable methods for growers.

7.1 Introduction

Phytophthora species result in crop losses and reduce the quality of greenhouse and nursery plants (Panabieres et al., 2016; Parke et al., 2019). Among these species, *Phytophthora nicotianae* has been identified as one of the most commonly encountered pathogens in nurseries and gardens (Baysal-Gurel et al., 2022). It is able to infect a wide range of woody and herbaceous ornamental plants, belonging to approximately 255 genera across 90 different plant families (liu et al., 2016), in a wide range of climates (Panabieres et al., 2016). In addition *P. nicotianae* is known as an economically important disease that causes stem and root rot on fruit crops (Grote & Claussen, 2001) and has become a devastating disease in the production of tobacco (Qu et al., 2016) and a major disease on pepper crops (Blaya et al., 2016).

P. nicotianae is considered a root pathogen, but it can also attack the aerial plant parts (Baysal-Gurel et al., 2022; Lamour, 2013; Panabieres et al., 2016). The main reason for the difficulty in controlling this pathogen is its ability to produce chlamydospores, survival structures that allow it to persist in unfavorable growth conditions (liu et al., 2016), as well as, asexual spores such as zoospores and sporangia that can be dispersed by splashing water, wind, or surface water, contributing to disease outbreaks (Qu et al., 2016). Furthermore, this pathogen is frequently recovered from recycled irrigation water and diseased nursery plants, due to several disease cycles on annual herbaceous plants within a growing season (Hu et al., 2008) and genetic variation can be generated through sexual reproduction, leading to the evolution of

novel virulence and pathogenicity *Phytophthora* strain (Panabieres et al., 2016). Additionally, disease control can be difficult due to the spread of the pathogen to other production nurseries or landscape through the movement of contaminated plants and/or growing media (Hu et al., 2008).

Management of *Phytophthora nicotianae* disease typically involves the use of chemical fungicides, however, resistance to fungicides has been increasingly recovered in nurseries (Hu et al., 2008). Furthermore, limitations on the use of these products under the European Directive 2009/128/EC, (the sustainable use of pesticides) (Lamichhane et al., 2016) and the inability to completely control the disease have created a need for the exploration and identification of new approaches of *Phytophthora* pathogen control.

Biocontrol through the application of microbial antagonists isolated from various environments is a viable alternative to the use of synthetic pesticides (Droby et al., 2022). Many biocontrol control strains have been found and some potential formulated bacterial and fungal bioagents have been evaluated for their performance in controlling *P. nicotianae* diseases (Guo et al., 2020). The majority of applications of antagonistic microorganisms have been conducted using selected strains of *Pseudomonas* sp. (Yang & Hong, 2020), *Bacillus* sp. (Guo et al., 2020), *Trichoderma* sp. (La Spada et al., 2020), *Chaetomium* sp. (Hung et al., 2015), *Glomus* sp. (Hung et al., 2015) and *Paenibacillus polymyxa* (Ren et al., 2012).

Moreover, the efficacy of BCAs varies depending on different factors such as the bacterial strain, the plant host species, timing of the biological agents, and the application modes (Aiello et al., 2020). Thus, for an effective integrated control strategy of *Phytophthora* diseases in nurseries, work should be done by testing different of BCA strains with different modes and times of application in combination with tested BCA formulations.

Therefore, the aims of this research are to isolate and efficiently screen and identify antagonistic bacteria against *P. nicotianae* and test them both *in vitro* and *in planta* on various ornamental plants with the goal of developing these organisms into an effective biopesticide.

7.2 Material and methods

7.2.1 Bacterial and *Phytophthora* isolates

Five bacterial microorganisms were isolated from environmental and food sources. Bacterial strain (MK), (G1) and (Cas) were isolated from kiwi, grape and castanea fruit, respectively, and (Pro), (A1) were isolated from contaminated plates.

(G1) was the only strain that was molecularly identified, and the identification was carried out by amplifying and analyzing the 16S ribosomal subunit DNA (rDNA) gene.

The antagonistic strain was stored at -20°C in 20% glycerol and in Luria-broth (LB) liquid medium.

Two isolates of *P. nicotianae* pathogen (P. 392; P. 306) were obtained from the collection of the Plant Pathology Laboratory; Department of Innovation in Biological, Agro-Food and Forest Systems, (DIBAF); University of Tuscia, Viterbo. These isolates were grown on carrot agar (CA) medium.

To retrieved pathogen aggressiveness, agar plug cut from the advancing margins of the colony were inoculated on rhododendron leaves and incubated at 25°C in a dark humid chamber for a week. Pieces were aseptically excised from the border between necrotic and apparently healthy tissue and were sub-cultured on selective (PARP) medium (Pimaricin + Ampicillin + Rifampicin + Pentachloronitrobenzene + Agar) and finally, purified cultures were obtained by single hyphal culture on CA medium.

7.2.2 Mechanisms of *Phytophthora* inhibition

A dual-plate assays was used to test the antagonistic biological control activities of five isolated bacteria against two isolates of *P. nicotianae* (P. 392; P. 306) by assessing their ability to produce extracellular antifungal metabolites and volatile organic compounds (VOCs) according to (Syed-Ab-Rahman et al., 2018a).

7.2.2.1 Dual culture methods with bacterial isolates

Bacterial anti-*Phytophthora* activity was quantitatively assessed by measuring the radial growth of *P. nicotianae* mycelium on PDA plates.

The dual-culture test was carried out by using two lines of bacterial suspension, equidistantly inoculated along the plate boundaries, and a plug (0,6 cm diameter) of one of *P. nicotianae* isolate placed in the center of the medium.

The percentage of growth inhibition (PGI) for each culture was expressed after incubation at 25°C for 7 days according to Zygodlo et al., (1994), using the formula:

$PGI (\%) = 100 [(GC-GT)/GC]$, where GC (growth control) represents the mean diameter of fungi grown in PDA, and GT (growth treatment) represents the mean diameter of fungi exposed to bacterial isolates in the dual plate assay.

7.2.2.2 Anti-*Phytophthora* Bacterial Volatile Compounds (BVCs) activities

Five bacterial isolates from different genera were tested for the effects of bacterial volatile organic compounds (BVCs) on hyphal development of two isolates of *P. nicotianae* (P. 392; P. 306) according to Ebadzadsahrai et al., (2020).

All bacterial isolates were streaked on PDA plates, except for the (Cas) isolate, which was streaked on KB medium because it directly changes PDA medium and turns it into dark. After streaking, the lid of each bacterial plate was replaced with a 'bottom' petri dish plate containing PDA inoculated with a 0.6 cm mycelial plugs harvested from the growing edge of *P. nicotianae* colonies. The plates were and then sealed together with tap, creating a sandwich plate, allowing free gas exchange between *Phytophthora* and bacteria, but preventing the exchange of non-volatile metabolites or physical contact. The plates were incubated at 25°C for one week.

Phytophthora negative controls were prepared in the same way but without bacteria in the bottom plate.

The BVCs anti-*Phytophthora* activity was calculated for each bacterial culture and expressed as percentage of growth inhibition (PGI).

7.2.3 Anti-*Phytophthora* activity of (Cas) bacterial culture filtrate (BCF) against *P. nicotianae*

For the detection of secondary metabolites, the (Cas) bacteria ($OD_{600} = 0,6$) was inoculated into LB and Potato Dextrose Broth (PDB) medium (25 mL) and incubated with shaking at room temperature for 5 days.

To remove the bacterial cell pellet, the supernatant was collected, and centrifuged at 4°C for 10 min at 10,000 rpm. The pH of the PDB supernatant was adjusted to 7.0 using 10M NaOH solution. Then, the cultures were filtered using 0.22 µm Millipore® filters to remove bacterial cells.

BCF was added to PDA medium to the final concentrations of 50% (v/v). PDA without BCF (0%) were used as a control. A mycelial plug from two isolates of *P. nicotianae* (P. 392; P.

306) was placed at the center of the PDA slide and incubated at 25°C in the dark for 7 days. Then the growth inhibition percentage was calculated as mentioned above.

7.2.4 Qualitative test *in vitro* screening for bacterial enzymatic and plant beneficial traits

1-Siderophore production

The method for assaying the production of siderophore by five bacterial strains involved using the Chrome Azurol S (CAS) agar overlay technique with some modifications, as described by Pérez-Miranda et al., (2007). The medium for a liter of overlay was composed of 60.5 mg Chrome Azurol S (CAS), 72.9 mg of hexadecyltrimethyl ammonium bromide (HDTMA), 30.24 of Piperazine-1,4-bis (2-ethanesulfonic acid) (PIPES) g, and 10 mL of 1 mM $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ in 10 mM HCl. Agarose (Bio-Rad Laboratories, Inc.) (1.5%, w/v) was used as gelling agent. The CAS agar medium was applied over LB agar plates that had been inoculated with the bacterial strains two days prior, and the plates were incubated at 25°C for 5 days.

2-Protease production

Protease activity was determined by using 10% skimmed milk agar plate method, as described by Brown & Foster, (1970). The method involved using a modified milk agar (pH 7.0) that contained (per liter): Ultra-high temperature processing (UHT) milk (100 mL), Tryptone (5.0 g), Yeast extract (2.5 g), Dextrose (1.0 g), and agar (15.0 g). The bacterial cultures were inoculated by spotting four 10 μL spots of overnight bacterial cultures OD= (0,3) on the agar plates, and the plates were subsequently incubated at 25°C for 1 day. Clear zones surrounding the bacterial colonies indicated a positive reaction for protease activity.

3-Cellulase production

Cellulolytic activity was determined by inoculation of 10 μL of a bacterial suspension, (OD= 0,3) into 1L of carboxy methyl cellulose (CMC) agar media plates, as described by Islam & Roy, (2018), The CMC agar media contained: 2g NaNO_3 ; 1g K_2HPO_4 ; 0.5g MgSO_4 ; 0.5g KCl ; 2 g Carboxymethyl cellulose; 0.2g peptone and 1.7% Agar.

The plates were then incubated at 25°C for 48 hr. Cellulolytic activity was visualized by flooding the plates with Lugol's iodine solution for 20 min. A cellulase positive producing bacteria will show a light orange to clear zone against the red (CMC) agar background. The diameter of the clear zone was measured.

4-Pectinase production

The detection of pectinase-producing bacteria was carried out by inoculating 10 μL of a water - diluted bacterial suspension inoculated by spot puncture on Pectinase Screening Agar Medium (PSAM), with some modifications according to KC et al., (2020). The PSAM medium was prepared with the following composition (g/L): $(\text{NH}_4)_2\text{HPO}_4$, 3.0; KH_2PO_4 , 2.0; K_2HPO_4 , 3.0; MgSO_4 , 0.1, pectin, 10.0; and agar, 25.0) Mannitol, 25.0; Yeast, 5.0 and Peptone, 5.0, and the pH of the media was adjusted to 4.5. The presence of characteristic clear zones upon flooding with iodine-potassium iodide solution around colonies after 3–4 days of incubation at 25 °C was considered to indicate pectolytic activity.

5-Phosphate solubilization

The isolation of phosphate-solubilizing bacterial isolates was performed on Pikovskaya's (PKV) agar medium, as described by Pikovskaya, (1948). The PKV agar medium consisted of g/L (yeast extract, 0.50; Dextrose, 10.00; Calcium Phosphate, 5.00; Ammonium Sulfate, 0.50; Potassium Chloride, 0.20; Magnesium Sulfate, 0.10; Manganese Sulfate. 0.0001; Ferrous Sulfate, 0.0001; and Agar, 15 dissolved in 1000 mL distilled water.

Four spots of bacterial suspensions (10 μ L) OD= 0,3 were pipetted on the agar plates. After 24 hr. of incubation at 25°C, a visible halo zone around the colonies was observed. The solubilization efficiency of the strain was calculated as the diameter of the entire visible halo zone divided by the diameter of the zone with bacteria colonies.

6-Hydrogen cyanide (HCN) production

The assessment of HCN was carried out according to Abd El-Rahman et al., (2019) with some modifications. Each bacterial isolate was streaked on King's B medium (100 ml) amended with 4.4 g/l glycine. Non-inoculated plates were used as a control. A sterile filter paper strip was dipped in a picric acid solution (0.5% picric acid in 2% sodium carbonate) and was placed in the upper lid of the Petri plate. The plates were then sealed off with Parafilm and Incubation was made at 25°C for 7 days. A color change of the filter paper strips from yellow to reddish-brown color was considered as an indication of HCN production.

7.2.5 pH measurement on bacterial inoculated soil

The bacterial (Cas) isolate (4 ml; OD= 0,6) was inoculated into 10 mL of sterile peat substrate (Brill -TYPical; France) soaked in 10 ml of sterile distilled water and incubated with shaking at room temperature. After 7 days, the pH was measured using a pH meter (HI223-Hanna instruments).

7.2.6 Ornamental Plant Protection Assay Against *P. nicotianae*

1-*P. nicotianae* infected *Petunia* plant cultivation

In planta experiments were conducted to assess the efficacy of the bacterial (G1) isolate in suppressing *P. nicotianae* on *Petunia hybrida* (nana compacta).

Petunia seeds were germinated in a sterile substrate (Triplo, Terriccio Effetto Tripla Crescita; TerComposti, Italy) in a plastic tray.

Four months old seedlings were transplanted into plastic pots with an autoclaved mixture (1:1, v/v) of peat substrate (Triplo, Terriccio Effetto Tripla Crescita; TerComposti, Italy) and perlite. The experiment consisted of eight different treatments: (1) control treatment; (2) (G1) bacterial treatment; (3, 4) *P. nicotiane* both isolates (P. 306; P. 392); (5,6) *P. nicotiane* both isolates (P. 306; P. 392) with bacterial (G1) strain; and (7,8) *P. nicotiane* both isolates (P. 306; P. 392) with the synthetic fungicide "Arpel WDG", applied in at the rate of 2500 mg.l⁻¹.

The experiment was carried out with fifteen *Petunia* plants for each treatment, which represents one replicate in a totally randomized design.

The strain (G1) was cultured for 2 days at 25°C on KB medium and subsequently diluted with distilled water to a concentration of 1×10^8 cfu/ml or absorbance at 600 nm = 0.3 and was inoculated four times at a rate of 4 ml/pot.

Pathogenic *P. nicotianae* of both isolates was inoculated as a soil drench with 2% of infected millet seed in each pot according to Jung et al., (1996). The progress of the wilt was monitored

at regular intervals for a period of 4 months. The disease incidence was determined using the formula $DI (\%) = \text{Number of wilted plants} / \text{Total number of plants} \times 100$

Additionally, the disease severity was assessed using an empirical scale to rate the severity of the pathogen's disease, which included the following values: 0- normal with no visible disease symptoms, 1- 4 range from slightly to severely yellowing to 5 - plant dead.

***In vitro* measurement of total cultivable bacterial counts in soil in two different types of pots**

The bacterial isolate (G1) was chosen for further studies to investigate its persistence in soil after many irrigations by using a classical soil baiting experiment inside two kinds of pots.

These activities could represent a first step towards the use BCAs formulated inside biodegradable carton pots for an eco-friendly control/management and a durable, sustainable method for growers in nurseries.

A series of four cellulose biodegradable carton pots (Relaxdays, 5.5x3.5x3.5 cm) and plastic pots (5.7x3.5x4.5cm) with a hole drilled at the bottom were filled with 20 ml/ pot of sterile peat soil (Triplo, Terriccio Effetto Tripla Crescita; TerComposti, Italy) and were put inside sterile jar sealed together inside plastic bags to form a humid chamber.

The soil was inoculated with 5 ml of concentrated (G1) bacterial suspension with an absorbance at 600 nm = 2,4 and were incubated at 25°C for a period of one month.

To characterize bacterial communities in soil and estimate the viable numbers of bacterial colonies present in pot, each week after irrigation, soil samples were taken, vortexed to homogenize and a series of decimal dilutions was conducted in a sterile saline solution. Then, the number of visibly growing colonies grown on KB medium and representing the original colony forming units per millilitre of diluted soil sample plated, or CFU/mL was checked under UV light.

2- *P. nicotianae* infected pansy plant cultivation

Bacterial isolate (Cas) was chosen for *in planta* experiments to assess its efficacy in suppressing *P. nicotianae* pathogen.

The treatment details of the pot bioassay were conducted as described above and included 8 treatments (1) controls (no inoculum); (2) inoculated with 2 isolates of *P. nicotianae* (P. 306; P. 392); (3) With Arpel WDG[®]-Chemical fungicide as positive control; 4) and with the BCAs alone or in combination with the *Phytophthora* pathogen.

The plant selected for inoculation was pansy plants (*Viola* × *wittrockiana* *Viola tricolor*), as this species grows rapidly from seed and is susceptible to root-infecting species of *P. nicotianae*. Eighteen single plant replications per treatment were arranged in a completely randomized design. The inoculation of BCA was carried out one weeks prior to soil drench of 2% of infected *Phytophthora* millet seed. Four times the inoculation of 4 ml of bacterial suspension (1×10^7 CFU/ml) or absorbance at 0,3 was used per pot, and after one month and half of treatment, the plants were evaluated for shoot length, plant height and disease incidence and severity.

7.2.7 Pathogenicity and Hypersensitivity test

7.2.7.1 Pathogenicity test on kiwi fruits

To check the pectinolytic activities of isolated bacteria, pathogenicity tests were performed using green kiwi fruits.

Fresh kiwi fruits were surface sterilized with 70% ethanol and left to dry under hooded chamber. For inoculation, artificial wounds approximately 2 mm deep were aseptically made using a pipette, and 20 µl of concentrated bacterial water suspension was inoculated onto healthy fruits. An equal volume of sterile distilled water was used on the control samples. The inoculated fruits were placed in sterile beaker, then wrapped using aluminum foil, and incubated at 27°C. After ten days, kiwi fruits were checked for symptom development.

7.2.7.2 Hypersensitivity Response (HR) on tobacco leaves

All bacterial isolates were tested for their ability to induce a hypersensitive reaction (HR) on tobacco leaves (*Nicotiana tabacum*). Plants were grown for 8 weeks in a growth chamber with a controlled temperature at 25°C ± 2 and relative humidity of 70% ± 5.

An aqueous bacterial suspension (OD₆₀₀ = 0,3) was infiltrated into the mesophyll using a hypodermic syringe against the bottom of tobacco leaf. Water was used as a negative control. The development of HR was monitored within 1-week post-infiltration.

7.2.8 Statistical analysis

All data were expressed as the mean ± SD or SE by measuring three or four independent replicates and were subjected to analysis of variance (ANOVA) using R studio Statistical Software (Version 1.2.5033). The mean values were compared using Tukey's HSD at P ≤ 0.05.

7.3 Results

7.3.1 Identification of Bacterial Isolates by 16S rDNA Amplicon Sequencing

The bacterial strain (G1) isolated from grapes were identified by 16S rDNA gene amplification and successfully sequenced. The strain was identified as *Pseudomonas fluorescens* by BLAST matching.

7.3.2 Screening of Bacterial Isolates for Antagonistic Activity

The biocontrol activity of five bacterial BCAs or their exudates was tested using an *in-vitro* dual plate assay. The results showed that the isolates exhibited varying levels of inhibition against *P. nicotianae*, and all five isolates displayed similar inhibitory effects against the *Phytophthora* isolates (P. 392, P. 306) (Figure 7.1).

These findings suggest that the isolates grown on PDA plates released extracellular metabolite(s) that hindered the growth of *P. nicotianae*. Additionally, the bacterial growth patterns suggest that mechanisms such as parasitism may also be involved in the biological control of the (MK), (A1) and (Pro) isolates. However, further research is needed to confirm this.

The most potent activity was observed for the (Cas) and *P. fluorescens* (G1) isolates, which inhibited the growth of *P. nicotianae* by 90%, and 70% respectively. These isolates were selected for further *in planta* and other analyses.

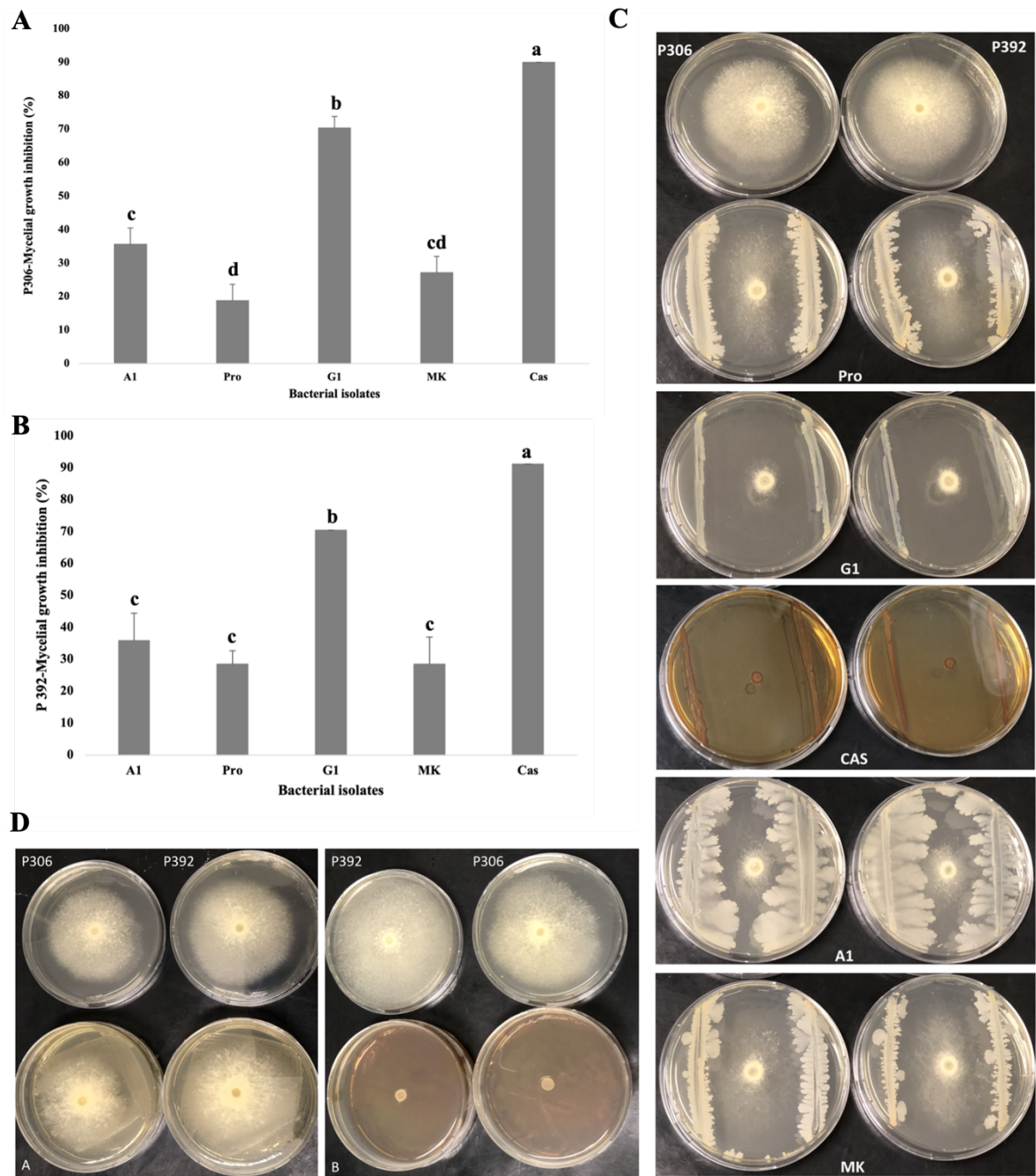


Figure 7. 1 Bacterial growth inhibition against *P. nicotianae*, (A, B) Percentage of inhibition of five bacterial isolates (A1, Pro, G1, Mk, Cas) against two isolates of *P. nicotianae* (P. 392 and P306). Data presents mean values $\pm$ standard deviation from three replicates. Bars labeled with the same letter are not significantly different from each other according to Tukey's HSD at $p < 0.05$. (C) Pictures of confrontation assay of anti-*Phytophthora* activity of the bacterial isolates against the two isolates of *P. nicotianae*. A representative control petri dish of *P. nicotianae* is presented at the top of the figure. (D) Pictures representing the effect of volatile metabolites on the growth of *P. nicotianae* by (Cas) bacterial isolate inoculated on A) KB and B) PDA medium.

7.3.3 Production of volatile metabolites

In the volatile metabolites test, the growth of *P. nicotianae* isolates was significantly reduced by only two bacterial isolates. The (Cas) bacterial strain, when tested on PDA medium, had a strong ability to inhibit the mycelial growth of *P. nicotianae* by producing volatile metabolites after seven days of incubation. The isolate turned the medium dark in color and showed complete growth inhibition on both pathogen isolates (P.306 and P.392) compared to the control. However, when inoculated on (KB) medium, the radial growth of *P. nicotianae* was not inhibited, with a (-0,77%) inhibition rate for isolate (P. 392), and a slightly promoted growth of (-9,73%) for (P. 306) isolate (Figure 7.1; D). On the other hand, the radial growth of both *P. nicotiane* isolates (P. 392) and (P. 306) was slightly inhibited by 6 % and 23 % respectively with the (G1) bacteria.

7.3.4 Anti-Phytophthora activity of (Cas) bacterial culture filtrate (BCF)

The culture filtrates of the (CAS) bacterial isolate demonstrated inhibitory activity against both *P. nicotianae* isolates (Figure 6.2). With a 50% concentration of BCF, the growth of *P. nicotianae* (P. 306) was inhibited by 42 % and 38 % when the bacteria were grown on LB and PDB medium, respectively. The growth of *P. nicotianae* was inhibited by less than 20% with both culture mediums. This confirms the presence of antibiosis that occurred during interactions with *P. nicotianae* pathogens, and suggests that low-molecular-weight diffusible compounds or antibiotics (Bae et al., 2015) are produced by the (Cas) isolate.

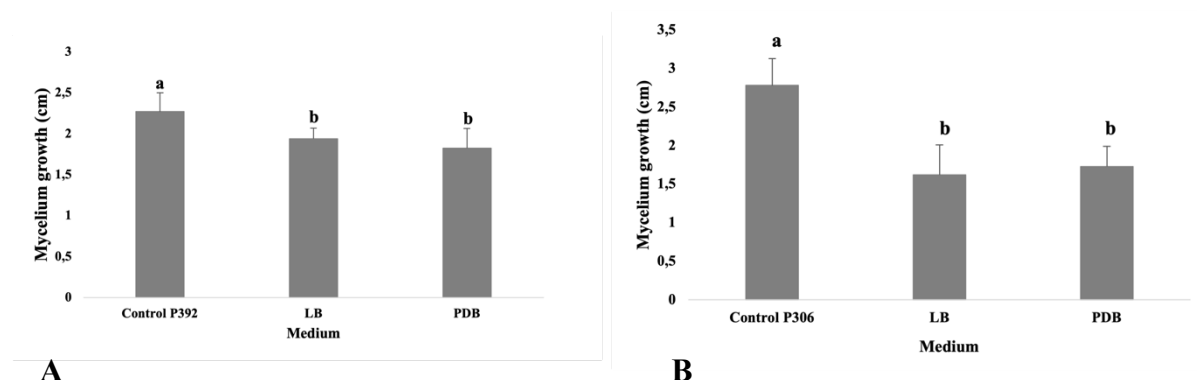


Figure 7. 2 Effects of (Cas) bacterial culture filtrate inoculated on LB and PDB medium on mycelium growth of both *P. nicotianae* isolates; (A) The size of mycelium growth of *P. nicotiane* P. 392 and (B) of *P. nicotiane* P392 after 7 days cultivation on PDA supplemented with 50% metabolites. Different letters indicate significant differences, determined by ANOVA with Tukey's HSD post hoc test ($p < 0.05$).

7.3.5 Cell Wall-Degrading Enzyme Activities

The isolates were tested for their ability to produce cell wall-degrading enzymes. Based on the calculation of the ratio of the diameter of the zone of clearance to the diameter of the colony, it was determined that these bacterial isolates demonstrated differences in their ability to degrade pectin, cellulose, and protein (Table; 7.1, 7.2, 7.3).

1-Pectinase production

The five bacterial strains exhibited varied levels of pectinase activity, as revealed by the formation of a clear zone on PSAM (Table 7.1). The strongest activity was observed for (Mk, Pro, Cas and A1) isolates.

Table 7. 1 Pectinase production by the bacterial isolates



Bacteria isolates	A1	Pro	G1	Cas	Mk
Clear zone; A (cm)	2,30	2,24	1,23	1,89	2,33
Colony; B (cm)	1,15	0,73	1,04	0,75	0,73
Ratio; A/B	2	3,07	1,18	2,52	3,19

2-Cellulase production

Bacterial isolates that can hydrolyze cellulose were screened for their cellulolytic potential on CMC agar plates. Three bacterial strains (Mk, Pro and A1) isolates exhibited cellulolytic activity, as revealed by the formation of a clear zone on the screening medium. However, no clarification zones were observed for (Cas) and *P. fluorescens* (G1) isolates indicating that none of these three bacterial strains synthesized cellulase (Table 7.2).

Table 7. 2 Cellulase production by the bacterial isolates



Bacteria isolates	A1	Pro	G1	Cas	Mk
Clear zone; A (cm)	2,26	2,27	1,05	0,94	2,38
Colony; B (cm)	0,64	0,63	1,05	0,94	0,62
Ratio; A/B	3,53	3,60	1	1	3,83

3-Protease production

All isolates produced distinct halos (clear zone > 1 cm) around the bacterial colonies on the plate, indicative of protease production. However, the (Cas) isolate did not seem to produce protease as no clarification zones were present (Table 7.3).

Table 7. 3 Protease production by the bacterial isolates



Bacteria isolates	A1	Pro	G1	Cas	Mk
Clear zone; A (cm)	2,36	2,26	1,94	0	2,20
Colony; B (cm)	1,76	1,49	1,41	0	1,46
Ratio; A/B	1,34	1,52	1,37	0	1,51

7.3.6 Plant Growth Promoting Traits

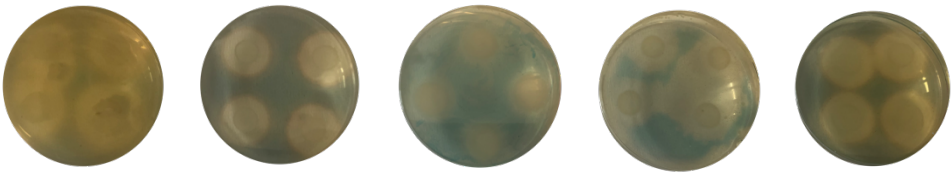
1-Phosphate Solubilization and HCN production

No clarification zones were observed for all tested isolates indicating that none of these five bacterial strains were able to solubilize phosphate. Furthermore, regarding HCN production, the paper color remained unchanged indicating that none of these strains were able to produce hydrogen cyanide.

2-Siderophore

Four bacterial strains induced a color change in Chromeazurol agar indicating that they can produce siderophores. The strongest activity was observed with (A1) isolate, and a yellow halo surrounding the bacterial colonies was present in (Pro, Cas, and Mk) isolates. However, the absence of discoloration with the (G1) isolate suggests that these isolates cannot produce siderophores (Table 7.4).

Table 7. 4 Siderophore production by the bacterial isolates



Bacteria isolates	A1	Pro	G1	Cas	Mk
Clear zone; A (cm)	2,28	1,61	0	1,62	1,89
Colony; B (cm)	1,01	0,85	0	0,8	0,96
Ratio; A/B	2,25	1,90	0	2,02	1,94

7.3.7 Ornamental Plant Protection Assay Against *P. nicotianae*

1-*P. nicotianae* infected *Petunia* plant cultivation

Data as depicted in Figure 7.3 illustrated the values of root and shoot length, root fresh and dry weight, and plant dry weight as the samples responded to different treatments. The results from

the *in-planta* test showed that the growth of *Petunia* plants treated with *P. fluorescens* (G1) strain was not stimulated. Overall, the treatment did not differ from the untreated control.

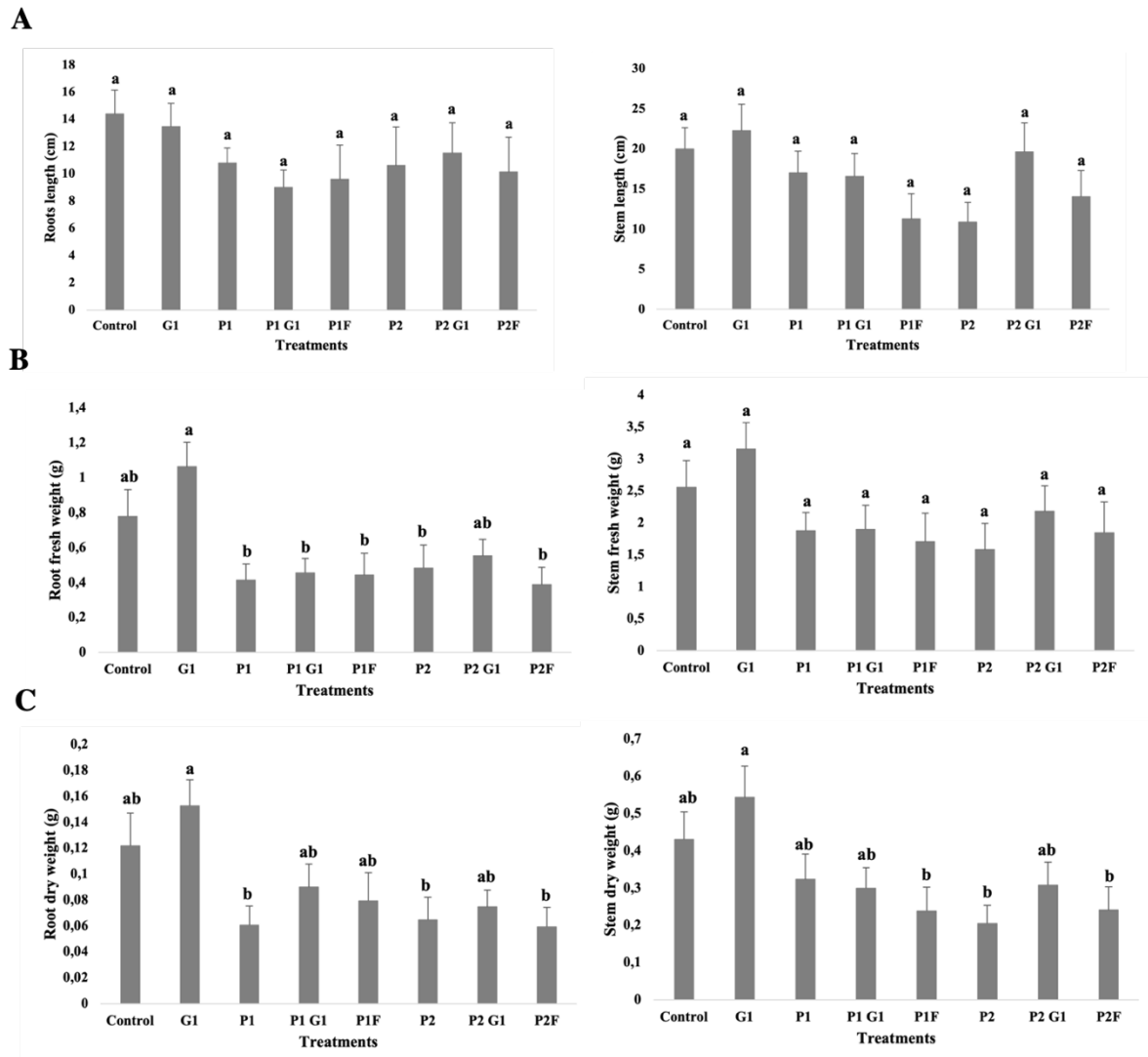


Figure 7.3 Effect of (G1) strain on disease severity of *Phytophthora* disease, under growth chamber conditions after 4 months inoculation using soil treatment methods. The top panel (A) shows the effect of inoculation of the isolate on root and stem length of *Petunia* plants. (B) on fresh weight (C) and dry weight (D). Bars represent the mean $\pm$ SE values. (Tukey Kramer's HSD, $P < 0.05$, $n = 15$).

At the end of the trial, both untreated plants and treated ones with *P. fluorescens* (G1) showed similar disease incidence and disease severity, thus indicating that the application of (G1) antagonistic bacteria was not effective in suppressing *P. nicotianae* disease.

Table 7. 5 Effect of (G1) bacterial isolate on *Phytophthora nicotianae* disease development and *Petunia* seedlings growth

Treatment	Disease severity	Disease incidence (%)
Control	2.50±0.45	13.3
G1	1.30±0.38	6.7
P1	2.40±0.45	6.7
P1G1	2.40±0.50	20.0
P1F	3.20±0.52	40.0
P2	2.00±0.59	33.3
P2G1	1.60±0.58	26.7
P2F	2.90±0.50	33.3

Disease severity was assessed on a 0 – 5 scale from 0 – no visible disease symptoms to 5 – plant dead Results are an average of four replicates ± Standard error.
P1: *Phytophthora* isolate (P306)
P2: *Phytophthora* isolate (P392)
P1F: *Phytophthora* isolate (P306) with Fungicide
P2F: *Phytophthora* isolate (P392) with Fungicide
G1: Bacterial isolate
P1G1: *Phytophthora* isolate (P306) with bacterial isolate
P2G1: *Phytophthora* isolate (P392) with bacterial isolate

***In vitro* measurement of total cultivable bacterial counts in soil in two different types of pots**

Culturing methods on KB medium were used to estimate (G1) soil bacterial numbers and its persistence inside biodegradable or carton pots (BPs) and plastic pots after irrigation. Results shown in (Figure 7.4) demonstrate that bacteria in soil were able to increase in number after one week of inoculation on carton pots and declined rapidly after 3 weeks application, whereas for plastic pots experiment, bacterial concentrations recovered from the soil appeared to be uniformly constant over time at roughly 10⁴ CFU.

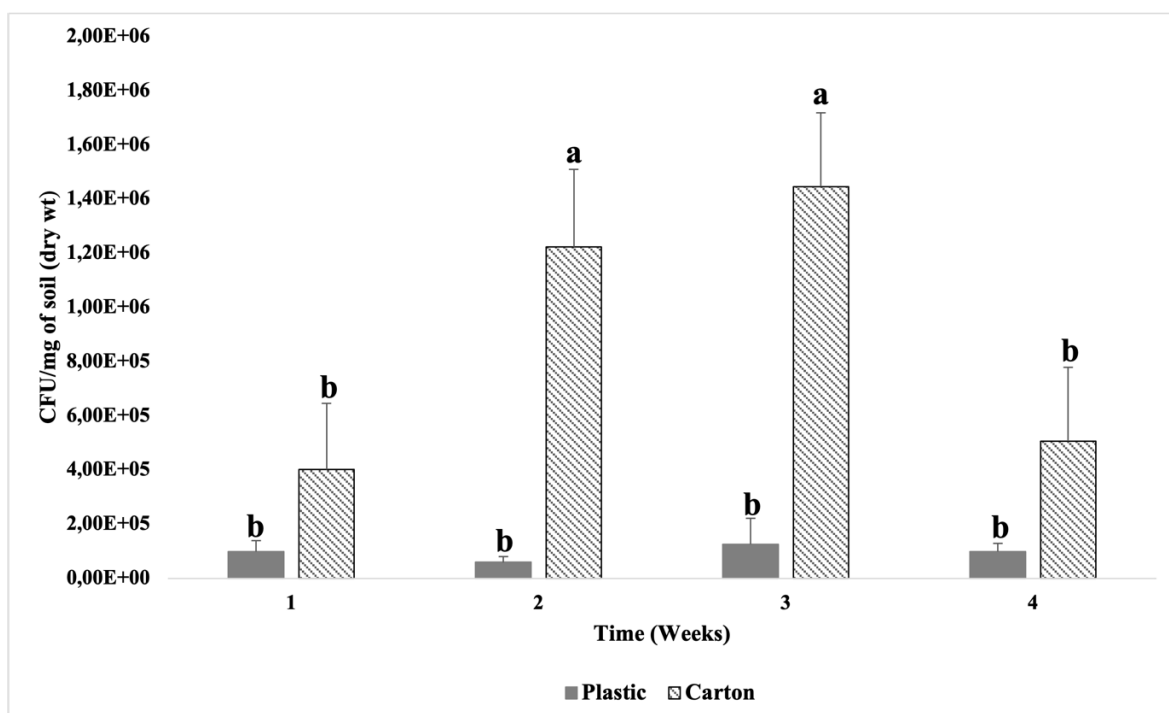


Figure 7. 4 Total cultivable bacterial counts in 2 types of pots: Plastic and Carton: Counts of cultivable bacteria (G1) isolate exposed to irrigation once per week are expressed as CFU per mg dry soil. The values and error bars are mean and S.D. of 4 replicates per treatment. Differing letters indicate significant differences ($P < 0.05$) between counts according to Tukey Kramer's HSD.

Furthermore, the control treatments plates checked under UV light demonstrated that there was no contamination with *P. fluorescence* in either the soil or irrigation water over the course of the two sets of experiments.

2-*P. nicotianae* infected Pansy plant cultivation

The (Cas) bacterial isolate was found to produce anti-*Phytophthora* diffusible as well as volatile metabolites, with evidence of considerable differences with the control ($p < 0.05$) regarding *P. nicotianae* mycelial growth inhibition. In addition, the bacterial isolate exhibited significant lateral roots growth compared to the control on cresson seeds grown on Woody Plant medium (Unpublished data) (Figure 7.5).

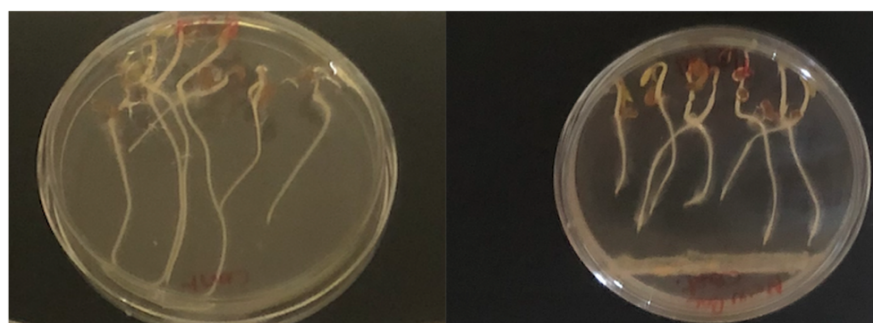


Figure 7. 5 Effect of (Cas) bacterial isolate on cresson seeds grown on Woody Plant agar medium comparing to control plate.

Thus, due to the beneficial effects in both the promotion of plant growth and the biological control of the pathogen, the selected (Cas) strain has been chosen to be tested *in planta*.

However, the results from *in planta* test showed that the plant growth parameters such as the increase in fresh and dry root and stem weight were not influenced by the application of (Cas) isolate. In addition, no significant differences in the stem and roots length of *Pansy* plants were found between the control and bacterial inoculated treatment (Figure 7.6; A, B, C).

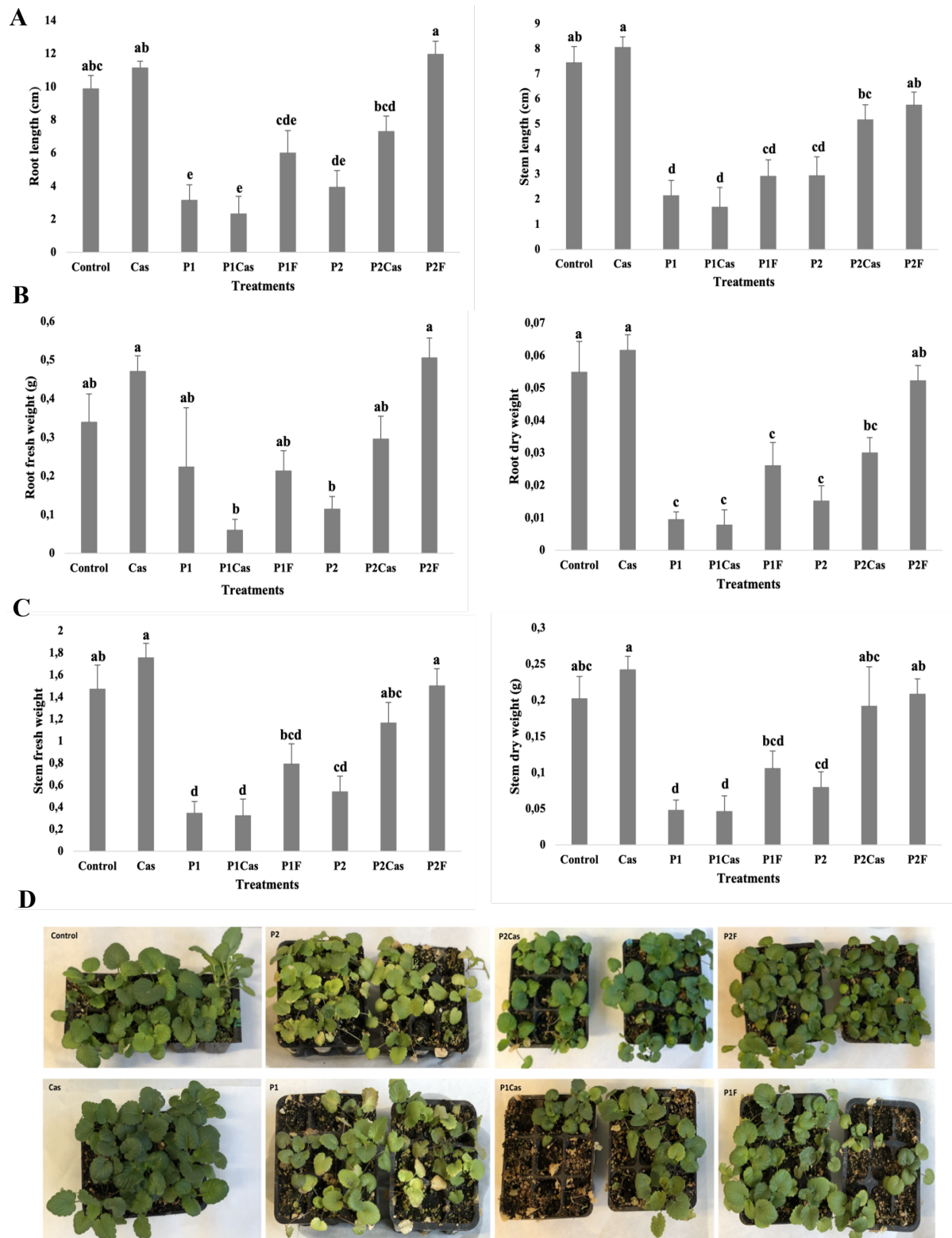


Figure 7. 6 Influence of different treatments with *P. nicotianae* and bacterial strain (Cas) on the growth parameters and disease severity of *Viola wittrockiana* under growth chamber conditions after 1,5 months. The top panel (A) shows the effect of soil inoculation of the isolate on root and stem length of *Viola* plants. and on fresh and dry (B) root weight (C) and stem weight (D) Figure representative of disease incidence and severity of eight different treatments after inoculation with *P. nicotianae* disease and (Cas) bacterial strain. The Symbol represents P1: *Phytophthora* isolate P306, P2: *Phytophthora*

isolate P392, P1F: *Phytophthora* isolate P306 with Fungicide, P2F: *Phytophthora* isolate P392 with Fungicide, Cas: Bacterial isolate, P1Cas: *Phytophthora* isolate P306 with bacterial isolate, P2Cas: *Phytophthora* isolate P392 with bacterial isolate and control treatment. Bars represent the mean $\pm$ SE values. (Tukey Kramer's HSD, $P < 0.05$, $n = 18$).

The results of the incidence and severity of treated *Viola* plants against *P. nicotianae* pathogen in the soil pot experiment are shown in (Figure 7.6, Table 7.6), where a visible yellowing symptom appeared only in pathogen inoculated plants (P1, P2) compared to other treatments (Figure 7.6).

Moreover, there were significant differences between non infected seedlings with all the other treatments. After 45 days, the (Cas) bacterial treatment significantly decreased the disease incidence to 17% and disease severity from 4 to 1 on only one pathogen isolate of *P. nicotianae* (P2), whereas the bacterial treatment did not effectively suppress the development of damage and mortality caused by *P. nicotianae* (P1) isolate (Table 7.6).

Furthermore, the lowest disease incidence (0%) was found in the control treatment and bacterial inoculation alone, indicating that the application of (Cas) strain are not Phytopathogenic to *Viola* plants.

Table 7. 6 Effect of (Cas) bacterial isolate on *P. nicotianae* disease development and *Viola* seedlings.

Treatment	Disease severity	Disease incidence (%)
Control	0.22 $\pm$ 0.10	0
Cas	0.06 $\pm$ 0.06	0
P1	4.00 $\pm$ 0.35	55.6
P1Cas	4.11 $\pm$ 0.4	77.8
P1F	3.00 $\pm$ 0.46	44.4
P2	3.83 $\pm$ 0.34	50
P2Cas	1.22 $\pm$ 0.43	16.7
P2F	0.83 $\pm$ 0.27	5.6

Disease severity was assessed on a 0 – 5 scale from 0 – no visible disease symptoms to 5 – plant dead Results are an average of four replicates $\pm$ Standard error. The following symbol represent:

P1: *Phytophthora* isolate (P306)

P2: *Phytophthora* isolate (P392)

P1F: *Phytophthora* isolate (P306) with Fungicide

P2F: *Phytophthora* isolate (P392) with Fungicide

Cas: Bacterial isolate

P1Cas: *Phytophthora* isolate (P306) with bacterial isolate

P2Cas: *Phytophthora* isolate (P392) with bacterial isolate

7.3.8 Pathogenicity and Hypersensitivity test

The results of bacteria inoculated on kiwi fruit showed water-soaked lesions, brown discoloration, and decay symptoms with a fermentation scent appeared on inoculated fruit in 7 days after inoculation with only (Cas) bacterial isolate suspension at 28°C (Figure 7.7), whereas the control fruit with the other strain (A1, Pro and G1) were asymptomatic.

Moreover, the potential antagonistic bacteria did not induce HR by producing necrosis on tobacco old leaves after 1 month inoculation.



Figure 7. 7 Effect of isolated bacteria on inoculated kiwi fruit.

7.4 Discussion

Conventionally, fungicides are the most effective strategy used to protect crops against *Phytophthora* pathogens. However, applications of synthetic fungicides and regular use of chemicals to control them usually take long timelines to be completely degraded, which negatively impacts the environment and presents health risks to non-target organisms (Pompimon et al., 2009). Additionally, it may lead to the development and selection of resistant *Phytophthora* strains.

Bacteria often exhibit beneficial plant attributes such as the promotion of plant growth and the ability to suppress plant pathogens acting as biocontrol agents (Jin et al., 2011; Syed-Ab-Rahman et al., 2018a).

In this study, five bacterial isolates (Figure 7. 1) were screened by dual culture plate assays for *in vitro* antagonism against *P. nicotianae* pathogen. The strain (Cas) showed the maximum zone of inhibition followed by (G1), the isolate belonging to *P. fluorescens*.

This present antagonistic activity of bacterial genera of *P. fluorescens* against *P. nicotianae* correlated with (Jin et al., 2011) from tobacco rhizosphere soil sample, which showed antagonistic activity against *P. nicotianae* with inhibition zones larger than 10 mm.

Similarly, Dong et al., (2012) isolated five *P. fluorescens* strains from rhizospheric soils of tobacco fields and screened for antagonistic activity against *P. nicotianae*.

Isolated bacteria have proven successful anti-*Phytophthora* activity against phytopathogens with the highest suppressive effect considered with some strains producing an inhibition zone of 13.0 mm (radius) with an inhibitory rate at 68.57%. In addition, it has showed a good biocontrol effect on tobacco black shank infection when tested on the tobacco plants grown in greenhouse.

Similarly, in the present study, (G1) strains exhibited a zone of inhibition against *P. nicotianae* with a mycelium inhibition percentage of around 70%. However, the challenge was to move away from *in vitro* to *in planta* studies, where (G1) did not stimulate plant growth or show any disease suppression, despite the fact that members of *Pseudomonas* genera are known to produce numerous bioactive compounds with anti-oomycete activities (Hashemi et al., 2021) and *P. fluorescens* strains have been known to be effective as biofertilizers (Barin et al., 2022) and biocontrol agents in agriculture (Couillerot et al., 2009).

The same phenomenon was observed with the (Cas) bacterial strain. Despite showing anti-*Phytophthora* activity in *in vitro* tests with the highest *Phytophthora* mycelium inhibition (100%) and modifying the root architecture of cresson seedlings to have more lateral roots

compared to uninoculated plant, the treatment with the (Cas) isolate did not stimulate plant growth in *in planta* experiments. Additionally, the biocontrol efficiency of the isolate was only proven against one *Phytophthora* isolate (P.392).

This result is consistent with Besset-Manzoni et al., (2019) who reported that *in vitro* tests do not always showing the best results *in planta* and *vice versa*.

Several factors might contribute to the loss of biocontrol effect. For example, the bacterial biological control agent may require a sufficient amount of time to colonize the soil due to the many biotic interactions in soil, lack of ecological competence, and allelopathy effect among organisms, where biochemical compounds influence the growth and development of others microorganisms either positively or negatively (Rodríguez-Romero et al., 2021).

Additionally, the diversity of sensitivity of plant pathogens to biocontrol agents (Bardin et al., 2015) and the host's physical, biological, and chemical features (Lahlali et al., 2022) may also affect the success of bacterial biological control treatment.

P. nicotianae was found to be the most prevalent species in floriculture nurseries, infecting *Pansy*, *Petunia*, and other ornamental plants (Hwang & Benson, 2005). Both plant species were chosen because they grow rapidly from seeds and are susceptible to *Phytophthora* root-infection. Furthermore, in this study, both *Phytophthora* isolates (P.306 and P.392) showed aggressiveness and diseases symptoms on *Pansy*, however, no effect was observed on *Petunia* plants. This might be due to a moderately resistant *Petunia* seed and variations in the aggressiveness of *P. nicotianae* isolates on *Pansy* seedlings.

Furthermore, to improve the efficiency of bacterial biocontrol products, commercial formulations are often needed, especially with *P. fluorescens* strains that lose their efficacy after long term storage (Guo et al., 2004).

The decrease in bacterial population is a common limitation for most plant growth promoting bacterial cell suspension species after inoculation in soil (Pellegrini et al., 2020).

To address this, the (G1) isolate of bacteria was inoculated inside biodegradable pots (BPs) as a durable, sustainable method for growers. Results showed a significant effect on bacterial persistence in soil and an increase in numbers after one week of inoculation on (BPs). The explanation could be related to the fact that (BPs) keep soil more aerated than plastic ones, which might also affect bacterial density due to an increase in soil temperature (Minuto et al., 2001).

This experiment could be considered as a preliminary new study to confirm the increase in bacteria biological control density in a (BPs). However, further studies are needed to develop the new idea of confinement of bacterial cells in a matrix or in capsules on pot surface for long-term storage. Additionally, as the pot types can influenced the incidence of root rot caused by *P. nicotianae* (Minuto et al., 2001), further studies are needed to test the combination effect of BCAs and (BPs) for their ability to control *Phytophthora* pathogens.

Moreover, enzyme production by BCAs plays an important role in disease control against *Phytophthora* phytopathogens. This study found that the major molecular mechanisms of parasitism by the (Mk, A1 and Pro) bacterial strains were by protease, pectinase and cellulase production.

However, in the case of the (Cas) isolate, bacterial mycelium inhibition was due to pectinase enzymatic production only, and it may also be related to the modification caused by the bacteria in the medium, especially acidification.

However, experiments using bacterial culture filtrate and soil pH revealed that even after pH adjustment, the (Cas) cell- free filtrate culture still inhibited *Phytophthora* colonial mycelium growth and bacterial inoculation did not significantly acidify the soils. These findings confirm that the major mode of action of this strain is antibiosis through the production of metabolic

substances, and it is possible that volatile compounds may be responsible for *Phytophthora* growth inhibition.

Recently, several studies have focused on the effects of bacterial metabolites on *Phytophthora* sp. growth. For example, a study by Guo et al., (2020) found that derived proteins from *Bacillus velezensis* strain Ba168 were effective in inhibiting *P. nicotianae* growth and causing damage to its cell walls and membranes. Similarly, Yang & Hong, (2020) found that strains of *Pseudomonas* species belonging to *Ps. granadensis* and *Ps. protegens* produced anti-*Phytophthora* secondary metabolites that reduced the radial growth of colonies of *P. nicotianae*, encystment, germination, and survival rates of zoospores, and foliage blight severity on annual *Vinca* plants.

The findings from the present study suggest that the (Cas) isolate has the potential to be developed as a biocontrol agent to control *Phytophthora* disease. However, molecular identification to reveal its pathogenicity and further studies with GC-MS analysis are needed to verify which compounds exhibit the anti-oomycete activities. The identification of the effective compound/s would be crucial in the preparation of a biocontrol formulation.

Furthermore, the (G1) bacteria isolated from a grape sample showed prominent protease activity on agar plates, which is consistent with other studies reporting that the production of extracellular proteases is a common feature among members of *P. fluorescens* (C. H. Liao & McCallus, 1998).

However, in present study, the (G1) strain did not show cellulase and pectinase activity. This may be because the enzyme activities of the isolated bacterial strain depend on carbon sources other than the carboxymethyl cellulose present in the agar medium used for bacterial growth. It may require glucose or glycerol as carbon and energy sources (Sethi et al., 2013).

Phosphorus, siderophore and hydrogen cyanide are recognized as important indirect mechanisms for plant growth and disease control (Otieno et al., 2015; Rijavec & Lapanje, 2016; Sayyed & Patel, 2011). However, this study showed that none of strains were able to solubilize phosphate and produce (HCN).

According to past studies, many bacterial genera have the ability to produce HCN including *Pseudomonas* species (Abd El-Rahman et al., 2019) and more specifically *P. fluorescens* (Voisard et al., 1989) and De Vrieze et al., (2020) identified the genes coding for the production of hydrogen cyanide (HCN) in *P. fluorescence* (S49) that exhibited antagonistic potential against *P. infestans*.

Regarding phosphate solubilizing, *P. fluorescens* strains have been shown to possess good phosphate solubilization activity (Otieno et al., 2015). However, the (G1) isolate in this study, was unable to solubilize phosphate. It is proposed that the genetic systems of this strains might be responsible for its lack of effective phosphate solubilizing abilities. Several recent studies have highlighted the important role of glucose metabolism genes in *P. fluorescens* in bacterial growth and inorganic phosphate solubilization (Otieno et al., 2015).

On the other hand, even though the (Cas) isolate causes acidification of the medium, which is a common mechanism by which most bacteria solubilize inorganic phosphates and release phosphate, this study, in contrast, demonstrated that the (Cas) isolate was unable to solubilize phosphate (Miller et al., 2010; Otieno et al., 2015).

Among the five strains, four bacterial strains were able to produce siderophore on CAS agar plates with a clear orange or yellow color zone, except for the (G1) isolate. *Pseudomonas* sp. have been known for their production of siderophore (Raaska et al., 1993). However, no, or very low production was observed with the (G1) isolate. This finding may be related that bacterial production ability for siderophore depends on culture medium (Raaska et al., 1993; Sasirekha & Srividya, 2016). Murugappan et al., (2012) reported that siderophore production by *Pseudomonas putida* was found to be 42% compared to other medium substrates and the maximum siderophore production was observed with MM9 medium.

7.5 Conclusion

In conclusion, *P. fluorescences* (G1) and (Cas) bacterial isolates and their metabolites show potential utility as a BCAs for the management of *P. nicotianae*. However, future studies should focus on identifying and characterizing the metabolic compounds synthesized by (Cas) isolate as biocontrol and growth-promoting agents. Additionally, further studies are needed to evaluate their anti-*Phytophthora* effects and persistence on the border of cartoon pot as a durable sustainable method for growers.

An increase in the sustainability of ornamental crop production and nurseries management is one of the main goals for this study. Therefore, the development of a bacterial biocontrol product against *P. nicotianae* should be primarily based on the direct screening of strains for their protective activity on plants against *Phytophthora* diseases, rather than on their *in vitro* antagonistic effects (Besset-Manzoni et al., 2019). However, under in *planta* test, further investigations will be required to determine if the selected bacterial strains are effective in the rhizosphere and have good root colonization. Moreover, it is essential to evaluate the biocontrol efficacy using the photosynthetic parameters such as carbon assimilation, leaf transpiration, and stomatal conductance on *Phytophthora*-inoculated plants with bacterial isolates. Additionally, comparative genome analysis could also be done in the future to reveal the potential uniqueness of BCAs strains versus other strains known as biocontrol agents.

7.6 References

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CHAPTER 8: CONCLUSION

8.1 Conclusion

Phytophthora species pose a major challenge to nursery production systems as they are capable of causing significant damage. They are prevalent in the nursery industry and the risk of their spread is expected to increase due to factors such as the movement of plants globally, climate change, and the introduction of susceptible host plants. These factors will have a significant impact on *Phytophthora* geographic distribution, virulence, and host range in the future, and it is likely that new combinations of host-pathogens or new *Phytophthora* hybrids will appear.

A European literature survey of nurseries revealed a high diversity of *Phytophthora* species, with a total of 77 identified, of which *P. ramorum* and *P. cinnamomi* were the most frequently isolated. Furthermore, many forest plant species typical of the Mediterranean region were found to be affected by more than ten *Phytophthora* species.

In Italian nurseries alone, 44 *Phytophthora* species were found associated with horticultural, forest and ornamental plant species. The high diversity of *Phytophthora* taxa found in Tuscany, Sardinia and Sicily nurseries may be related to plant trading and the geographic and climatic characteristics of this region.

Phytophthora ramorum, and *P. lateralis* were the only two *Phytophthora* species found in Italian plant nurseries that are included on the EPPO quarantine list. However, their presence in Italian nurseries confirms that they were not stopped despite strict quarantine regulations.

In general, nursery producers are faced with a number of challenges that impact their production, including high costs of phytosanitary treatments, strict regulations for eradicating invasive plant pathogens, and the burden of maintaining biosecurity measures.

In light of the global impact of *Phytophthora* diseases, it is crucial to prevent primary infections. This can be achieved through implementing effective management strategies to reduce the risk of infestation by *Phytophthora* pathogens, as well as developing simple, efficient, early, and cost-effective detection methods such as point-of-care (POC) (Baldi & La Porta, 2020) and new nucleic acid-based detection methods. These methods are particularly important for hazardous species when implementing certification schemes for pathogen-free planting material.

Detection and identification of *Phytophthora* species in nurseries is essential as it can reveal the presence of alien invasive pathogens and the associated risks. Understanding the epidemiology of the individual detected *Phytophthora* species will aid in the development of targeted disease management strategies. These strategies are crucial in preventing potential outbreaks and invasions in new region caused by these pathogens.

Moreover, in order to prevent the spread of disease, there is an urgent need for increased efforts from government agencies, international health organizations, forest nurseries, citizens and volunteers to raise awareness about the dangers posed by new introductions of *Phytophthoras*. It is also essential to provide tools, transfer knowledge, and provide training to farmers. Social networks can also play a role in addressing these problems and help to reduce the threats in practice.

In this study, a detailed questionnaire using Google forms was prepared to collect data on *Phytophthora* infections in Italian nurseries. The questionnaire was sent via email to 65 Italian nurseries (Appendix G) and a journal article for growers was also prepared (Appendix H).

Unfortunately, no responses were received from the questionnaire study. However, to increase engagement, it may be better to post the survey on a specific social media channel or embed it on relevant website pages for nursery growers, making it easily accessible as an online decision

tool, similar to the "Clean Water3" website in the USA. In this way, growers would be able to answer questions about their operations and facilities and receive a disease risk score, helping them to make necessary changes to reduce their risk of *Phytophthora* infections (Parke et al., 2019).

Within the continued evolution of *Phytophthora* populations, the biological control approach for managing this disease within the framework of integrated plant protection strategies is the best practical and one of the most environmentally sustainable methods, it involves inhibiting plant pathogens, enhancing plant immunity, and/or promoting plant growth. However, to improve the sustainability of this approach, it is important to further understand the interactions between biological control agents and the environment, as well as to develop of new eco-friendly products formulation that can deliver plant extracts, microbial biological agent and plant growth promoting strains.

This study aimed to identify new bacterial microorganisms and plant extracts that could be used as biological control agents against *Phytophthora* diseases.

By using conventional methods that target cultivable microorganisms, the goal was to screen and select potential strains of biological control agent and plant growth promoter bacteria. However, it is unclear which individual trait or set of traits can predict the best BCAs, as some of the selected bacteria like *Gluconobacter kondonii* may enter into parasitic interactions with plants. This can be due to changes in the host physiology or the environment that may trigger pathogenicity in endophyte bacteria that were previously considered as mutualist and plant growth promoters (Ansari et al., 2016). Therefore, it is essential to assess the risk of every potential bacterial biocontrol agent to avoid using potential plant pathogens as agricultural inputs.

Moreover, despite the anti-*Phytophthora* activities shown in *in vitro* tests with the highest *Phytophthora* mycelium inhibition (100%) with some isolates, in accordance with other studies, *in planta* experiments in growth chambers have shown that the strains expressing the best activities *in vitro* are not always the strains showing the best results *in planta* (Besset-Manzoni et al., 2019).

The complementarity of the two systems chosen (*in-vitro* culture media, and whole plants) has helped in selecting and identifying potential modes of action and has provided a desirable characteristic in a bacterial BCAs against *Phytophthora* species.

However, future studies should focus on:

- 1) Screening a collection of bacterial strains and characterizing them at the genomics level.
- (2) Studies on how the BCAs affect the survival or germination of sporangia, zoospore, and other *Phytophthora* survival structures.
- (3) Investigating BCAs survival in soil and colonization of root systems, taking into consideration the integrated study of soil-plant systems and interactions with other microorganisms that may result in multispecies pathogenic microbial interactions (Lamichhane & Venturi, 2015)
- 4) Care should be taken towards plant resistance development by BCAs bacterium against *Phytophthora* diseases.
- 5) Attention must be paid to the process of selecting BCAs against *Phytophthora sp.* including issues such as curative and protective modes of action, the development of these organisms into an effective biopesticide large-scale production, formulation, preservation conditions, shelf life, and application methods that might be improved on various crops and under different climatic condition.

Furthermore, with the hope of reducing the overutilization of fungicides in agricultural soils, plant extracts can show great potential as novel, low cost, and environmentally friendly

botanical fungicides. However, a great variability between extraction protocols and plants influences the fungicidal properties. It is often quite difficult to compare the results obtained from different studies, due to the variation in extract compositions depending on the geographical region, the variety, age of the plant, the biotic and abiotic stress, and the method of extraction of the extract. Therefore, extraction techniques need to be done according to the target bio-compounds in a sustainable way. From this study, one aqueous plant extract, *Tulbaghia violacea*, closely related to the genus *Allium* with marasminin the main abundant compound, has demonstrated a potential bio fungicide by combining *in vitro* and *in planta* protocols and by targeting different steps of the fungal and phytophthora pathogen life cycle (spore germination, mycelial growth, and plant invasion). However, future studies with *Tulbaghia* treatment must be tested *in planta* in a formulated form under both controlled environments and field conditions.

Finally, this study draws attention to the significant increase in the number of *Phytophthora* species in European plant nurseries and emphasizes the need to implement effective biosecurity measures to minimize the ecological and economic threat posed to forests, food chains, natural ecosystems, and urban areas.

The application of BCAs and plant extracts in an integrated management could be considered a satisfactory level for sustainable and environmentally management strategies of *Phytophthora* disease. However potential bottlenecks in *in planta* application methods should be tackled early in the selection process.

Additionally, in the future, to help optimize the usage of bio fungicide, more research and project concerning combinations of biological control treatments, involving a low-dose fungicides, plant extracts and biocontrol agent aligned with the cultural practices, cultivar resistance, and early detection of *Phytophthora* disease through modelling or image-based detection systems can go a long way towards controlling *Phytophthora* diseases.

On the other hand, biological control application as a site-specific spray and the use of adequate sufficient doses, especially within the precision-agriculture and ‘smart farming’ approach (Roberts et al., 2021) can give advantages to decreasing costs associated with biological control application, where high application rates over larger land areas are not economically-feasible.

8.2 References

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APPENDIX A

Phytophthora and host diversity in Italian nurseries

<i>P. acerina</i> (Clade 2)				
YEAR OF INFECTION	HOST	REGION	TYPE OF NURSERIES	REFERENCE
2019-2020	<i>Alnus incana</i>	Friuli Venezia Giulia	Forest	Bregant et al., 2021
<i>P. alni subsp. uniformis</i> (Clade 7)				
YEAR OF INFECTION	HOST	REGION	TYPE OF NURSERIES	REFERENCE
NA	<i>Alnus cordata</i>	Tuscany	Forest	Santini et al., 2003
<i>P. asparagi</i> (Clade 6a)				
YEAR OF INFECTION	HOST	REGION	TYPE OF NURSERIES	REFERENCE
2004	<i>Agave attenuata</i>	Sicily		Cacciola et al, 2006
NA	<i>Dracaena sp.</i>	NA		Badalà et al. 2009
NA	<i>Phormium tenax</i>	NA		Badalà et al. 2009
<i>P. bilorbang</i> (Clade 6b)				
YEAR OF INFECTION	HOST	REGION	TYPE OF NURSERIES	REFERENCE
2019-2020	<i>Phyllirea latifolia</i>	Sardinia	Forest	Bregant et al., 2021
<i>P. cactorum</i> (Clade 1)				
YEAR OF INFECTION	HOST	REGION	TYPE OF NURSERIES	REFERENCE
2019-2020	<i>Castanea sativa</i>	Sardinia	Forest	Bregant et al., 2021
(1993-1994) (last 5 year)	<i>Juglans regia</i>	Lazio-central Italy		Belisario et al., 1995; 1997, 2007
2004	<i>Photinia fraseri</i> cv. Red Robin	Marche		Vettraino et al., 2006-2005
2006	<i>Prunus armeniaca</i>	Sicily		Pane el al., 2009
Last 5 years	<i>Quercus sp.</i>	Central Italy		Belisario et al., 2007
Last 5 years	<i>Rhododendron sp.</i>	Central Ital		Belisario et al., 2007
2002	<i>Fragaria sp.</i>	Basilicata		Camele et al., 2006
Last 5 years	<i>Viburnum sp.</i>	Central Italy		Belisario et al., 2007

2003-2004 / NA	<i>Viburnum tinus</i>	Tuscany		Belisario et al., 2004; Migliorini et al., 2015
2012-2013	<i>Diospyros kaki</i>	Calabria		Prigigallo et al., 2016
<i>P. cambivora (Clade 7)</i>				
YEAR OF INFECTION	HOST	REGION	TYPE OF NURSERIES	REFERENCE
2012-2013	<i>Rosmarinus officinalis</i>	Apulia		Prigigallo et al., 2015
2012-2013	<i>Petunia parviflora</i>	Apulia		Prigigallo et al., 2015
2012-2013	<i>Lobelia erinus</i>	Apulia		Prigigallo et al., 2015
2012-2013	<i>Dahlia campanulata</i>	Apulia		Prigigallo et al., 2015
2012-2013	<i>Impatiens hawkeri</i>	Apulia		Prigigallo et al., 2015
2012-2013	<i>Prunus mahaleb</i>	Apulia		Prigigallo et al., 2015
<i>P. crassamura-(Clade 6)</i>				
YEAR OF INFECTION	HOST	REGION	TYPE OF NURSERIES	REFERENCE
2011	<i>Picea abies</i>	Sardinia		Scanu et al., , 2015
<i>P. cinnamomi (Clade 7)</i>				
YEAR OF INFECTION	HOST	REGION	TYPE OF NURSERIES	REFERENCE
Last 5 years	<i>Quercus sp.</i>	Central Italy		Belisario et al., 2007
1996	<i>Vaccinium corymbosum</i>	Piedmont		Tamietti, 2003
2019-2020	<i>Abies alba</i>	Veneto	Forest	Bregant et al., 2021
NA	<i>Arbutus unedo</i>	Tuscany		Migliorini et al., 2015
2019-2020	<i>Castanea sativa</i>	sardinia		Bregant et al., 2021
2019-2020	<i>Fagus sylvatica</i>	Veneto	Forest	Bregant et al., 2021
2012-2013	<i>Grevillea lanigera</i>	Apulia		Prigigallo et al., 2015
Last 5 years-(2019, 2020)	<i>Juglans regia</i>	Central Italy _Sardinia		Belisario et al., 2007- Bregant et al., 2021
2011	<i>Kalmia latifolia L ‘Olympic Fire</i>	Piedmont		Garibaldi et al., 2012
NA	<i>Laurus nobilis</i>	Tuscany		Migliorini et al., 2015
NA	<i>Myrtus communis</i>	Tuscany		Migliorini et al., 2015

NA	<i>Platanus × acerifolia</i>	Lazio		Pilotti et al., 2014
2013-2016 (2019, 2020)	<i>Quercus ilex</i>	Sardinia	Forest	Seddaiu et al., 2020- Bregant et al., 2021
2013-2016	<i>Quercus suber</i>	Sardinia	Forest	Seddaiu et al., 2020
Last 5 years	<i>Viburnum sp.</i>	Central Italy		Belisario et al., 2007
2003-2004	<i>Viburnum tinus</i>	Tuscany		Belisario et al., 2004
NA	<i>Chamaecyparis</i>	NA		Parrini et al., 1997
1982	<i>Fortunella japonica</i>	NA		Jung et al., 2016
1982	<i>Fortunella japonica x Citrus aurantifolia</i>	NA		Jung et al., 2016
2007	<i>Olea europea</i>	NA		Jung et al., 2016
2011	<i>Pinus pinea</i>	NA		Jung et al., 2016
2009	<i>Thuja occidentalis</i>	NA		Jung et al., 2016
<i>P. cinnamomi</i> -like (Clade 7)				
YEAR	HOST	REGION		REFERENCE
2012-2013	<i>Grevillea lanigera</i>	Apulia		Prigigallo et al., 2015
2012-2013	<i>Cercis siliquastrum</i>	Apulia		Prigigallo et al., 2015
2012-2013	<i>Bougainvillea glabra</i>	Apulia		Prigigallo et al., 2015
<i>P. citricola</i> senso stricto (Clade 2)				
YEAR	HOST	REGION		REFERENCE
1982	<i>Citrus sp.</i>	NA		Jung et al., 2016
1982	<i>Fortunella japonica x Citrus aurantifolia</i>	NA		Jung et al., 2016
1982-2013	<i>Fortunella japonica</i>	NA		Jung et al., 2016
<i>P. citricola</i> (Clade 2)				
YEAR	HOST	REGION		REFERENCE
1977-2010	<i>Citrus aurantium</i>	NA		Jung et al., 2016
1982-1985	<i>Citrofortunella microcarpa x citrus aurantium</i>	Sicily		Magnano di San Lio et al., 1986
1982-1985	<i>Citrofortunella microcarpa x citrus volkameriana</i>	Sicily		Magnano di San Lio et al., 1986

2003-2004	<i>Cytisus scoparius</i>	Sicily	Pane et al., 2005
2012/2013	<i>Dahlia campanulata</i>	Apulia	Prigigallo et al., 2015
1982-1985	<i>Fortunella margarita x citrus aurantium</i>	Sicily	Magnano di San Lio et al., 1986
1982-1985	<i>Fortunella margarita x citrus volkameriana</i>	Sicily	Magnano di San Lio et al., 1986
2012/2013	<i>Lobelia erinus</i>	Apulia	Prigigallo et al., 2015
NA	<i>Myrtus communis</i>	Southern Italy	Cacciola et al., 1997
1977-2010	<i>Poncirus trifoliata x Citrus sinensis</i>		Jung et al., 2016
2012/2013	<i>Prunus mahaleb</i>	Apulia	Prigigallo et al., 2015
NA	<i>Viburnum tinus</i>	Tuscany	Migliorini et al., 2015
1982-1985	<i>Fortunella japonica x citrus aurantium</i>	Sicily	Magnano di San Lio et al., 1986
1982-1985	<i>Fortunella japonica-x citrus volkameriana</i>	Sicily	Magnano di San Lio et al., 1986
1982-2013	<i>Citrus sp.</i>	NA	Jung et al., 2016

P. citrophthora (Clade 2)

YEAR OF INFECTION	HOST	REGION	TYPE OF NURSERIES	REFERENCE
NA	<i>Arbutus unedo</i>	Tuscany		Migliorini et al., 2015
NA	<i>Buxus sempervirens</i>	Tuscany		Migliorini et al., 2015
NA	<i>Ceanothus thyrsiflorus</i>	Tuscany		Migliorini et al., 2015
	<i>Citrus aurantium</i>	Apulia		Ipolito et al, 2002 -2004
1982-1985	<i>Citrofortunella microcarpa x citrus aurantium</i>	Sicily		Magnano di San Lio et al., 1986
1982-1985	<i>Citrofortunella microcarpa x citrus volkameriana</i>	Sicily		Magnano di San Lio et al., 1986
NA	<i>Convolvulus cneorum</i>	Southern Italy		Cacciola et al., 1997
last 6 year +2004	<i>Rosa indica cv. Major rootstock</i>	Sicily		Salamone et al., 2011
NA	<i>Euonymus fortunei</i>	Tuscany		Migliorini et al., 2015
1982-1985	<i>Fortunella japonica x citrus aurantium</i>	Sicily		Magnano di San Lio et al., 1986

1982-1985	<i>Fortunella japonica-x citrus volkameriana</i>	Sicily		Magnano di San Lio et al., 1986
1982-1985	<i>Fortunella margarita x citrus aurantium</i>	Sicily		Magnano di San Lio et al., 1986
1982-1985	<i>Fortunella margarita x citrus volkameriana</i>	Sicily		Magnano di San Lio et al., 1986
2019-2020	<i>Laurus nobilis</i>	Sardinia	Forest	Bregan et al., 2021
2004	<i>Mandevilla × amabilis 'Alice du Pont'</i>	Sicily		Pane et al., 2010
2004	<i>Mandevilla splendens</i>	Sicily		Pane et al., 2010
2004	<i>Mandevilla sanderi 'Alba'</i>	Sicily		Pane et al., 2010
2004	<i>Mandevilla 'My Fair Lady'</i>	Sicily		Pane et al., 2010
2004	<i>Mandevilla × amabilis 'Scarlet Pimpernel'</i>	Sicily		Pane et al., 2010
2005	<i>Pandorea jasminoides</i>	Sicily		Pane et al., 2008
2006	<i>Prunus armeniaca</i>	Sicily		Pane et al., 2009
NA	<i>Rhamnus sp.</i>	Southern Italy		Cacciola et al., 1997
2012/2013	<i>Rosmarinus officinalis</i>	Apulia		Prigigallo et al., 2015
NA	<i>Citrus aurantium</i>	Basilicata		Salama Eid et al., 2008
NA	<i>Carrizo citrange rootstocks</i>	Basilicata		Salama Eid et al., 2008
NA	<i>Citrus × volkameriana</i>	Basilicata		Salama Eid et al., 2008
NA	<i>Citrus macrophylla</i>	Basilicata		Salama Eid et al., 2008
1990-2010	<i>Olea europea</i>	NA		Jung et al., 2016
<i>P. cryptogea (Clade 8)</i>				
YEAR	HOST	REGION		REFERENCE
2015	<i>Musa acuminata</i>	Sicily		Pane et al., 2018
NA-(2012-2013)	<i>Convolvulus cneorum</i>	Southern Italy-Apulia		Cacciola et al., 1997-Prigigallo et al., 2015
2004	<i>Lantana camara</i>	Sicily		Cacciola et al., 2005
2004	<i>Mandevilla × amabilis 'Alice du Pont'</i>	Sicily		Pane et al., 2010
2004	<i>Mandevilla splendens</i>	Sicily		Pane et al., 2010
2004	<i>Mandevilla sanderi 'Alba'</i>	Sicily		Pane et al., 2010

2004	<i>Mandevilla 'My Fair Lady'</i>	Sicily		Pane et al., 2010
2004	<i>Mandevilla × amabilis 'Scarlet Pimpernel'</i>	Sicily		Pane et al., 2010
NA	<i>Prunus laurocerasus</i>	Tuscany		Migliorini et al., 2015
2013-2016	<i>Quercus ilex</i>	Sardinia	Forest	Seddaiu et al., 2020
2013-2016	<i>Quercus suber</i>	Sardinia	Forest	Seddaiu et al., 2020
Last 5 years	<i>Quercus laevis</i>	Central Italy		Belisario et al., 2007
Last 5 years	<i>Rhododendron sp.</i>	Central Italy		Belisario et al., 2007
2012-2013	<i>Rosmarinus officinalis</i>	Apulia		Prigigallo et al., 2015
2013	<i>Salvia officinalis</i>	Liguira		Garibaldi et al., 2014
(2012-2013)-2001-2013	<i>Salvia sp.</i>	Apulia-Sicily-Liguira		Prigigallo et al., 2015-Cacciola et al., 2002-Bertetti et al., 2014
NA-2001	<i>Pistacia lentiscus</i>	Sardinia		Jung et al., 2016
2004	<i>Callistephus sp.</i>	NA		Jung et al., 2016
2004	<i>Ceanothus sp.</i>	NA		Jung et al., 2016
1997-2004	<i>Cistus sp.</i>	NA		Jung et al., 2016
1998	<i>Dimorphanthera sinuata</i>	NA		Jung et al., 2016
1997-2004	<i>Pittosporum sp.</i>	NA		Jung et al., 2016
1982-2013	<i>Citrus sp.</i>	NA		Jung et al., 2016
2012-2013	<i>Diospyros kaki</i>	Calabria		Prigigallo et al., 2016
<i>P. drechsleri</i> (Clade 8a)				
YEAR OF INFECTION	HOST	REGION	TYPE OF NURSERIES	REFERENCE
2003, 2004	<i>Cytisus scoparius</i>	Sicily		Pane et al., 2005
2004	<i>Escallonia laevis</i>	NA		Jung et al., 2016
<i>P. gonapodyides</i> (Clade 6)				
YEAR OF INFECTION	HOST	REGION	TYPE OF NURSERIES	REFERENCE
2008-2010	<i>Quercus ilex</i>	NA		Jung et al., 2016
<i>P. hedraiaandra</i> (Clade 1)				
YEAR OF INFECTION	HOST	REGION	TYPE OF NURSERIES	REFERENCE

2004	<i>Viburnum tinus</i>	Tuscany		Belisario et al., 2006
Last 5 years	<i>Viburnum sp.</i>	Central Italy		Belisario et al., 2007
<i>P. hydropathica (Clade 9)</i>				
YEAR OF INFECTION	HOST	REGION	TYPE OF NURSERIES	REFERENCE
2012	<i>Viburnum tinus</i>	Lazio		Vitale et al., 2014
<i>P. inundata (Clade 6)</i>				
YEAR OF INFECTION	HOST	REGION	TYPE OF NURSERIES	REFERENCE
2015	<i>Musa acuminata</i>	Sicily		Pane et al., 2018
1998-2004	<i>Syagrus romanzoffiana</i>	NA		Jung et al., 2016
<i>P. italica (Clade 1)</i>				
YEAR OF INFECTION	HOST	REGION	TYPE OF NURSERIES	REFERENCE
1990	<i>Myrtus communis</i>	Sardinia		Belisario et al., 1993- Cacciola et al., 1996
<i>P. lacustris (Clade 6)</i>				
YEAR OF INFECTION	HOST	REGION	TYPE OF NURSERIES	REFERENCE
1999	<i>Prunus persica</i>	Emilia Romagna	Fruit tree	Nechwatal et al., 2013
<i>P. lateralis (Clade 8)</i>				
YEAR OF INFECTION	HOST	REGION	TYPE OF NURSERIES	REFERENCE
2012/2013	<i>Cyclamen persicum</i>	Apulia		Prigigallo et al., 2015
2012/2013	<i>Tagetes erecta</i>	Apulia		Prigigallo et al., 2015
2012/2013	<i>Petunia parviflora</i>	Apulia		Prigigallo et al., 2015
2012/2013	<i>Punica granatum</i>	Apulia		Prigigallo et al., 2015
<i>P. meadii (Clade 2)</i>				
YEAR OF INFECTION	HOST	REGION	TYPE OF NURSERIES	REFERENCE
2012/2013	<i>Mentha sp.</i>	Apulia		Prigigallo et al., 2015
2012/2013	<i>Petunia parviflora</i>	Apulia		Prigigallo et al., 2015
2012/2013	<i>Lobelia erinus</i>	Apulia		Prigigallo et al., 2015
2012/2013	<i>Impatiens hawkeri</i>	Apulia		Prigigallo et al., 2015

<i>P. meadi</i> like (Clade 2)				
YEAR OF INFECTION	HOST	REGION	TYPE OF NURSERIES	REFERENCE
2012/2013	<i>Dahlia campanulata</i>	Apulia		Prigigallo et al., 2015
<i>P. mediterranea</i> sp. nov. <i>Phytophthora mediterranea</i> (Clade 7c)				
YEAR OF INFECTION	HOST	REGION	TYPE OF NURSERIES	REFERENCE
2019- 2020	<i>Myrtus communis</i>	Sardinia	Forest	Bregant et al., 2021
<i>P. megasperma</i> (Clade 7c)				
YEAR OF INFECTION	HOST	REGION	TYPE OF NURSERIES	REFERENCE
2002	<i>Cytisus scoparius</i>	Central Italy		Vettraino et al., 2003
2019-2020	<i>Laurus nobilis</i>	Sardinia		Bregant et al., 2021
2011	<i>Abies alba</i>	NA		Jung et al., 2016
1990-2010	<i>Olea europea</i>	NA		Jung et al., 2016
<i>P. megasperma sensu stricto</i> (Clade 7c)				
YEAR OF INFECTION	HOST	REGION	TYPE OF NURSERIES	REFERENCE
2013	<i>Celtis australis</i>	Umbria		Luongo et al., 2015
<i>P. multivora</i> (Clade 2)				
YEAR OF INFECTION	HOST	REGION	TYPE OF NURSERIES	REFERENCE
2013 - 2015	<i>Cytisus scoparius</i>	Sicily		Puglisi et al., 2016
2013 - 2015	<i>Hedera helix</i>	Sicily		Puglisi et al., 2016
2013 - 2015	<i>Polygala myrtifolia</i>	Sicily		Puglisi et al., 2016
1998, 2004	<i>Hibiscus rosa-sinensis</i>	NA		Jung et al., 2016
<i>P. nicotianae</i> (Clade 1)				
YEAR OF INFECTION	HOST	REGION	TYPE OF NURSERIES	REFERENCE
2007	<i>Agapanthus</i> sp.	Liguria		Mammella et al., 2011, Mammella et al., 2013
2012-2013	<i>Allium schoenoprasum</i>	Apulia		Prigigallo et al., 2015
2007	<i>Anemone americana</i>	Liguria		Mammella et al., 2011, Mammella et al., 2013

2005	<i>Anthurium sp.</i>	Sicily	Mammella et al., 2011, Mammella et al., 2013
NA	<i>Arbutus unedo</i>	Southern Italy- Tuscany	Cacciola et al., 1997-Migliorini et al., 2015
2012-2013	<i>Armeria maritima</i>	Apulia	Prigigallo et al., 2015
2011-2013	<i>Bignonia venusta</i>	Sicily	Aiello et al., 2018
2006, 2010	<i>Bougainvillea sp.</i>	NA	Jung et al., 2016
2012	<i>Buxus sempervirens</i>	Central Italy-Tuscany	Luongo et al., 2013-Migliorini et al., 2015
NA	<i>Callistemon citrinus</i>	Southern Italy	Cacciola et al., 1997
2004	<i>Callistephus sp.</i>	NA	Jung et al., 2016
2002	<i>Ceanothus sp.</i>	Sicily	Mammella et al., 2011, Mammella et al., 2013
NA	<i>Ceanothus thyrsiflorus</i>	Tuscany	Migliorini et al., 2015
2009	<i>Chamaerops humilis</i>	Sicily	Faedda et al., 2011
NA	<i>Chamaleucium uncinatum</i>	Apulia-Sicily	Biasi et al., 2016
2004	<i>Choisya ternata</i>	Liguira	Mammella et al., 2011, Mammella et al., 2013
2011-2013	<i>Cistus creticus</i>	Sicily	Aiello et al., 2018
2011-2013	<i>Cistus salvifolius</i>	Sicily	Aiello et al., 2018
NA	<i>Cistus sp.</i>	Southern Italy	Cacciola et al., 1997
NA-2011-2013	<i>Citrus aurantium</i>	Apulia -Sicily- Calabria	Ippolito et al., 2002-Ippolito et al., 2004- Eid et al., 2008- Aiello et al., 2018
2010	<i>Citrus limon</i>	NA	Jung et al., 2016
1982-1985	<i>Citrofortunella microcarpa x citrus aurantium</i>	Sicily	Magnano di San Lio et al., 1986
1982-1985	<i>Citrofortunella microcarpa x citrus volkameriana</i>	Sicily	Magnano di San Lio et al., 1986
2006	<i>Citrus sinensis x Poncirus</i>	NA	Jung et al., 2016
2004	<i>Cleome hassleriana</i>	NA	Jung et al., 2016

2012-2013-NA	<i>Convolvulus mauritanicus</i>	Apulia	Prigigallo et al., 2015; Biasi et al., 2016
NA-2004	<i>Correa reflexa</i>	Sicily	Biasi et al., 2016; Mammella et al., 2011, Mammella et al., 2013
2012/2013	<i>Cyclamen persicum</i>	Apulia	Prigigallo et al., 2015
2004	<i>Cyclamen sp.</i>	Sicily-Liguira	Mammella et al., 2011, Mammella et al., 2013
2012/2013	<i>Dahlia campanulata</i>	Apulia	Prigigallo et al., 2015
2008	<i>Daphne odora</i>	Piedmont	Garibaldi et al., 2009-Bertetti et al., 2009
2004-2006	<i>Dipladenia sp.</i>	NA	Jung et al., 2016
NA	<i>Dodonaea viscosa cv. Purpurea</i>	Sicily	Biasi et al., 2016
2005	<i>Dodonaea viscosa cv. Purpurea</i>	Sicily	Mammella et al., 2011, Mammella et al., 2013
2009	<i>Edgeworthia papyrifera</i>	Piedmont	Garibaldi et al., 2010
NA	<i>Elaeagnus angustifolia</i>	Tuscany	Migliorini et al., 2015
NA	<i>Escallonia laevis</i>	Southern Italy	Cacciola et al., 1997
1987-1988	<i>Eucalyptus globulus</i>	Lazio	Belisario 1990-1993
1987-1988	<i>Eucalyptus delegarensis</i>	Lazio	Belisario 1990-1993
1987-1988	<i>Eucalyptus regnans</i>	Lazio	Belisario 1990-1993
2009	<i>Feijoa sellowiana</i>	NA	Jung et al., 2016
1990	<i>Forsythia viridissima</i>	Campania	Pane and Magnano di San Lio, 1994
1982-1985	<i>Fortunella japonica x citrus aurantium</i>	Sicily	Magnano di San Lio et al., 1986
1982-1985	<i>Fortunella japonica-x citrus volkameriana</i>	Sicily	Magnano di San Lio et al., 1986
1982	<i>Fortunella japonica</i>	NA	Jung et al., 2016
1982-1985	<i>Fortunella margarita x citrus aurantium</i>	Sicily	Magnano di San Lio et al., 1986
1982-1985	<i>Fortunella margarita x citrus volkameriana</i>	Sicily	Magnano di San Lio et al., 1986

Last 5 years	<i>Fragaria vesca</i>	Central Italy		Belisario et al., 2007
2004	<i>Gardenia augusta</i>	NA		Jung et al., 2016
2011-2013	<i>Grevillea gracilis</i>	Sicily		Aiello et al., 2018
2011-2013	<i>Grevillea juniperina</i>	Sicily		Aiello et al., 2018
2012/2013	<i>Grevillea lanigera</i>	Apulia		Prigigallo et al., 2015
NA	<i>Grevillea rosmarinifolia</i>	Southern Italy		Cacciola et al., 1997
NA	<i>Hebe</i> × <i>Veronica buxifolia</i>	Sicily		Biasi et al., 2016
NA	<i>Hebex andersonii</i>	Southern Italy		Cacciola et al., 1997
NA	<i>Hedera helix</i>	Southern Italy		Cacciola et al., 1997
2019-2020	<i>Helichrysum italicum</i>	Sardinia	Forest	Bregant et al., 2021
2004	<i>Hibiscus sp.</i>	Calabria		Mammella et al., 2011- Mammella et al., 2013
NA	<i>Hibiscus rosa-sinensis</i>	Southern Italy		Cacciola et al., 1997
2000	<i>Howea sp.</i>	Sicily		Mammella et al., 2011, Mammella et al., 2013
2007	<i>Impatiens wallerana</i>	Liguria		Mammella et al., 2011, Mammella et al., 2013
2000	<i>Lavandula angustifolia</i>	Piedmont-Sicily		Biasi et al., 2016-Mammella et al., 2011, Mammella et al., 2013
2000-2019- 2020	<i>Lavandula officinalis</i>	Southern Italy- Marche-Apulia - Sicily-Liguria- Sardinia		Cacciola et al., 1997-Nardi et al., 2000-Biasi et al., 2016- Bregant et al., 2021
2012-2013; NA; Last 5years	<i>Lavandula sp.</i>	Apulia-Central Italy		Prigigallo et al., 2015, Biasi et al., 2016- Belisario et al., 2007
NA	<i>Lavandula stoechas</i>	Sicily		Biasi et al., 2016
NA	<i>Leptospermium sp.</i>	Southern Italy		Cacciola et al., 1997
2004	<i>Limonium sinensis</i>	Liguria		Mammella et al., 2011, Mammella et al., 2013
2012/2013	<i>Lobelia erinus</i>	Apulia		Prigigallo et al., 2015
2004	<i>Mandevilla</i> × <i>amabilis</i> 'Alice du Pont'	Sicily		Pane et al., 2010

2004	<i>Mandevilla splendens</i>	Sicily		Pane et al., 2010
2004	<i>Mandevilla sanderi 'Alba'</i>	Sicily		Pane et al., 2010
2004	<i>Mandevilla 'My Fair Lady'</i>	Sicily		Pane et al., 2010
2004	<i>Mandevilla × amabilis 'Scarlet Pimpernel'</i>	Sicily		Pane et al., 2010
2012/2013	<i>Mentha sp.</i>	Apulia		Prigigallo et al., 2015
2004	<i>Metrosideros polymorphus</i>	NA		Jung et al., 2016
1990-(2002, 1991,2007), 2019-2020	<i>Myrtus communis</i>	Southern Italy Sardinia--Sicily- Sardinia-Liguira- Tuscany-Sicily- Sardinia		Cacciola et al. 1997-Belisario et al., 1993- Biasi et al., 2016- Migliorini et al., 2015- Mammella et al., 2011, Mammella et al., 2013- Bregant et al., 2021
NA-2012/2013	<i>Olea europea</i>	Sicily -Apulia		Cacciola et al., 2005- Prigigallo et al., 2015
2005	<i>Pandorea jasminoides</i>	Sicily		Pane et al., 2008-Mammella et al., 2011, Mammella et al., 2013
NA	<i>Passiflora caerulea</i>	Southern Italy		Cacciola et al., 1997
2012/2013	<i>Petunia parviflora</i>	Apulia		Prigigallo et al., 2015
2012/2013	<i>Petunia sp.</i>	Apulia		Prigigallo et al., 2015
2004	<i>Phormium tenax</i>	Liguira		Mammella et al., 2011, Mammella et al., 2013
NA-2019- 2020	<i>Pistacia lentiscus</i>	Southern Italy- Sardinia	Forest	Cacciola et al., 1997- Bregant et al., 2021
1996-NA-	<i>Pittosporum sp.</i>	Sicily-Southern Italy		Mammella et al., 2011, Mammella et al., 2013- Cacciola et al., 1997
NA-2007	<i>Polygala myrtifolia</i>	Liguira		Biasi et al., 2016, Mammella et al., 2011 Mammella et al., 2013
2011-2013	<i>Polygala myrtifolia×oppositifolia</i>	Sicily		Aiello et al., 2018
1977-2010	<i>Poncirus trifoliata x Citrus sinensis</i>	NA		Jung et al., 2016

2006-2005-	<i>Prunus armeniaca</i>	Sicily, Calabria		Pane et al., 2009, Mammella et al., 2011, Mammella et al., 2013
NA	<i>Prunus lusitanica</i>	Tuscany		Migliorini et al., 2015
1999	<i>Prunus persica</i>	NA		Jung et al., 2016
2000	<i>Rhamnus alaternus</i>	Sicily		Mammella et al., 2011, Mammella et al., 2013
NA	<i>Rhamnus sp.</i>	Southern Italy		Cacciola et al., 1997
Last 5 years	<i>Rhododendron sp.</i>	Central Italy		Belisario et al., 2007
NA	<i>Rosmarinus officinalis</i>	Southern Italy-Sicily		Biasi et al., 2016; Cacciola et al., 1997
NA-Last 5 years	<i>Rosmarinus sp.</i>	Sicily-Central Italy		Biasi et al., 2016- Belisario et al., 2007
NA	<i>Ruta graveolens</i>	Sicily		Biasi et al., 2016
2003	<i>Skimmia japonica cv. Rubella</i>	Piedmont		Garibaldi et al., 2004-Bertetti et al., 2004
NA	<i>Solanum lycopersicum</i>	Sicily		La Spada et al., 2020
NA	<i>Citrus aurantium</i>	Basilicata, Calabria, Sicily		Salama Eid et al., 2008
NA	<i>Carrizo citrange rootstocks</i>	Basilicata, Calabria, Sicily		Salama Eid et al., 2008
NA	<i>Citrus × volkameriana</i>	Basilicata, Calabria, Sicily		Salama Eid et al., 2008
NA	<i>Citrus macrophylla</i>	Basilicata, Calabria, Sicily		Salama Eid et al., 2008
2012/2013	<i>Tagetes erecta</i>	Apulia		Prigigallo et al., 2015
NA	<i>Thuja occidentalis</i>	Tuscany		Migliorini et al., 2015
NA	<i>Thymus vulgaris</i>	Liguria		Martini et al., 2006
2009	<i>Trachycarpus fortunei</i>	Sicily		Cacciola et al., 2011
2012-2013	<i>Diospyros kaki</i>	Calabria		Prigigallo et al., 2015
<i>P. niederhauserii</i> (Calde 7b)				
YEAR OF INFECTION	HOST	REGION	TYPE OF NURSERIES	REFERENCE

2011-2013	<i>Grevillea gracilis</i>	Sicily		Aiello et al., 2018
2011-2013	<i>Grevillea thelemanniana</i> × <i>oppositifolia</i>	Sicily		Aiello et al., 2018
2012-2013	<i>Convolvulus cneorum</i>	Apulia		Prigigallo et al., 2015
2012-2013	<i>Salvia</i> sp.	Apulia		Prigigallo et al., 2015
2012-2013	<i>Olea europea</i>	Apulia		Prigigallo et al., 2015
2012-2013	<i>Diospyros kaki</i>	Calabria		Prigigallo et al., 2015
2012-2013	<i>Cyclamen persicum</i>	Apulia		Prigigallo et al., 2015
2012-2013	<i>Cyclamen persicum</i>	Apulia		Prigigallo et al., 2015
2012-2013	<i>Fuchsia magellanica</i>	Apulia		Prigigallo et al., 2015
2012-2013	<i>Dahlia campanulata</i>	Apulia		Prigigallo et al., 2015
NA	<i>Acacia dealbata</i>	Sicily		Abad et al., 2014
NA	<i>Hedera helix</i>	Sicily-Marche		Abad et al., 2014
2008-NA	<i>Pistacia lentiscus</i>	Sardinia	Forest	Scanu et al., 2011-Abad et al., 2014
2003	<i>Cistus salvifolius</i>	Sicily		Cacciola et al., 2009-Abad et al., 2014
2003	<i>Callistemon citrinus</i>	Sicily		Cacciola et al., 2009-Abad et al., 2014
2003	<i>Banksia speciosa</i>	Liguria		Cacciola et al., 2009-Abad et al., 2014
2003	<i>Banksia baxteri</i>	Liguria		Cacciola et al., 2009-Abad et al., 2014
2003	<i>Banksia prionotes</i>	Liguria		Cacciola et al., 2009-Abad et al., 2014
2010-NA	<i>Acacia dealbata</i>	Liguria-Sicily		Faieda et al., 2013-Abad et al., 2014
1982-2013	<i>Citrus</i> sp.	NA		Jung et al., 2016
NA	<i>Banksia</i> sp.	Sicily		Abad et al., 2014
<i>P. niederhauserii</i> -like (Clade 7b)				
YEAR OF INFECTION	HOST	REGION	TYPE OF NURSERIES	REFERENCE
2012-2013	<i>Cyclamen persicum</i>	Apulia		Prigigallo et al., 2015

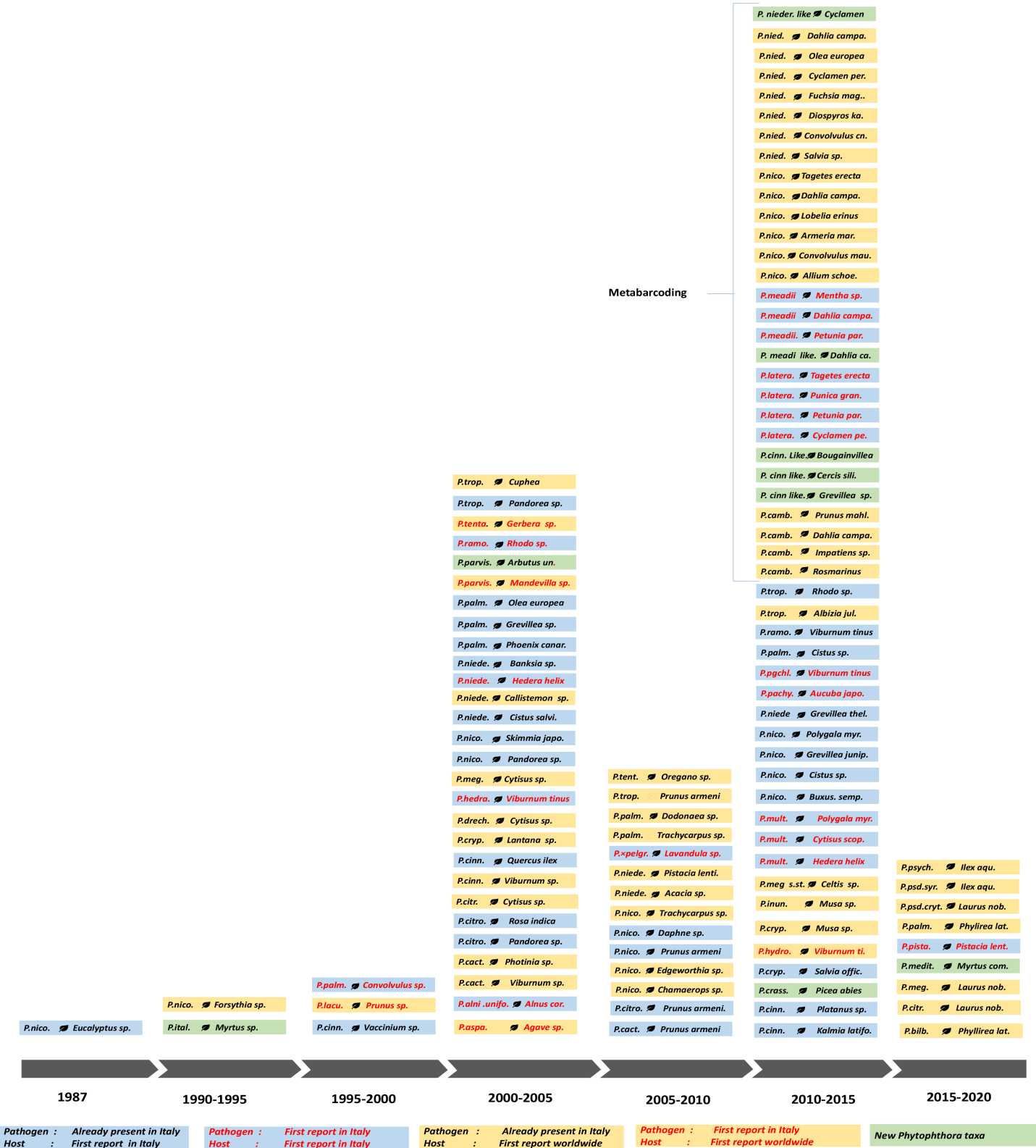
<i>P. pachyplora</i> (Clade 2)				
YEAR OF INFECTION	HOST	REGION	TYPE OF NURSERIES	REFERENCE
2012-2014	<i>Aucuba japonica</i>	Tuscany		Ginetti et al., 2015
<i>P. palmivora</i> (Clade 4)				
YEAR OF INFECTION	HOST	REGION	TYPE OF NURSERIES	REFERENCE
2010	<i>Dodonaea viscosa</i> cv. <i>Purpurea</i>	Sicily		Aiello et al., 2011
2004-2006	<i>Phoenix canariensis</i>	Sicily		Pane et al., 2007
2000-2003	<i>Grevillea alpina</i>	Sicily		Cacciola et al., 2003
2000-2003	<i>Grevillea juniperina</i>	Sicily		Cacciola et al., 2003
2000-2003	<i>Grevillea lavandulacea</i>	Sicily		Cacciola et al., 2003
2000-2003	<i>Grevillea rosmarinifolia</i>	Sicily		Cacciola et al., 2003
2000-2003	<i>Grevillea lavandulacea</i> × <i>rosmarinifolia</i>	Sicily		Cacciola et al., 2003
2000-2003	<i>Grevillea baueri</i> × <i>lavandulacea</i>	Sicily		Cacciola et al., 2003
2009	<i>Trachycarpus fortunei</i>	Sicily		Cacciola et al., 2011
NA	<i>Olea europea</i>	Sicily		Cacciola et al., 2000
NA	<i>Prunus lusitanica</i>	Tuscany		Migliorini et al., 2015
NA	<i>Pittosporum tobira</i>	Tuscany		Migliorini et al., 2015
NA	<i>Cupressus sempervirens</i>	Tuscany		Migliorini et al., 2015
NA	<i>Buxus sempervirens</i>	Tuscany		Migliorini et al., 2015
NA	<i>Arbutus unedo</i>	Tuscany		Migliorini et al., 2015
NA	<i>Ceanothus thyrsiflorus</i>	Tuscany		Migliorini et al., 2015
NA	<i>Convolvulus cneorum</i>	Southern Italy		Cacciola et al., 1997
2011-2013	<i>Cistus creticus</i>	Sicily		Aiello et al., 2018
2011-2013	<i>Cistus salvifolius</i>	Sicily		Aiello et al., 2018
2011-2013	<i>Dodonaea viscosa</i> cv. <i>Purpurea</i>	Sicily		Aiello et al., 2018
Last 5 years	<i>Hedera helix</i>	Central Italy		Belisario et al., 2007
Last 5 years	<i>Lavandula</i> sp.	Central Italy		Belisario et al., 2007
Last 5 years	<i>Pittosporum</i> sp.	Central Italy		Belisario et al., 2007

NA	<i>Simmondsia chin</i>	Apulia		Magnano di San Lio et al., 1986
NA	<i>Chamaedorea elegans</i>	Sicily		Magnano di San Lio et al., 1986
NA	<i>Olea europea</i>	Sicily		Cacciola et al., 2005-Cacciola et al., 2011
2019-2020	<i>Phyllirea latifolia</i>	Sardinia	Forest	Bregant et al., 2021
2004-2005	<i>Agave attenuata</i>	NA		Jung et al., 2016
2009-2010	<i>Chamaerops humilis</i>	NA		Jung et al., 2016
2004-2008	<i>Cyclamen persicum</i>	NA		Jung et al., 2016
1996	<i>Pittosporum tenuifolium</i>	NA		Jung et al., 2016
<i>P. parvispora (Clade 7)</i>				
YEAR OF INFECTION	HOST	REGION	TYPE OF NURSERIES	REFERENCE
2019-2020	<i>Arbutus unedo</i>	Sardinia	Forest	Bregant et al., 2021
2004	<i>Mandevilla sp.</i>	Sicily		Pane et al., 2010
2008	<i>Arbutus unedo</i>	Sardinia	Forest	Scanu et al., 2014
<i>P. pistachiae (Clade 7)</i>				
YEAR OF INFECTION	HOST	REGION	TYPE OF NURSERIES	REFERENCE
2019-2020	<i>Pistacia lentiscus</i>	Sardinia	Forest	Linaldeddu et al., 2020-Bregant et al., 2021
<i>P. × pelgrandis (Clade 1)</i>				
YEAR OF INFECTION	HOST	REGION	TYPE OF NURSERIES	REFERENCE
2007	<i>Lavandula stoechas</i>	Liguria		Faedda et al., 2013
<i>P. plurivora (Clade 2)</i>				
YEAR	HOST	REGION		REFERENCE
2013-2016	<i>Quercus ilex</i>	Sardinia	Forest	Seddaiu et al., 2020
2013-2016	<i>Quercus suber</i>	Sardinia	Forest	Seddaiu et al., 2020
2019-2020	<i>Alnus incana</i>	Friuli Venezia Giulia	Forest	Bregant et al., 2020
<i>P. pseudocryptogea (Clade 8)</i>				

YEAR OF INFECTION	HOST	REGION	TYPE OF NURSERIES	REFERENCE
2019-2020	<i>Laurus nobilis</i>	Sardinia		Bregant et al., 2021
<i>P. pseudosyringae</i> (Clade 3)				
YEAR OF INFECTION	HOST	REGION	TYPE OF NURSERIES	REFERENCE
2012/2013	<i>Cercis siliquastrum</i>	Apulia		Prigigallo et al., 2015
2019-2020	<i>Ilex aquifolium</i>	Sardinia	Forest	Bregant et al., 2021
<i>P. psychrophila</i> (Clade 3)				
YEAR	HOST	REGION		REFERENCE
2019-2020	<i>Ilex aquifolium</i>	Sardinia	Forest	Bregant et al., 2021
<i>P. ramorum</i> (Clade 8c)				
YEAR OF INFECTION	HOST	REGION	TYPE OF NURSERIES	REFERENCE
2002	<i>Rhododendron yakushimanum</i>	Piedmont		Gullino et al., 2003
2013-2014	<i>Viburnum tinus</i>	Tuscany		Ginetti et al., 2015
2013	<i>Viburnum tinus</i>	Tuscany		Ginetti et al., 2014
<i>P. syringae</i> (Clade 8)				
YEAR OF INFECTION	HOST	REGION	TYPE OF NURSERIES	REFERENCE
NA	<i>Thuja occidentalis</i>	Tuscany		Migliorini et al., 2015
<i>P. taxon Pgchlamydo</i> (Clade 6)				
YEAR OF INFECTION	HOST	REGION	TYPE OF NURSERIES	REFERENCE
NA	<i>Viburnum tinus</i>	Tuscany		Ginetti et al., 2014
2012-2013	<i>Cercis siliquastrum</i>	Apulia		Prigigallo et al., 2015
<i>P. tentaculata</i> (Clade 1)				
YEAR OF INFECTION	HOST	REGION	TYPE OF NURSERIES	REFERENCE
2007	<i>Origanum vulgare</i>	Liguria		Martini et al., 2009
2002	<i>Gerbera jamesonii</i>	Campania		Cristinzio et al., 2006
<i>P. tropicalis</i> (Clade 2)				
YEAR	HOST	REGION		REFERENCE

2014	<i>Albizia julibrissin</i>	Tuscany	Luongo et al., 2016
2012	<i>Rhododendron catawbiense</i>	Lazio	Luongo et al., 2013
2006	<i>Prunus armeniaca</i>	Sicily	Pane et al., 2009
2005	<i>Pandorea jasminoides</i>	Sicily	Pane et al., 2008
2003-2004	<i>Cuphea</i>	Sicily	Cacciola et al., 2006

APPENDIX B



Appendix B, Figure 2 First report of *Phytophthora*'s host association in Italy and worldwide.

P. acerina = *P. acer*, *P. alni subsp. uniformis* = *P. alni .unifo*, *P. asparagi* = *P. aspa*, *P. bilorbang* = *P. bilb*, *P. cactorum* = *P. cact.*, *P. cambivora* = *P. camb.*, *P. cinnamomi* = *P. cinn.*, *P. cinnamomi-like* = *P. cinn like*, *P. citricola* = *P. citr.*, *P. citricola senso stricto* = *P. citr s.st.*, *P. citrophthora* = *P. citro.*, *P. cryptogea* = *P. cryp.*, *P. crassamura* = *P. crass.*, *P. drechsleri* = *P. drech.*, *P. gonapodyides* = *P. gona.*, *P. hedraiandra* = *P. hedra.*, *P. hydropathica* = *P. hydro.*, *P. inundata* = *P. inun.*, *P. italica* = *P. ital.*, *P. lacustris* = *P. lacu.*, *P. lateralis* = *P. latera.*, *P. meadii* = *P. meadii*, *P. meadi like* = *P. meadi like*, *P. megasperma* = *P. mega.*, *P. mediterranea sp.* = *P. medit.*, *P. megasperma senso stricto* = *P. meg s.st.*, *P. multivora* = *P. mult.*, *P. nicotianae* = *P. nico.*, *P. niederhauserii* = *P. niede.*, *P. niederhauserii -like* = *niede. like*, *P. palmivora* = *P. palm.*, *P. plurivora* = *P. pluri.*, *P. pachyplora* = *P. pachy.*, *P. parvispora* = *P. parvis.*, *P. × pelgrandis* = *P. ×pelgr.*, *P. pistachiae* = *P. pista.*, *P. pseudocryptogea* = *P. .psd.cryt.*, *P. pseudosyringae* = *P. psd.syr.*, *P. psychrophila* = *P. psych.*, *P. taxon pgchlamydo* = *P. pgchl.*, *P. ramorum* = *P. ramo.*, *P. syringae* = *P. syri.*, *P. tentaculata* = *P. tenta.*, *P. tropicalis* = *P. trop.*

APPENDIX C:

Phytophthora sp. recovered in European nurseries until 2021

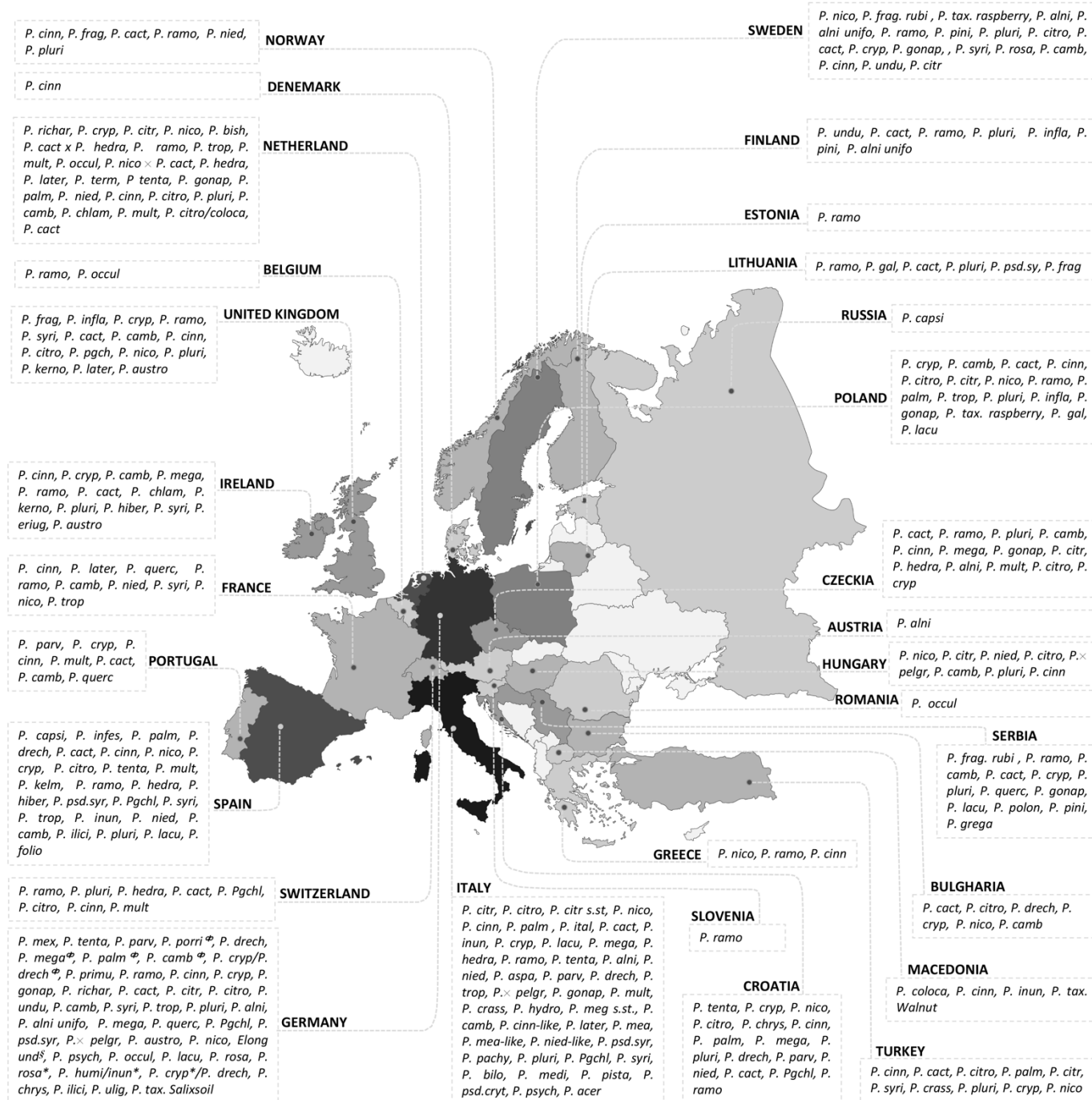
Table 1. Total number of *Phytophthora* species recovered from **Italian** nurseries, characterization of each species according to the functional traits used for the functional diversity analysis, status in Europe, and year of arrival, which was used for the residence time analysis.

Species	countries	Host Range	Host (Gymno/Angio)	Status	Time first record in nurseries (Italy)	Time First record Country (Italy)	Time of arrival Europe	Temp max	Temp min *	Temp Optimum	Asexual survival structures	Reproductive mode	Persistence of sporangia	first record time in Europe (Santini et al., 2013)
<i>Phytophthora acerina</i> B. Ginetti, T. Jung, D. E. L. Cooke, S. Moricca sp. Nov (2013)	Italy	Specialist	Angiosperm	Alien	2021	2013	2013	32°C	6° C	25°C	hyphal swellings-hyphal aggregations	homothallic	Persistent	Italy 2013
<i>Phytophthora alni</i> Brasier & S.A. Kirk (2004) ssp. <i>uniformis</i>	Italy	Specialist	Angiosperm	Hybrid-alien	2001	2001	*1996	30°C	3° C	24–27°C	No structure	homothallic	Persistent	UK: 1993-sweedon, 1996
<i>Phytophthora asparagi</i> Saude & Hausbeck 2008	Italy	Generalist	Ang/Gymn	Alien	2006	1996	NA	30°C-35 °C sardinian isolate	5° C	25°C		homothallic	Persistent	
<i>S. Aghighi, G.E. Hardy, J.K. Scott & T.I. Burgess 2015</i>	Italy	Generalist	Ang/Gymn	Cryptic	2021	2014	1999-france?	32.5°C.	4° C	25°C	hyphal swellings-chlamydospores (sardinian isolate)	homothallic	Persistent	Australia 2012, France, 1999
<i>Phytophthora cactorum</i> (Lebert & Cohn) J. Schröt. (1886),	Italy	Generalist	Ang/Gymn	Alien	1995	1954 ? Chesnut	Germany	30°C.	4° C	25°C	Chlamydospores some isolate	homothallic	Caduous	Germany 1870, Switzerland, 1904(Yung)
<i>Phytophthora cambivora</i> (Petri) Buisman (1927)	Italy	Generalist	Angiosperm	Alien	2015	1917	Italy 1917	32°C.	2° C	22–24°C	hyphal swellings	Heterothallic	Persistent	Portugal, 1838
<i>B. Scanu, A. Deidda & T. Jung sp. nov. (2015)</i>	Italy	Generalist	Ang/Gymn	Alien	2015	2015	2015	35°C	5° C	25°C	hyphal swellings	homothallic	Persistent	Italy, 2015
<i>Phytophthora cinnamomi</i> Rands (1922)	Italy	Generalist	Ang/Gymn	Alien				30°C	9° C	27°C,	hyphal swellings-chlamydospores	Heterothallic	Persistent	Portugal, 1838
<i>Phytophthora citricola</i> Sawada (1927)	Italy	Generalist	Ang/Gymn	Alien	1986			30°C	3° C	24°	No structure	homothallic	Persistent	Italy, 1959
<i>Phytophthora citrophthora</i> Sawada (1927)	Italy	Generalist	Ang/Gymn	Alien	1986			33°C	6° C	24–28°C	Chlamydospores	Heterothallic-sterile	Persistent	Portugal, 1832
<i>Phytophthora cryptogea</i> PetHybridge and Lafferty (1919)	Italy	Generalist	Angiosperm	Alien				30°C	3° C	24°C	hyphal swellings	Heterothallic	Persistent	UK, 1915
<i>Phytophthora drechsleri</i> Tucker (1931)	Italy	Generalist	Angiosperm	Alien	2005			36°C	3° C	27–33°C	hyphal swellings	Heterothallic	Persistent	Greece,1968
<i>Phytophthora hedraiaandra</i> De Cock & Man in 't Veld (2004)	Italy	Generalist	Angiosperm	Alien	2006			30°C	6° C	22–24°C	Chlamydospores	homothallic	Caduous	Netherlands, 2004
<i>Phytophthora hydropathica</i> Hong and Gallegly (2010)	Italy	Generalist			2014			40°C		30°C		Heterothallic	Persistent	
<i>Phytophthora inundata</i> Brasier, Sanch. Hern. & S.A. Kirk (2003)	Italy	Generalist	Angiosperm	Alien				36°C	3° C	27–30°C	hyphal swellings	Heterothallic	Persistent	UK, 1970
<i>Phytophthora italica</i> Cacciola, Magnano di San Lio, and Belisario (1996)	Italy	Specialist	Angiosperm		1996			35°C	10° C	26°C	No structure	homothallic		Italy, 1990
<i>Phytophthora lacustris</i> Brasier, Cacciola, Nechwatal, Jung & Bakonyi (2013)	Italy	Generalist	Angiosperm	Cryptic	2013			37°C	3° C	28–30°C	hyphal swellings-intercalar	Heterothallic-sterile	Persistent	
<i>Phytophthora lateralis</i> Tucker & Milbrath (1942)	Italy	Specialist	Gymnosperm	Alien				25°C	3° C	20°C	chlamydospores	homothallic-rare oogonia	Persistent	France, 1998

<i>Phytophthora meadii</i> McRae (1918)	Italy	Generalist	Angiosperm		2015			32°C	6°C	25–30°C	hyphal swellings small-chlamydospores (rare)	Heterothalic/Heterothalic	Cadusous	
<i>Phytophthora mediterranea</i> Bregant, Mulas and Linaldeddu sp. nov.	Italy	Specialist	Angiosperm		2021	2021	2021	37 °C	10 °C	32 °C	hyphal swellings-chlamydospores	Heterothalic	Persistent	
<i>Phytophthora megasperma</i> Drechsler (1931)	Italy	Generalist	Ang/Gymn	Cryptic				27°C	3°C	18°C	hyphal swellings	homothallic	Persistent	UK, 1933
<i>Phytophthora multivora</i> Scott & Jung (2009)	Italy	Generalist	Angiosperm	Alien				32.5°C	5°C	25°C	hyphal swellings	homothallic	persistent	
<i>Phytophthora nicotianae</i> Breda de Haan (1896)	Italy	Generalist		Alien	1986			33°C	9°C	24°C	hyphal swellings-chlamydospores	Heterothalic	Persistent/Cadusous	
<i>Phytophthora niederhauserii</i> Z.G. Abad & J.A. Abad (2014)	Italy	Generalist		Alien				37°C	10°C	30°C	hyphal swellings	Heterothalic	Persistent	
	Italy			Alien										
<i>Phytophthora pachypleura</i> Henricot, Pérez Sierra & Jung (2014)	Italy	Specialist	Angiosperm		2015	2015		35°C	5°C	25°C	hyphal swellings	homothallic	Persistent	UK; 2008
<i>Phytophthora palmivora</i> (E.J. Butler) E.J. Butler (1919)	Italy	Generalist		Alien	1986			33°C	9°C	24–30°C	hyphal swellings-chlamydospores	Heterothalic	Cadusous	
<i>Phytophthora parvispora</i> Scanu and Denman (2014)	Italy	Generalist/specialist	Ang/Gymn	Alien				37°C	10°C	27°C	hyphal swellings-chlamydospores	Heterothalic	Persistent	South-East Asia
<i>Phytophthora pistaciae</i> Mirabolfathy, sp. Nov	Italy	Specialist	Angiosperm		2020			35°C	5°C	30°C	hyphal swellings	homothallic	persistent	
<i>Phytophthora 3pelgrandis</i> W.F. Gerlach, Nirenberg & Grajenhan nothosp. nov.	Italy	Specialist	Angiosperm		2013			32.5°C	7.5 °C	27.5° C	hyphal swellings	homothallic	Persistent	Germany
<i>Phytophthora plurivora</i> Jung & Burgess (2009)	Italy	Generalist	Ang/Gymn	Expected to be Native	2017			32.5°C	5°C	25°C	No structure	homothallic	Persistent	
<i>Phytophthora pseudocryptogea</i> Safaiefarahani, Mostowfizadeh, Hardy, and Burgess (2015)	Italy	Generalist	Angiosperm		2021			35°C	3°C	25°C	hyphal swellings	Heterothalic	Persistent	
<i>Phytophthora pseudosyringae</i> T. Jung & Delatour (2003)	Italy	Generalist	Angiosperm	Native	2015			25°C	5°C	20°C	hyphal swellings	homothallic	Persistent/Cadusous	Germany, 1996
<i>Phytophthora psychrophila</i> Jung & Hansen (2002)	Italy	Specialist	Gymnosperm	Native	2021			21°C	3°C	15°C	Chlamydospores	homothallic	Persistent/Cadusous	Germany
<i>Phytophthora ramorum</i> Werres De Cock & Man in 't Veld (2001)	Italy	Generalist	Ang/Gymn	Alien	2003			27°C	9°C	15–21°C,	hyphal swellings-chlamydospores	Heterothalic	Cadusous	Germany, 1995
<i>Phytophthora syringae</i> (Kleb.) Kleb. (1909)	Italy	Generalist	Angiosperm	Native	2015			23–25°C	5°C	18–20°C	hyphal swellings	homothallic	Persistent	Germany, 1905
<i>Phytophthora chlamydospora</i> Brasier and Hansen (2015)	Italy	Generalist		Cryptic	2014			36–37°C		25–28°C	hyphal swellings-chlamydospores	self-sterile	Persistent	
<i>Phytophthora tentaculata</i> Kröber & Marwitz (1993)	Italy	Generalist		Alien	2006			30°C	6°C	20–25°C	hyphal swellings-chlamydospores	homothallic	Cadusous	
<i>Phytophthora tropicalis</i> Aragaki & Uchida (2001)	Italy	Generalist		Alien				33°C	6°C	27–30°C	Chlamydospores	Heterothalic	Cadusous	
<i>Phytophthora gonapodyides</i> (H. E. Petersen) Buisman (1927)	Italy	Generalist	Ang/Gymn	Cryptic				30°C	3°C	24–27°C	hyphal swellings	sterile	Persistent	

status in Europe based on Santini *et al.* (2013)

status in Europe based on direct and indirect evidence proposed by Jung *et al.* 2015



Appendix C: Figure 1: Map showing the total number of *Phytophthora* sp. distribution according to European countries until 2021.

P. acerina = *P. acer*, *P. alni subsp. uniformis* = *P. alni .unifo*, *P. asparagi* = *P. aspa*, *P. bilorbang* = *P. bilb*, *P. cactorum* = *P. cact.*, *P. cambivora* = *P. camb.*, *P. cinnamomi* = *P. cinn.*, *P. cinnamomi-like* = *P. cinn like*, *P. citricola* = *P. citr.*, *P. citricola senso stricto* = *P. citr s.st.*, *P. citrophthora* = *P. citro.*, *P. cryptogea* = *P. cryp.*, *P. crassamura* = *P. crass.*, *P. drechsleri* = *P. drech.*, *P. gonapodyides* = *P. gona.*, *P. hedraiandra* = *P. hedra.*, *P. hydropathica* = *P. hydro.*, *P. inundata* = *P. inun.*, *P. italica* = *P. ital.*, *P. lacustris* = *P. lacu.*, *P. lateralis* = *P. latera.*, *P. meadii* = *P. meadii*, *P. meadi like* = *P. meadi like*, *P. megasperma* = *P. mega.*, *P. mediterranea sp.* = *P. medit.*, *P. megasperma senso stricto* = *P. meg s.st.*, *P. multivora* = *P. mult.*, *P. nicotianae* = *P. nico.*, *P. niederhauserii* = *P. niede.*, *P. niederhauserii-like* = *niede. like*, *P. palmivora* = *P. palm.*, *P. plurivora* = *P. pluri.*, *P. pachyplora* = *P. pachy.*, *P. parvispora* = *P. parvis.*, *P. × pelgrandis* = *P. ×pelgr.*, *P. pistachiae* = *P. pista.*, *P. pseudocryptogea* = *P. .psd.cryt.*, *P. pseudosyringae* = *P. psd.syr.*, *P. psychrophila* = *P. psych.*, *P. taxon pgchlamydo* = *P. pgchl.*, *P. ramorum* = *P. ramo.*, *P. syringae* = *P. syri.*, *P. tentaculata* = *P. tenta.*, *P. tropicalis* = *P. trop.*

APPENDIX D

Table 1: Number of Plant extract families									
Family	Journals article number	Family	Journals article number	Family	Journals article number	Family	Journals article number	Family	Journals article number
Acanthaceae	2	Cactaceae	1	Fagaceae	4	Nepenthaceae	1	Primulaceae	3
Achariaceae	2	Campanulaceae	1	Gentianaceae	1	Nitrariaceae	1	Pteridaceae	1
Acoraceae	4	Cannabaceae	1	Geraniaceae	1	Nyctaginaceae	1	Ranunculaceae	9
Actinidiaceae	1	Capparaceae	2	Gesneriaceae	1	Nymphaeaceae	1	Rhamnaceae	3
Adoxaceae	4	Caprifoliaceae	3	Grossulariaceae	1	Oleaceae	7	Rhizophoraceae	1
Amaranthaceae	11	Caricaceae	2	Hylocomiaceae *	1	Onagraceae	2	Rosaceae	8
Amaryllidaceae	18	Caryophyllaceae	5	Hypericaceae	4	Orchidaceae	1	Rubiaceae	8
Amblystegiaceae	1	Celastraceae	1	Iridaceae	3	Oxalidaceae	1	Rutaceae	10
Anacardiaceae *	5	Chloranthaceae	3	Juglandaceae	3	Paeaniaceae	2	Salicaceae	6
Annonaceae	6	Clusiaceae	3	Juncaceae	3	Papaveraceae	8	Sapindaceae	4
Apiaceae	6	Combretaceae	5	Lamiaceae	33	Passifloraceae	2	Sapotaceae	2
Apocynaceae	10	Convolvulaceae	5	Lardizabalaceae	1	Paulowniaceae	1	Saururaceae	1
Aquifoliaceae	1	Cornaceae	1	Lauraceae	8	Pedaliaceae	1	Scapaniaceae *	1
Araceae	2	Crassulaceae	1	Lecythidaceae	1	Pelliaceae *	1	Schisandraceae	1
Araliaceae	4	Cucurbitaceae	8	Leguminosae	37	Pentaphylacaceae	1	Scrophulariaceae	4
Arecaceae	2	Cupressaceae	8	Lepidoziaceae *	1	Peraceae	1	Solanaceae	14
Aristolochiaceae	1	Cyperaceae	1	Liliaceae	1	Phyllanthaceae	3	Sphagnaceae *	1
Asparagaceae	10	Daphniphyllaceae	1	Linaceae	1	Phytolaccaceae	1	Staphyleaceae	1
Asteraceae	52	Dicranaceae	1	Loranthaceae	2	Pinaceae	9	Styracaceae	2
Balsaminaceae	1	Dipterocarpaceae *	2	Lythraceae	5	Piperaceae	6	Taxaceae	1
Barbeyaceae	1	Dryopteridaceae	1	Magnoliaceae	5	Pittosporaceae	1	Theaceae	2
Bartramiaceae	1	Ebenaceae	2	Malvaceae	5	Plagiotheciaceae *	1	Tropaeolaceae	1
Berberidaceae *	2	Elaeagnaceae	1	Melanthiaceae	1	Plantaginaceae	4	Urticaceae	2
Bignoniaceae	4	Elaeocarpaceae	1	Meliaceae	9	Poaceae	21	Verbenaceae	6
Boraginaceae	2	Equisetaceae	2	Mniaceae *	1	Polygalaceae	1	Vitaceae	6
Brassicaceae	13	Ericaceae	2	Moraceae	4	Polygonaceae	14	Winteraceae	3
Bursaceae	1	Eucommiaceae	1	Moringaceae	3	Polytrichaceae *	1	Zingiberaceae	13
Buxaceae	2	Euphorbiaceae	11	Myrtaceae	15	Portulacaceae	1	Zygophyllaceae	3

*: Represent bryophyte species

APPENDIX E

Table 1: Journal article regarding *Allium* extract effect on different *Phytophthora* sp.

<i>Phytophthora</i> sp.	Year	Material used	Solvent	Number of cultivars	Experimental setup	Main finding	Reference
<i>Phytophthora drechsleri</i>	1992	NA	NA	NA	<i>In vitro</i>	At 2,5 ppm of "Ajone" a compound derived from <i>Allium sativum</i> was effective for inhibit sporangia germination, however higher concentration (20 ppm) was effective for mycelium growth and zoospore germination of <i>P. drechsleri</i> .	(A. U. P. Singh et al., 1992)
<i>Phytophthora infestans</i>	2004	Garlic bulbs	No Solvent (Fresh juice)	1	<i>In vitro</i> , <i>In planta</i> (Potato tuber)	Allicin reduce <i>P. infestans</i> spore germination <i>In vitro</i> and disease in blighted tubers. However, it is effectively reduced by prophylactic treatments with garlic extract than by curative treatments post infection.	(Curtis et al., 2004)
<i>Phytophthora capsici</i>	2006	Above-ground plant (Dried)	Ethanol (99.5%)	1	<i>In vitro</i> , <i>In planta</i>	Garlic at concentrations of 5 and 10 $\mu\text{l ml}^{-1}$ inhibited mycelial growth by 7.55 and 13.73 % respectively. in <i>In vivo</i> test <i>Phytophthora</i> blight of pepper was reduced 39.8% by garlic	(Demirci & Dolar, 2006)
<i>Phytophthora infestans</i>	2006	NA	NA	NA	<i>In planta</i>	To manage tomato late blight a mixture of plant extract has been test it where Bordeaux mixture and neem oil were the most efficient treatment.	(Diniz et al., 2006)
<i>Phytophthora infestans</i>	2006	Bulbs, Fresh	Ethyl alcohol (95 %)	1	<i>In vitro</i> , <i>In planta</i> : Greenhouse (Ethiopia)	Concentrations of 0.5 % 2 %, 4 %, 6 % and 8 % significantly ($p < 0.05$) inhibited the growth of the mycelia, the germination of zoospore and sporangia of <i>P. infestans</i> , the 2 % garlic crude extracts showed curative effect in <i>in planta</i> test	(Bekepe et al., 2006)
<i>Phytophthora infestans</i>	2007	Bulbs (dried)	water	1	<i>In vitro</i> , leaf essay,	Extract of lemon grass leaves was the best one in controlling both late followed with	(Abd-El-Khair & Haggag, 2007)

					<i>In planta:</i> (Field)	<i>Allium sativum</i> that reduced the disease severity by 81.2%.	
<i>Phytophthora infestans</i>	2007	NA	NA	NA	<i>In vitro,</i> <i>In planta:</i> (Field)	Envirepel a commercial formulation of garlic extract [0,5%]. Have 7% inhibition of sporangia germination with no inhibition of mycelial growth and foliar blight on tomato plants.	(Dorn et al., 2007)
<i>Phytophthora cinnomomi</i> <i>Phytophthora nicotianae</i>	2007	NA- (commercially garlic extract)	NA	NA	<i>In Vitro</i>	Mycelium growth of tested soil borne fungal pathogen has been inhibited at 10% levels of garlic extract. However, efficacy of the treatment depends on root substrate and soil type.	(Sealy et al., 2007)
<i>Phytophthora infestans</i>	2008	NA	NA	NA	<i>In vitro,</i> Leaf essay, <i>In planta</i>	Allicin effectively controlled seed-borne <i>Phytophthora</i> leaf blight and encapsulation formulations for reducing the pathogen inoculum potential in planting material. (EC50) values of 64.2 and 25.9 mg·mL ⁻¹ , respectively of garlic extract against mycelium growth and zoospore germination has been demonstrated in addition to preventive activity against the <i>P. capsici</i> in <i>in Planta</i> test but no curative effect.	(Slusarenko et al., 2008)
<i>Phytophthora capsici</i>	2008	Bulbs (fresh)	No Solvent (Fresh Juice)	1	<i>In vitro,</i> <i>In planta</i>	Paint formulations involving (<i>Ocimum sanctum</i> L.) and bulb extract of garlic at 100 g/l, linseed oil and <i>T. harzianum</i> formulation at 100 g/l rice starch solution were found controlling <i>Phytophthora</i> disease.	(Li & Zhihui, 2008)
<i>Phytophthora rot (citrus)</i>	2011	bulbs	NA	1	<i>In planta:</i> (Field -India)	Water extracts of <i>Allium sativum</i> had minimum inhibitory concentration (MICs) of 1.65 mg/ml while acetone extracts 0.78mg/ml both extracts effectively	(Gaur et al., 2011)
<i>Phytophthora infestans</i>	2014	bulbs	Water, Acetone, Ethyl acetate	1	<i>In vitro,</i> <i>In Planta</i>		(Ngadze, 2014)

<i>Phytophthora infestans</i>	2015	bulbs, Fresh	Water	1	<i>In planta:</i> (Field-Slovenia)	reduced potato late blight in the greenhouse. No satisfactory efficacy against the late blight.	(Bohinc et al., 2015)
<i>Phytophthora capsici</i>	2016	bulbs	Water	28 (China)	<i>In vitro</i> , Leaf essay, <i>In Planta</i>	Diversity in allicin abundance among cultivars affect four pathogenic fungus the highest and lowest concentration respectively are the following 3.98 mg g ⁻¹ 0,66 mg. g ⁻¹ with plant growth effect however, at higher concentrations may affect cucumber seedlings growth.	(Hayat et al., 2016)
<i>Phytophthora infestans</i>	2016	bulbs	Water	1	Leaf essay, <i>In Planta</i>	<i>Allium sativum</i> more effective than <i>Eucalyptus camaldulensis</i> and <i>Azadirachta indica</i> on tomato plants	Dubey et al., 2016)
<i>Phytophthora palmivora</i>	2016	bulbs	Methanol	1	<i>In vitro</i>	NA	(Sukrasno et al., 2016)
<i>Phytophthora cactorum</i>	2019	Flesh, roots, byproduct	Water	4	<i>In vitro</i>	Effect of <i>Allium</i> extracts and active ingredients on <i>Phytophthora</i> and other crop pathogens. The inhibitory effect is higher when <i>Alium sativum</i> cultivar was used.	(Jang et al., 2019)
<i>Phytophthora infestans</i>	2020	Cloves, dried	Water, Ethanol (95%), Methanol (95%)	1	<i>In vitro</i>	Essential oils the most effective than crude extracts in restricting the pathogen growth and Ethanol most suitable extraction solvent.	(Mugao et al., 2020b)

APPENDIX F

Table 1: Journal article regarding some biological control agent causing disease symptoms on plant.

Screened bacterial microorganisms	Type: (Pathogen/BCA)	Disease Symptoms	Target pathogen(s)	Country of origin; Isolated plants	Reference
<i>Burkholderia cepacia</i>	Pathogen	Fruit rot		China; Apricot	(Fang et al., 2009)
<i>Burkholderia cepacia</i> XXVI	BCA		<i>Colletotrichum gloeosporioides</i>	Mexico; Mango	(de los Santos-Villalobos et al., 2012)
<i>Burkholderia gladioli</i>	BCA		<i>Rhodotorula pilimanae</i> , <i>Penicillium digitatum</i> , <i>Penicillium expansum</i> , <i>Botrytis cinerea</i> , <i>Aspergillus flavus</i> , <i>Aspergillus ochraceus</i> and <i>Alternaria alternata</i>		(Scuderi et al., 2009)
<i>Burkholderia gladioli</i>	Pathogen	Internal browning		China; Sweetpotato	(X. X. Zhang et al., 2020)
<i>Bacillus altitudinis</i>	Pathogen	Soft rot		Egypt; Apple and Pear	(Elbanna et al., 2014)
<i>Bacillus altitudinis</i>	BCA		<i>Potato common scab (Streptomyces spp.)</i>	China; Potato	(B. Li et al., 2019)
<i>Bacillus amyloliquefaciens</i>	Pathogene	Soft rot		China; Potato	(L. Wang et al., 2017)
<i>Bacillus amyloliquefaciens</i>	BCA		<i>Rhizopus stolonifer</i> , <i>Penicillium variable</i> , <i>Fusarium verticillioides</i>	Democratic Republic of Congo; Maiz	(Kulimushi et al., 2018)
<i>Bacillus pumilus</i>	Pathogen	Soft rot		Mali; Potato	(Bathily et al., 2010)

<i>Bacillus pumilus</i> LX11	BCA		<i>Sclerotium rolfsii</i>	China; Peanut	(M. Xu et al., 2020)
<i>Serratia marcescens</i> ETR17	BCA		Root rot disease	India; Tea	(Dhar Purkayastha et al., 2018)
<i>Serratia marcescens</i>	Pathogen	Soft rot		China; Ginger rhizomes	(Huang et al., 2020)
<i>Pseudomonas fluorescens</i>	Pathogen	Stem rot		China; Melon	(Zhang et al., 2016)
<i>P. brassicacearum</i> ; <i>P. fluorescens</i> complex	Pathogen	Necroses		Pacific Northwest of the USA; Tomato	(M. Yang et al., 2020)
<i>Pseudomonas fluorescens</i>	BCA		Tomato spotted wilt virus (TSWV)	India, Tomato	(Kandan et al., 2005)
<i>Pseudomonas aeruginosa</i> NJ-15	BCA		<i>Fusarium oxysporum</i> , <i>Trichoderma harzianum</i> , <i>Alternaria alternata</i> , <i>Macrophomina phaseolina</i>	India	(Bano & Musarrat, 2003)
<i>Pseudomonas aeruginosa</i>	Pathogen	Plant mortality		Colorado; Arabidopsis - Sweet basil	(Walker et al., 2004)
<i>Fusarium equiseti</i>	BCA		<i>Fusarium avenaceum</i> and <i>Peyronellaea pinodella</i>	Germany; Pea	(Šišić et al., 2017)
<i>Fusarium equiseti</i>	Pathogen	Fruit rot		Thailand; Cantaloupe	(Nuangmek et al., 2019)
<i>Paenibacillus polymyxa</i>	BCA		<i>Pythium ultimum</i> , <i>Rhizoctonia solani</i> , and <i>Fusarium oxysporum</i>	South Korea; Barley	(Jeong et al., 2019)
<i>Paenibacillus polymyxa</i>	Pathogen	Stem rot		China; Dragon fruit	(R. Y. Zhang et al., 2017)

APPENDIX G: Nursery questionnaire

20/05/2022

questionario sulla gestione dei vivai

questionario sulla gestione dei vivai

Buongiorno, con questo questionario vogliamo raccogliere informazioni sulla gestione dei vivai per fornire soluzioni per realizzare strutture produttive sempre più sostenibili. Il Suo contributo è molto importante per il successo di questo studio, bastano pochi minuti del suo tempo.

Il questionario è organizzato in 3 parti:

- Informazioni generali
- Informazioni sul vivaio
- Malattie delle piante

La ringraziamo molto per l'aiuto e l'attenzione che vuole concederci
Dania Tabet, dottoranda presso l'Università degli Studi della Tuscia, Viterbo

***Campo obbligatorio**

Parte 1: Informazioni personali

1. 1.1-Località in cui è ubicato il vivaio (CAP): *

2. 1.2-Lei è *

Seleziona tutte le voci applicabili.

- ☐ Proprietario del vivaio
☐ Gestore

3. 1.3-Titolo di studio *

Seleziona tutte le voci applicabili.

- ☐ Diploma
☐ Laurea
☐ Master
☐ Nessuno

4. 1.4-Sesso

Seleziona tutte le voci applicabili.

- ☐ Femmina
☐ Maschio

5. 1.5-Da quanti anni gestisce il vivaio? *

6. 1.6-Numero di impiegati "Maschio"

Seleziona tutte le voci applicabili.

	<5	5-10	10-20	>20
Familiari	☐	☐	☐	☐
Part -time	☐	☐	☐	☐
Full time	☐	☐	☐	☐

7. 1.7-Numero di impiegati "Femmina"

Seleziona tutte le voci applicabili.

	>5	5-10	10-20	>20
Famillari	☐	☐	☐	☐
Part-time	☐	☐	☐	☐
Full time	☐	☐	☐	☐

8. Quali sono i modi interattivi in cui preferisci per ottenere consigli tecnici

Seleziona tutte le voci applicabili.

- ☐ Servizio fitosanitario
- ☐ Seminario all'università
- ☐ Social media (Facebook, YouTube, Twitter, WhatsApp ...)
- ☐ Esposizione
- ☐ Giornale scientifico
- ☐ Sito web o applicazione

Parte 2: Informazioni vivaio

9. 2.1-Tipologia del vivaio *

Seleziona tutte le voci applicabili.

- ☐ Vivaio produttivo
- ☐ Garden
- ☐ Vivaio produttivo e Garden

10. 2.2-Numero di ettari

Seleziona tutte le voci applicabili.

	<5 ha	5-10 ha	>10 ha
Ettari in pieno campo	☐	☐	☐
Ettari in vaso	☐	☐	☐
Ettari in coltura protetta	☐	☐	☐

11. 2.3-Numero di piante prodotte annualmente *

12. 2.4-Tipologia di piante prodotte

Seleziona tutte le voci applicabili.

	10-30%	30-60%	60-90%	100%
Piante ornamentali	☐	☐	☐	☐
Alberi da frutto	☐	☐	☐	☐
Piante ortive	☐	☐	☐	☐
Piante forestali	☐	☐	☐	☐
Piante tropicali	☐	☐	☐	☐
Fiori	☐	☐	☐	☐
Piante aromatiche	☐	☐	☐	☐

13. Altro

14. 2.5-Numero di Piante acquistate dall' Italia *

15. 2.6-Numero di Piante acquistate dall' Europa *

16. 2.7-Numero di Piante acquistate da Extra Europa *

17. 2.8-Tipologia di piante importate

Seleziona tutte le voci applicabili.

	10-30%	30-60%	60-90%	100%
Piante ornamentali	☐	☐	☐	☐
Alberi da frutto	☐	☐	☐	☐
Piante ortive	☐	☐	☐	☐
piante forestali	☐	☐	☐	☐
Piante tropicali	☐	☐	☐	☐
Fiori	☐	☐	☐	☐
Piante aromatiche	☐	☐	☐	☐

18. Altro

19. 2.9-Numero di Piante esportate e Paese di destinazione *

20. 2.10-Sorgente delle acqua utilizzata per l'irrigazione *

Seleziona tutte le voci applicabili.

- ☐ Acquedotto
- ☐ Pozzo
- ☐ Acqua meteorica
- ☐ Altro: _____

21. 2.11-Turno di irrigazione

Seleziona tutte le voci applicabili.

	1 volta al giorno	2 volte al giorno	1 volta /2 giorni	1 volta /3 giorni	in continuo
Primavera	☐	☐	☐	☐	☐
Estate	☐	☐	☐	☐	☐
Autunno	☐	☐	☐	☐	☐
Inverno	☐	☐	☐	☐	☐

22. 2.12-Le acque reflue vengono recuperate?

Seleziona tutte le voci applicabili.

- ☐ SI
- ☐ No

23. Se Si come?

24. 2.13-Indicare i prodotti/dosi /modalità per la concimazione/fertirrigazione? *

25. 2.14-Indicare periodo e tempi di concimazione/fertirrigazione *

26. 2.15- Indicare i prodotti fungicidi /insetticidi utilizzati e periodo di applicazione *

27. 2.16-Vengono effettuate le analisi per verificare la presenza di patogeni *

Seleziona tutte le voci applicabili.

	Mai	1 volta anno	1 volta ogni 2 anni	1 volta ogni 3 anni	Raramente
Nel suolo	☐	☐	☐	☐	☐
Nell'acqua	☐	☐	☐	☐	☐

28. 2.17-Quali tipi di superfici sono utilizzate nella area di produzione interna *

Seleziona tutte le voci applicabili.

- ☐ Cemento
☐ Plastica
☐ Tessuto
☐ Terreno nudo
☐ Ghiaia, conchiglie, pacciamme,
☐ N/D (nessuna area di produzione interna)

29. Altro

30. 2.18-Si procede alla sterilizzazione di

Seleziona tutte le voci applicabili.

	Si	No
Terericii per invasare le piante	☐	☐
Substrato per nuove semine	☐	☐
Suolo	☐	☐
Vasi	☐	☐
Cassoni per la semina	☐	☐

31. 2.19-quale metodo si usa per sterilizzare i materiali?

32. 2.20-Che tipo di terriccio è utilizzato nei vasi *

Parte 3:
Malattie
delle piante

Definizioni: moria dei semenzali= morte delle piantine; appassimento delle piante= perdita di turgore; Marciumi radicali= marcescenza dell'apparato radicale

33. 3.1-Ha riscontrato problemi *

Seleziona tutte le voci applicabili.

	Raramente	10%	25%	50%	75%	100%	causato da Phytophthora/Pyth (no)
Moria dei semenzali	☐	☐	☐	☐	☐	☐	☐
Marciumi radicali	☐	☐	☐	☐	☐	☐	☐
Appassimento delle piante	☐	☐	☐	☐	☐	☐	☐

34. 3.2-Come è intervenuto? *

35. 3.3-Ha effettuato analisi fitopatologiche per verificare l'agente causale dei sintomi da Lei riscontrati?

Seleziona tutte le voci applicabili.

- ☐ Attraverso privati
☐ Attraverso università o enti di ricerca
☐ Attraverso il servizio fitosanitario regionale
☐ NO

36. 3.4-A quanto stima la perdita delle piante per malattia (%piante morte anno)? *

37. 3.5-Le piante morte o deperienti come vengono smaltite? *

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Google Moduli

APPENDIX H:

Unpublished journal article example for nursery growers

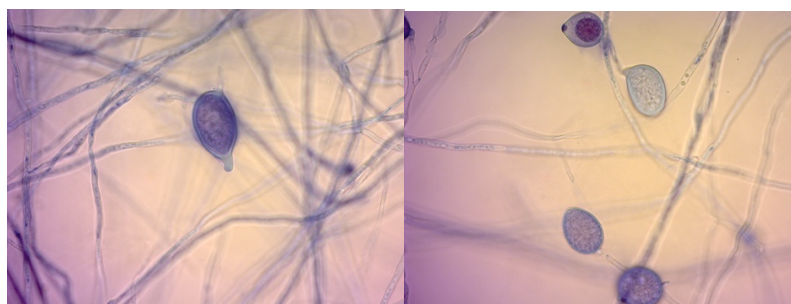
Phytophthora a worldwide nursery threat

Phytophthora, also known as water moulds, is a virulent soil-borne plant pathogen that resembles fungi but is closely related to brown algae. There are approximately 150 known species of *Phytophthora*.

It is a significant global problem, causing extensive plant losses worldwide.

A recent survey has shown a wide distribution and a high diversity of *Phytophthora* species in forest and ornamental nurseries across European countries. Some of the detected *Phytophthora* species are considered quarantine and can pose significant threats to ornamental plants, agriculture, aquaculture, urban ecosystems, and natural forest environments.

Phytophthora species can infect any part of a plant at any stage of its growth. Airborne *Phytophthora* sp. infect the leaves, shoots, fruits and the bark of branches and stems, producing deciduous sporangia (an enclosure in which spores are formed and stored) in moist conditions which are spread through rain and wind splatter. Soil borne *Phytophthora* sp., under wet soil conditions, infects the fine roots, bark of the suberized roots, and collar region by releasing motile zoospores (short-lived, flagellated zoospores that can swim through thin films of water) from the sporangia.



Sporangia of *Phytophthora* sp. containing zoospores

The typical symptoms of *Phytophthora* are generally nonspecific, and can often be mistaken for normal abiotic stress or other pathogens. These symptoms include chlorosis, root and crown rot, dieback, stem canker, trunk gummosis, foliar blight and leaf lesions, stunting, wilting and death of plants. Some plants may show specific symptoms caused by *Phytophthora* such as leaf reddening on *Pistacia lentiscus*, premature defoliation on high-bush blueberry, wilting and death of plants without leaf drop on *Skimmia japonica* and brown to black foliar lesions, necrosis of the petioles, and browning of the stems on *Viburnum tinus* and *Aucuba japonica*.

Detection of *Phytophthora* is usually based on a visual inspection for symptoms. However, in some case, *Phytophthora* can be recovered from asymptomatic plants where above-ground plant organs appeared visually healthy.

For this reason, the ability to detect soil-borne pathogens is a critical element in the management of these organisms, especially in the absence of visible symptoms. Therefore, it is important for growers and nursery manager to directly contact plant pathologists and phytosanitary inspection services for an accurate disease diagnosis.

Protective measures in nurseries are essential in preventing the establishment of *Phytophthora* disease. The best measures to reduce the risk of *Phytophthora* development include:

1) Good cultural practices, such as sanitation, avoiding the movement of soil and contaminated plant material, preventing direct contact between soil and plant containers, disinfecting plant seed trays and containers, and removing plant debris. It is important to note that the effectiveness of disinfectants and the recovery of *Phytophthora* species from contaminated surfaces depend on the concentration, treatment time, and type of pots and saucer surface that can affect the efficacy of the disinfectant and the recovery of *Phytophthora* species, as different surfaces may have varying levels of porosity and roughness, which can impact the ability of the disinfectant to penetrate and effectively kill the pathogen

2) Treating irrigation water by filtration and disinfection with sodium hypochlorite or calcium chlorite can help in reducing pathogen infection as irrigation water is often a major source of *Phytophthora* diversity in nurseries.

3) Avoiding soil compaction by using an appropriate, sustainable and healthy potting mediums with diverse soil biota, and avoiding the use of disinfected soil or reusing of plant growth substrates. However, it is important to ensure that temperatures are high enough to kill pathogens when composting.

4) Using healthy plant material and proper nutrient management to reduce *Phytophthora* infection.

5) Frequently applying synthetic fungicides, or using resistance breeding plants as current hygiene and monitoring practices in nurseries are not sufficient to eliminate the risk of spreading this harmful pathogen. “Propamocarb” is a commonly used chemical fungicide that should be used preventatively. However, it is becoming increasingly difficult to control diseases due to the low durability of fungicides and the emergence of new genotypes with different mating types and resistance to chemical fungicides.

Thus, it is essential to promote the use of Integrated Pest Management (IPM) and alternative approaches to synthetic pesticides, such as biological control and low -risk compounds, the use of fungicide in alternation with different modes of action, controlling pesticides losses, the use of host plant defense inducers and cover crops as a potential tools for suppression of soil borne diseases with the aim of achieving a sustainable and ecologically sound use of plant protection measures in nurseries.
