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Research and Development of new Biological Alternatives to Control Downy Mildew of Grapevine (*Plasmopara viticola*) and Implement of new Integrated Pest Management Strategies

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LIST OF SYMBOLS AND ABBREVIATIONS

^{1}H NMR	Proton nuclear magnetic resonance
ACN	Acetonitrile
AZ78	*Lysobacter capsici* AZ78
BCA	Biocontrol agent
CAA	Carboxylic acid amides
CC	Column Chromatography
CD_3OD	Deuterated methanol
$CDCl_3$	Deuterated chloroform
CFDA	Carboxy-fluorescein diacetate
CH_2Cl_2	Dichloromethane or methylene chloride
CH_3CN	Acetonitrile
$CHCl_3$	Chloroform
DAB	3,3’-diaminobenzidine
DAPG	2,4-Diacetylphloroglucinol
DMP	Dihydromaltophylin
DPPG	Dipropylphloroglucinol
DSS	Decision Support Systems
EO	Essential Oil
EPPO	European and Mediterranean Plant Protection Organization
ESIMS	Electrospray ionization mass spectra
EtOAc	Ethyl acetate
EtOH	Ethanol
FH	Homogeneous Fraction

F_{IS}	Fixation Index
FRAC	Fungicide Resistance Action Committee
F_{ST}	Genetic differentiation Index
H_2O	Water
H_2SO_4	Sulphuric acid
H_e	Expected heterozygosity
H_o	Observed Heterozygosity
HPLC	High-Performance Liquid Chromatography
HSAF	Heat Stable Antifungal Factor
HWE	Hardy-Weinberg Equilibrium
ILS	Internal Lane Standard
IPM	Integrated Pest Management
LBA	Luria Bertani Agar
LC/MS TOF	Liquid chromatography/mass spectrometry time of flight
mBCA	microbial Biocontrol Agent
MCMC	Markov Chain Monte Carlo
MeOH	Methanol
MP	Maltophylin
NA	Nutrient Agar
Na_2SO_4	Anhydrous sodium sulphate
NMR	Nuclear Magnetic Resonance
OSBPI	Oxysterol Binding Protein Inhibitor
QiI	Quinone inside Inhibitors
QoI	Quinone outside Inhibitors
QoSI	Quinone outside Inhibitor Stigmatellin binding site

QTL	Quantitative Trait *Loci*
RH	Relative Humidity
rt	Retention time
TLC	Thin Layer Chromatography
UV	Ultraviolet

ABSTRACT

The plant pathogenic oomycete *Plasmopara viticola* represents one of the major threats to viticulture, causing heavy and diffused yield losses if not properly controlled. Nowadays, the control of *P. viticola* relies mainly on repeated fungicide application, with a remarkable impact on the environment and human health. For these reasons, many active ingredients have been excluded from the market, or their use has been drastically limited. On the other hand, the high occurrence of mutations in *P. viticola* populations leads to an outburst of resistant mechanisms worldwide towards single-site chemical fungicides, making appropriate disease management even more challenging. Hence, there is urgent need for new sustainable candidates, such as plant extracts or biocontrol agents, to widen the portfolio of active ingredients available on the market and provide new tools to farmers to develop efficient plant protection strategies. At the same time, it is essential to deepen the knowledge on the structure of *P. viticola* populations and constantly monitor the occurrence of fungicide resistances.

In this challenging scenario, the aim of the thesis is to present and characterize new sustainable biofungicides for the control of *P. viticola* and to offer an insight on the populations´ structure in the Trentino Alto Adige region, with a further investigation on the resistance to fungicides.

Glechoma hederacea aqueous plant extract has significantly inhibited activity towards *P. viticola*. Moreover, the purification of the extract by chromatographic techniques lead to the isolation of six pure metabolites with anti-oomycete activity, identified as carvacrol, caffeic acid and methyl caffeate, the flavonoids cirsimaritin and apigenin, and the polyphenolic acid rosmarinic acid. Furthermore, Gowan proposed three active ingredients as potential biofungicides, namely the plant extracts 12035 and 12110 and the bacterial BioControl Agent (BCA) *Lysobacter capsici* AZ78.

Following their remarkable plant protection efficacy and rain fastness, the mode of action has been partially characterized. All the candidates showed acute toxic activity on the sporangia and the induction of reactive oxygen species production in grapevine leaf discs. Moreover, 12110 and *L. capsici* AZ78 stimulated the production of callose on the stomata´s guard cells. Finally, the metabolites responsible for the *L. capsici* AZ78 anti-oomycete activity were isolated and identified, namely dihydromaltophylin and maltophylin.

On the other hand, *P. viticola* samples have been collected in 18 vineyards with different disease management strategies in Trentino Alto Adige in 2021 and 2022. Genotyping has been performed on the isolates to identify the structure of *P. viticola* populations in the region using 22 microsatellite *loci*. However, the analysis did not highlight a genetic differentiation among the different *P. viticola* populations, according to location or disease management strategy. Finally, 11 single-site chemical fungicides were tested on the isolates to assess the presence of fungicide resistances. The bioassay highlighted frequent resistant isolates towards cymoxanil in the Region and mandipropamid in Alto Adige province. A moderate adaptation was shown in isolates treated with ametoctradin, amisulbrom, and fluopicolide. On the other hand, the isolates were susceptible to azoxystrobin, dimetomorph, zoxamide and oxathiapiprolin.

This study presents several valid candidates for the control of *P. viticola*, with multi-level mode of action, which can represent a promising alternative to conventional fungicides. However, further studies on product´s formulation need to be performed, since it plays a crucial role in disease management in commercial vineyards. Moreover, the investigation on *P. viticola* populations displayed a very limited genetic variability in Trentino Alto Adige, but at the same time diffused adaptation towards single-site fungicide, suggesting an extremely high risk of outburst of resistant

isolates. This underscores the importance of detailed and constant monitoring, which is a key proactive measure in the fight against *P. viticola*.

CHAPTER 1. INTRODUCTION

The cultivation of grapevines (*Vitis vinifera*) represents a cornerstone of global agriculture, supporting the production of table grapes and wine. Globally, viticulture encompasses approximately 7.3 million hectares, producing 64 million tons of grapes in 2024, with over 50% of this yield directed toward wine production. This sector is economically significant, particularly in Europe and other wine-producing regions, where it constitutes a vital cultural and financial asset.

However, grapevines are notoriously vulnerable to disease. Among the most devastating threats is *Plasmopara viticola*, the causal agent of grapevine downy mildew. Introduced into Europe in the late 19th century during efforts to combat the phylloxera crisis, *P. viticola* rapidly spread across the continent, causing extensive economic losses. Its diffusion across the continent was rapid, with France reporting its destructive potential as early as 1893, when the disease wiped out half of the country's grape production. Since then, managing this relentless pathogen has become a crucial aspect for growers, requiring constant vigilance and significant investment in disease control management.

For instance, in 1893, French vineyards experienced a 50% reduction in grape production due to this pathogen. The management of *P. viticola* remains a critical priority in viticulture, necessitating a multifaceted approach to disease mitigation.

Plasmopara viticola is an obligate biotrophic oomycete that infects grapevine tissues, completing its life cycle entirely within the host. Its lifecycle includes both sexual and asexual reproductive stages, contributing to its adaptability and persistence. The pathogen overwinters as oospores in senescent leaves, which serve as inoculum for primary infections in the spring. Under optimal conditions - characterized by temperatures between 20–25°C and high relative humidity -

oospores germinate to produce sporangia. These sporangia release zoospores, motile propagules equipped with flagella that enable chemotactic movement toward stomata on grapevine leaves (Gessler *et al*., 2011). Within 30 minutes, the encysted zoospores germinate and produce germ tubes, which enter the plant tissue via stomata openings and develop an intercellular mycelial network inside the mesophyll tissue.

Upon entry through the stomata, zoospores transition into a sessile phase, forming structures such as appressoria and hyphae to colonize the intercellular spaces of host tissues. Hyphae extract nutrients via specialized structures known as haustoria, facilitating rapid pathogen proliferation. Symptomatology becomes evident within 5–7 days post-infection, initially manifesting as chlorotic, oil-spot lesions on the adaxial surface of leaves. These lesions correspond to sporangiophore development on the abaxial surface, where sporangia are produced, facilitating secondary spread via wind and rain. With favorable climatic conditions, several secondary infections may occur, leading to devastating damages to the crop, such as tissue necrosis, defoliation, and reduced photosynthetic capacity, with berries and shoots also displaying deformities and decay. While damage to the canopy may have a limited direct economic effect, inflorescences are extremely susceptible, especially until fruit set, and the attack of *P. viticola* to these organs brings a direct impact on yield quantity and quality (Koledenkova *et al*., 2023).

Historically, it has been demonstrated that a single *P. viticola* population was imported in Europe during the Phylloxera crisis and then expanded throughout the continent, until reaching Asia, Australia and South America. However, European populations are characterized by an extremely low genetic diversity and a weak genetic structure, indicating that the populations are panmictic at this scale (Fontaine *et al*., 2013) but also at field and regional scale (Gobbin *et al*., 2003; Gobbin *et al*., 2006; Rouxel *et al*., 2012; Delmas *et al*., 2017; Maddalena *et al*., 2020). The low genetic diversity

may be a clear suggestion of the occurrence of a demographic bottleneck during the epidemic expansion of *P. viticola*, with an extremely rapid dispersion of the pathogen across the continent, via the transport of infected plant material which favored sporangia dispersal. Already moving to Eastern Europe, a spatially distinct cluster had been detected, even though with low genetic differentiation. Fontaine *et al*., (2019) stated that Europe was devastated by a specific genetic cluster of *P. viticola sp. aestivalis* and then European populations were the source of for secondary introduction in other continents, such as China, South Africa and Australia (Yin *et al*., 2014; Taylor *et al*., 2019; Fontaine *et al*., 2021). Interestingly, populations in warmer climates might be different to those in regions with more favorable conditions, probably due to selective pressure leading to bottlenecks caused by unfavorable climatic conditions (Koopman *et al*., 2007).

A study conducted on Italian *P. viticola* populations using 19 microsatellite markers, confirmed again the poor genetic diversity and weak structure on the spatial scale, with complete absence of clone (i.e. isolates sharing identical genotypes), highlighting, however, a slight differentiation in two sub-populations (Maddalena *et al*., 2020).

Research on the genetic diversity and population structure of *Plasmopara viticola* has revealed several important insights. North American populations exhibit higher genetic diversity, likely due to a founder effect, while regions in Europe where the pathogen was initially introduced also show relatively high genetic variation compared to other European areas. Within Europe, most of the genetic diversity is concentrated at small spatial scales, such as within vineyards. Although data on long-distance dispersal remain unavailable, its potential influence cannot be disregarded. Primary infections play a key role in initiating epidemics, while zoosporic infections sustain disease cycles throughout the growing season. The population structure of *P. viticola* across Europe is weak but

significant, and the populations function as panmictic units with frequent sexual recombination occurring during winter (Gouveia *et al*., 2024).

Genetic characterization of *P. viticola* is essential for understanding its epidemic dynamics and evolutionary processes in the field. The pathogen's high mutation rate and efficient asexual sporulation likely drive its rapid adaptation to single-site fungicides (Chen *et al*. 2007; Delmas *et al*., 2017; Toffolatti *et al*., 2018). Furthermore, studies suggest that *P. viticola* undergoes rapid evolution through soft sweeps, where adapted alleles repeatedly emerge from standing genetic variation (Chen *et al*., 2007; Delmas *et al*., 2017). These combined factors highlight the importance of investigating the genetic mechanisms underlying the pathogen's adaptability to develop more sustainable management strategies.

At the moment, the management of this pathogen relies on a multi-level approach. The combination of agronomical practices, the selection of less susceptible genotypes and a complex, integrated, smart and precise management of the fungicides present on the market.

Genetic resistance to *P. viticola* has been identified in wild American and Asian grape species, which co-evolved with this pathogen. Breeding programs leverage these resistance traits to develop hybrid cultivars that integrate resistance with desirable agronomic traits, such as high yield and optimal organoleptic properties. While European Vitis vinifera cultivars are highly susceptible to the pathogen, several American (*V. rotundifolia*, *V. rupestris*, *V. labrusca*, *V. riparia*, *V. cinerea*) and Asian species (*V. amurensis*, *V. piasezkii*, *V. coignetiae*) exhibit varying levels and mechanisms of resistance. Efforts to develop resistant varieties began in the 19th century, with breeders crossing susceptible *V. vinifera* cultivars with resistant *Vitis* species (Zyprian *et al*., 2016; Sargolzaei *et al*., 2020). The introgression of quantitative trait *loci* (QTLs) associated with resistance, such as *Rpv*

genes, has facilitated the development of cultivars with durable resistance (see Chapter 4.3.1 for further details).

To date, researchers have identified 31 QTLs associated with moderate to high levels of resistance (see Table 13, Chapter 4.3.1). However, most commercially available resistant varieties rely on resistance conferred by *loci* such as *Rpv1*, *Rpv3*, *Rpv10*, and *Rpv12* (Sargolzaei *et al*., 2020; VIVC, 2022). Adopting resistant grapevine varieties offers growers significant benefits, including reduced fungicide applications, lower labor costs, and increased yields.

Despite these advantages, market acceptance of resistant varieties remains limited. Wines produced from these varieties constitute a small fraction of the market, and consumer perception often associates them with lower quality. Although studies have demonstrated that wines from resistant varieties can match or even surpass traditional wines in quality, consumer education remains essential (Espinoza *et al*., 2018). Legal restrictions further complicate the situation: in many regions, the cultivation of resistant varieties is not permitted for wine production under protected designations of origin (Dagostin *et al*., 2011; Pertot *et al*., 2017). Moreover, switching to resistant varieties represents a considerable investment for farmers, since susceptible grapevines have to be replaced in the vineyards, leading to a lack of grape production for the first years after the planting. Hence growers may wait until the already present grapevines come to the natural end of their productive lives, before replacing them with resistant genotypes (Pedneault and Provost, 2016).

Another challenge is the emergence of *P. viticola* isolates capable of overcoming resistance conferred by certain QTLs, posing a serious risk to the durability of these varieties (Wingerter *et al*., 2021).

Currently, the management of *P. viticola* primarily depends on frequent and repeated fungicide applications to maintain sustainable yields, especially under conditions that favor disease development. Growers generally follow a phenology-based schedule for fungicide application, beginning at grapevine bud break and continuing until veraison. In Europe, nearly 70% of all fungicide applications occur in vineyards (Wingerter *et al.,* 2021).

Chemical fungicides remain integral to *P. viticola* management, however the vast majority of the active ingredients registered present on the market are not allowed in organic viticulture.

Copper-based fungicides are among the oldest tools for managing downy mildew and remain crucial, especially in organic viticulture. Copper compounds such as copper hydroxide, oxychloride, and sulfate act as multi-site protectants, forming a defensive barrier on plant surfaces (Koledenkova *et al*., 2022). However, long-term use of copper has raised environmental and health concerns. Its accumulation in soil and groundwater poses risks of phytotoxicity, while excessive exposure in humans is linked to liver diseases and neurological conditions (Ballabio *et al*., 2018). As a result, the European Union has progressively lowered the allowed amount of this active ingredient, and therefore researchers are working relentlessly to reduce copper usage in agriculture (Thuerig *et al*., 2018).

In addition to copper, other multi-site contact fungicides such as dithiocarbamates (e.g., mancozeb, metiram, and propineb), quinones (e.g., dithianon), and phthalimides (e.g., captan and folpet) were commonly used for preventive treatments. These fungicides are effective when applied before infection, but their low mobility limits protection to treated tissues, leaving new tissues vulnerable.

The introduction of systemic and cytotropic fungicides has revolutionized downy mildew management. These fungicides, which include carboxylic acid amides (e.g., mandipropamid and

dimetomorph), phenylamides (e.g., metalaxyl-m and benalaxyl), and complex III inhibitors (e.g., strobilurins, cyazofamid, and amisulbrom), offer long-lasting protection and curative activity (Blum *et al*., 2010; Fontaine *et al*., 2019; Mounkoro *et al*., 2019). Moreover, they shield new growth and resist being washed off by precipitation, making them particularly valuable during peak susceptibility periods, such as bloom.

Recently, oxathiapiprolin, a member of the Oxysterol Binding Protein Inhibitor (OSBPI) group, has emerged as a promising addition to the fungicide arsenal (Massi *et al*., 2021).

Plasmopara viticola, like other oomycetes, is considered a high-risk pathogen due to its remarkable evolutionary adaptability. This adaptability is fueled by their complex life cycle, which includes both sexual and asexual reproduction, and their ability to complete multiple infection cycles within a single growing season (Poeydebat *et al*., 2022). Additionally, the repeated application of single-site fungicides intensifies selection pressure, favoring the survival of tolerant populations. Notably, resistance to many widely used single-site fungicides has been reported soon after their market introduction (Ma and Michailides, 2005).

Fungicide resistance, a critical challenge in plant pathology, refers to the acquired and inheritable decrease in a fungal or oomycete pathogen's sensitivity to a specific fungicide (Background Information, FRAC). This resistance arises through five primary mechanisms: alterations to the target site that prevent fungicide binding, overproduction of the target protein, activation of alternative metabolic pathways to bypass the fungicide's inhibition, metabolic breakdown of the fungicide's active ingredient, and active export or exclusion of the fungicide from pathogen cells (Deising *et al*., 2008).

During pathogen evolution, mutations naturally occur, some of which can confer increased tolerance to fungicides. Initially, resistant individuals within a pathogen population represent only a minor threat. However, when resistant individuals proliferate and dominate, fungicide treatments may fail, leading to significant management challenges. The development of resistance is influenced by the fungicide's mode of action, the pathogen's biological and epidemiological characteristics, and the agronomic practices employed (Massi *et al*., 2021).

Complex III inhibitors, a crucial class of fungicides, have been extensively used in disease management. These fungicides are classified into three groups by the Fungicide Resistance Action Committee (FRAC): Quinone outside Inhibitors (QoIs), which include strobilurins and famoxadone; Quinone inside Inhibitors (QiIs), such as cyazofamid and amisulbrom; and Quinone inside-outside stigmatellin binding site Inhibitors (QoSIs), represented by ametoctradin (Cherrad *et al*., 2018).

QoI fungicides disrupt mitochondrial respiration by targeting the Qo site of the cytochrome bc1 complex, halting electron transfer and ATP synthesis (Bartlett *et al*., 2002). However, resistance to QoIs emerged rapidly following their introduction. Globally, *P. viticola* isolates resistant to QoIs have been identified, with all resistant isolates carrying the G143A mutation, which substitutes glycine with alanine at codon 143 of the cytochrome b gene (Gullino *et al*., 2004; Chen *et al*., 2007; Furuya *et al*., 2010; Colcol and Baudoin, 2016).

Among QoSIs, resistance to ametoctradin has been observed in *P. viticola* populations in France. Two studies identified resistance mechanisms involving a single-point mutation in cytochrome *b* (S34L substitution) and alternative oxidase activity (Mounkoro *et al*., 2019; Fontaine *et al*., 2019). Interestingly, the S34L substitution has also been linked to cyazofamid resistance in *P. viticola* isolates (Cherrad *et al*., 2018).

Carboxylic acid amide (CAA) fungicides, including dimethomorph and mandipropamid, are single-site fungicides that inhibit the biosynthesis of oomycete cell walls (Corio-Costet, 2012). Reports of CAA-resistant *P. viticola* isolates have surfaced worldwide (Aoki *et al*., 2015; Nanni *et al*., 2016; Ghule and Sawant, 2018; Toffolatti *et al*., 2018). A mutation in the *PvCesA3* gene (G1105S) is suspected to underlie this resistance (Blum *et al*., 2010). Nonetheless, additional recessive genes also contribute to resistance, reducing its prevalence in the field (Corio-Costet, 2012).

Phenylamides, first introduced in 1977, include fungicides such as metalaxyl, metalaxyl-m, and mefenoxam (Gisi and Sierotzki, 2015). Resistance to phenylamides has been associated with a Y382F mutation in the RNA polI gene, which was recently identified in *P. viticola* isolates resistant to mefenoxam (Randall *et al*., 2014).

More recently, resistance to oxathiapiprolin, a member of the Oxysterol Binding Protein Inhibitor (OSBPI) class, has been detected in *P. viticola* populations in France and Italy. This resistance is linked to specific amino acid substitutions: G770V, N837L, and L863W in the OSBP gene, and N837I in the *PvORP1* gene (Mboup *et al*., 2022; Massi *et al*., 2022).

Lastly, the C239S substitution, known to confer resistance to zoxamide in *Phytopthora sojae*, has been detected for the first time in *P. viticola* isolates in North-East Italy (Cherrad *et al*., 2024).

The rapid emergence of fungicide resistance in *P. viticola* underscores the pathogen's evolutionary potential and the challenges of managing it. The pathogen's reproductive strategies, including frequent recombination events during sexual reproduction and its ability to complete numerous infection cycles in a growing season, enhance its adaptability and accelerate the development of resistance.

Repeated reliance on single-site fungicides exacerbates these challenges, intensifying selection pressure and facilitating the proliferation of resistant strains. Despite their effectiveness, these fungicides must be used judiciously, often in rotation or combination with multi-site fungicides or with fungicides with a different mode of action, to mitigate resistance development. Finally, resistance monitoring is crucial to keep track of the state of the art in the field and consequently adjust the disease management strategies accordingly (Massi *et al*., 2021).

Managing fungicide resistance requires an integrated approach that combines the use of diverse fungicide classes, adoption of cultural practices to reduce disease pressure, and the incorporation of decision support systems (DSS) to optimize fungicide application timing. DSS tools leverage environmental and pathogen-specific data to forecast infection risks, enabling growers to apply treatments more strategically and minimize unnecessary fungicide use (Bove *et al*., 2020; Brischetto *et al*., 2021).

In addition to integrated chemical strategies, there is growing interest in alternative approaches, such as the use of biocontrol agents and breeding for resistant grapevine varieties. Biocontrol agents (BCA), including microbial antagonists and plant-derived compounds, offer potential for reducing reliance on chemical fungicides, although challenges like environmental stability and cost must be addressed for widespread adoption (Thuerig *et al*., 2018).

Literature offers countless examples of plant extracts, microbial and fungal BCA that had, to some extent, inhibitory activity towards *P. viticola*. Among plant extracts, main metabolites with anti-oomycete activity were mainly belonging to flavonols, flavonoids, terpenes and stilbenes, while mBCA and fBCA were characterized by the production of secondary metabolites with antifungal activity (Nadalini and Puopolo, 2024, also see Chapter 4.5.1). Biofungicides represent an excellent opportunity as partners in integrated disease management strategies since they are often characterized

by multi-level mode of action, acting directly against the pathogen but also eliciting plant response and can be applied preventively (Perazzolli *et al*., 2008; 2011).

Unfortunately, while the majority of the studies were based on germination tests or leaf disc bioassays, very few candidates made it successfully to field conditions, preserving satisfying plant protection efficacy. In fact, one of the critical aspects is their rain fastness and resistance to degradation by heat or UV-light, which require more frequent applications, alongside with their higher economical costs compared to traditional fungicides (Dagostin *et al.,* 2011; Gessler *et al.,* 2011; Pertot *et al.,* 2017). Fungicide formulation will surely play a crucial role in protecting active ingredient efficacy and persistence on the plants.

The management of *Plasmopara viticola* necessitates an integrated pest management approach that combines genetic, chemical, cultural, and biological methods. Advances in molecular biology and genomics are facilitating the identification of novel resistance mechanisms and the development of pathogen-resistant cultivars. Precision viticulture tools, including remote sensing technologies and predictive modeling, enable early disease detection and optimized fungicide application.

The future of *P. viticola* management will depend on the continued development of sustainable, integrated solutions. By leveraging advancements in fungicide chemistry, pathogen biology, and precision agriculture, the viticulture industry can work to counteract resistance and ensure effective disease control. Collaboration among researchers, policymakers, and industry stakeholders will be critical to achieving this goal and maintaining the viability of grape production in the face of evolving challenges.

CHAPTER 2. AIM OF THE THESIS

The aim of this PhD project was to offer deeper and broader insights regarding the development and implementation of integrated pest management strategies to control grapevine downy mildew, *Plasmopara viticola*.

The specific aims of this work were to investigate the genetic structure of *P. viticola* and the occurrence of resistance to single-site fungicides in the Trentino Alto Adige region, under different disease management strategies (CHAPTER 3), to review the state of the art of the research upon the biocontrol of *P. viticola* (CHAPTER 4), and thus investigate and characterize novel candidates to be potentially proposed as biofungicides, based on plant extracts and a microbial biocontrol agent (CHAPTER 5, CHAPTER 6, CHAPTER 7).

CHAPTER 3.

LOW GENETIC DIVERSITY AND ADAPTATION TO SINGLE-SITE FUNGICIDE OF *PLASMOPARA VITICOLA* POPULATIONS IN TRENTINO SOUTH TYROL (ITALY)

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Abstract

Downy mildew of grapevine, caused by the plant pathogenic oomycete *Plasmopara viticola*, is one of the most devastating diseases for viticulture worldwide. In this study, for the first time, the population structure and the genetic variability of *P. viticola* population in Trentino South Tyrol on a regional level, one of the leading Italian winegrowing regions, was investigated by genotyping 307 isolates collected in 2021 and 2022 from 18 vineyards, characterised by three different disease management strategies, at 13 *loci* using microsatellite markers. Structure analysis of *P. viticola* population of Trentino South Tyrol highlighted the presence one single population with significantly reduced genetic diversity, as in the case of an introduced and spatially expanding pathogen, and a panmictic behaviour, with only 5 out of 13 *loci* diverging from Hardy-Weinberg Equilibrium.

Fungicide sensitivity leaf disc bioassays on 11 active ingredients showed widespread adaptation to mandipropamid, cymoxanil, ametoctradin and amisulbrom with isolates frequently exceeding baseline EC_{50} thresholds across all management strategies and rarely confirmed on both years, with the exception of mandipropamid. This first comprehensive study of *P. viticola* in Trentino South Tyrol provides critical insights into its biology and resistance dynamics.These findings

underscore the need for and importance of the future to reduce the selection of pathogen strains able to overcome the mode of action of the few active principles currently applied to control *P. viticola*, as well as, to preserve novel molecules and the durability of disease resistant varieties.

Keywords: *Plasmopara viticola, population genetics, fungicide resistance, genotyping, SRR analysis.*

3.1 Introduction

The plant pathogenic oomycete *Plasmopara viticola* (Berk. & Curt) Berl. & de Toni, the causal agent of grapevine downy mildew, represents one of the major threats for viticulture worldwide. In fact, *P. viticola* is a polycyclic pathogen that causes remarkable losses both in terms of yield and quality as it can attack leaves, inflorescences and berries, leading to tissue necrosis (Gessler *et al*., 2011). *P. viticola* was accidentally introduced from North America through infected plant material used for rootstocks in order to control the devastating effect of phylloxera on *Vitis vinifera* caused by *Daktulosphaira vitifoliae* Fitch. It was first detected in France in 1878 and then rapidly spread in Europe, which offers the perfect climatic condition for the development, reproduction and diffusion of this pathogen, given the mild temperatures, the humid climate and the frequent precipitations during the vegetative season (Toffolatti *et al*., 2018).

The mating system of *P. viticola* is heterothallic and requires distinct sexual compatibility or mating types (Lamour & Kamoun, 2009). P. viticola is a plant pathogen characterized by a polycyclic life cycle, which includes a variable number of asexual cycles and a sexual phase, increasing the allele recombination and mutation rate and thus contributing to a rapid and dynamic evolution of resistance to single-site fungicides or break down of grapevine resistance genes (Chen *et al*., 2007; Peressotti *et al*., 2010; Delmotte *et al*., 2014; Delmas *et al*., 2017). Appropriate disease control strategies are crucial to achieve a sustainable yield under favorable conditions for the development of the disease.

In the last decades, farmers relied on repeated applications of copper-based fungicides or of single-site chemical fungicides. Nowadays, the main single-site fungicide classes used to control *P. viticola* infections are: a) carboxylic acid amides (CAA), such as mandipropamid and dimetomorph (Blum *et al*., 2010); b) phenylamides, like metalaxyl-m, and c) complex III inhibitors, such as strobilurins (Quinone Outside Inhibitors, QoI), cyazofamid and amisulbrom (Quinone inside

Inhibitors, QiI), ametoctradin (Quinone outside Stigmatellin binding site Inhibitors, QoSI) (Blum *et al*., 2010; Fontaine *et al*., 2019; Mounkoro *et al*., 2019; Massi *et al*., 2021; Koledenkova *et al*., 2022).

In the last decades, *P. viticola* isolates resistant to single-site fungicides with different modes of action have been recorded worldwide. For instance, shortly after the introduction on the market of Quinone Outside Inhibitors (QoIs), *P. viticola* resistant isolates carrying the G143A mutation at the *cyt b*, were detected in Italy, France, Japan, Brazil and the United States (Gullino *et al*., 2004; Chen *et al*., 2007; Furuya *et al*., 2010; Santos *et al*., 2020; Campbell *et al*., 2021). Among the Quinone inside and outside inhibitor, stigmatellin binding site (QoiSI), studies in France and in India recorded resistance to ametoctradin, both involving a single point mutation in the cytochrome *b*, by S34L substitution (Fontaine *et al*., 2019; Mounkoro *et al*., 2019; Sagar *et al*., 2024) and to cyazofamid (Cherrad *et al*., 2018).

The carboxylic acid amide (CAA) fungicides, including dimethomorph and mandipropamid, are single-site fungicides inhibiting cell wall biosynthesis of oomycetes (Corio-Costet *et al*., 2012). Recently, several CAA resistant *P. viticola* isolates have been reported worldwide (Aoki *et al*., 2015; Nanni *et al*., 2016, Ghule and Sawant 2018; Toffolatti *et al*., 2018). The mutation G1105S at the *PvCesA3* gene reportedly causes resistance (Blum *et al*., 2010), however, several other recessive genes are involved in resistant *P. viticola* isolates, decreasing the risk of resistance in the field (Corio-Costet 2012).

In the last decades, seven molecules belonging to the phenylamides group have been released, among which metalaxyl, metalaxyl-m and mefenoxam (Gisi and Sierotzki, 2015). Recently, a single point mutation was identified as Y382F in the RNA polI gene, associated with resistance to mefenoxam in *P. viticola* isolates (Randall *et al*., 2014).

Plasmopara viticola isolates resistant to oxathiapiprolin have been detected in France and Italy. In these cases, the resistance was caused by the amino acids substitutions G770V, N837L and L863W on the *OSBP* gene (Mboup *et al*., 2022; Cherrad *et al*., 2024) and N837I at PvORP1 gene (Massi *et al*., 2022).

Lastly, the C239S substitution, known to confer resistance to zoxamide in *Phytophthora sojae,* has been detected for the first time in *P. viticola* isolates in North-East Italy (Cherrad *et al*., 2024).

Managing fungicide resistance in vineyards requires a thorough understanding of the pathogen's population genetic structure, including reproductive mode, gene flow, and effective population size (Delmotte *et al*., 2006). Several studies have already demonstrated that the European *P. viticola* population introduced from North America has been subjected to the invasive pathogen founder effect leading to a loss of genetic diversity (Rouxel *et al*., 2012; Fontaine *et al*., 2021). In fact, only one of the five native North American lineages established in Europe, spreading followingly to Asia, South Africa, Australia and South America, as a consequence of the transport and transport of European grapevine plants. (Fontaine *et al*., 2021).

The genetic population structure and the pathogen epidemics were studied at continental (Gobbin *et al*., 2006; Fontaine *et al*., 2013; Li *et al*., 2016), regional (Gobbin *et al*., 2003; Delmas *et al*., 2017; Maddalena *et al*., 2020) and field or single plant scale (Gobbin *et al*., 2003; Matasci *et al*., 2010), showing the panmictic behavior of *P. viticola* populations and highlighting the role of sexual and asexual reproduction.

To date, only few studies investigated the population structure of *P. viticola* in Italy, even though it is one of the leading wine producing countries. These studies took into consideration only a few regions (Gobbin *et al*., 2003), or a limited number of isolates within broad European research

(Fontaine *et al*., 2013) and were mainly based on the analysis of a very limited number of *loci* (5 to 8 SSR). In 2020, Maddalena *et al*. (2020) broadly investigated the genetic structure of *P. viticola* population, at 31 *loci* on samples collected over 12 Italian regions homogeneously distributed over the country, however eight regions were not included in the study, such as Trentino-South Tyrol.

Indeed, no recent studies focusing on the genetic structure of *P. viticola* populations in Trentino-South Tyrol, one of the most renowned Italian regions for wine making, are currently available. Moreover, in the recent years, research took into consideration a limited number of isolates or active ingredients in this region, leaving still a considerable gap in the knowledge regarding *P. viticola* populations and the adaptation to single site chemical fungicides (Gullino *et al*., 2004; Toffolatti *et al*., 2011; Nanni *et al*., 2016, Massi *et al*., 2022; Nanni *et al*., 2024).

In this study, we propose a comprehensive investigation on *P. viticola*, with the goal of filling a knowledge gap about the *P. viticola* population in the Trentino-South Tyrol region, investigating: i) the genetic structure of the pathogen, analyzing 307 isolates sampled in 2021 and 2022 in 18 different vineyards, characterized by three different management strategies, at 13 *loci* using microsatellite markers; and ii) the occurrence of *P. viticola* isolates resistant to single-site chemical fungicides testing a wide range of different mode of action classes such as CAA (mandipropamid and dimetomorph), benzamides (fluopicolide and zoxamide), phenylamides (metalaxyl-m), quinone inhibitors (QoI, QiI, QoSI; azoxystrobin, ametoctradin and amisulbrom), cymoxanil and oxathiapiprolin (oxysterol binding protein homologue inhibition, OSBPI).

The information gathered could play a crucial role in understanding the pathogen evolution, even at a local scale, in order to reduce the selection of isolates able to overcome the mode of action of the few active ingredients currently applied to control *P. viticola*, and thus implementing disease management strategies to preserve novel molecules and the long-term durability of resistant varieties.

3.2 Materials and Methods

3.2.1 Experimental design and field sampling

For the present study, 18 vineyards were selected in the two Autonomous Provinces of Trento and Bozen-Bolzano (Italy), undergoing three different disease management strategies, i.e. integrated and two organic strategies at different amount of copper input per hectare (Table 1).

Table 1 Geographical information and disease management of the sampled vineyards.

Province	**Location**	**Code**	**GPS Coordinates**	**Disease management**	**Variety**
Bolzano	Terlan	Ter_O	46°32'16"N 11°15'06"E	Organic 4 kg Cu ha^{-1} $year^{-1}$	Pinot Blanc
	Tramin	Tr_O	46°21'09"N 11°14'36"E		
	Magreid	Mag_O	46°16'33"N 11°12'37"E		
	Terlan	Ter_O+	46°32'12"N 11°15'15"E	Organic 3 kg Cu ha^{-1} $year^{-1}$	
	Tramin	Tr_O+	46°24'32"N 11°15'00"E		
	Magreid	Mag_O+	46°14’31"N 11°13’38"E		
	Terlan	Ter_I	46°31'56"N 11°15'15"E	Integrated	
	Tramin	Tr_I	46°20'27"N 11°15'01"E		
	Magreid	Mag_I	46°18'44"N 11°12'44"E		
Trento	San Michele a.A.	SaM_O	46°11'58"N 11°08'16"E	Organic 4 kg Cu ha^{-1} $year^{-1}$	Chardonnay
	Valle dei Laghi	Vdl_O	46°03'50"N 10°59'33"E		
	Rovereto	Rov_O	45°52'50"N 11°00'16"E		
	San Michele a.A.	SaM _O+	46°11'56"N 11°06'33"E	Organic 3 kg Cu ha^{-1} $year^{-1}$	
	Valle dei Laghi	Vdl_O+	46°01'55"N 10°57'00"E		
	Rovereto	Rov_O+	45°52'07"N 11°02'24"E		
	San Michele a.A.	SaM _I	46°13’39"N 11°09’34"E	Integrated	
	Valle dei Laghi	Vdl _I	46°03'58"N 10°59'26"E		
	Rovereto	Rov _I	45°45'10"N 10°59'20"E		

Vineyards were located along the Adige Valley and near Garda Lake, in the main areas of interest for winemaking in the region, at an altitude ranging from 70m to 400m above sea level.

Grapevine leaves presenting visible *P. viticola* symptoms (oil spots) were collected during two consecutive vegetative seasons comprising a total of 315 samples (180 in 2021 and 135 in 2022). Leaves were individually put in sterile plastic bags and incubated at 95 ± 5% relative humidity (RH) overnight, to induce *P. viticola* sporulation. Five leaves per vineyard were used for the fungicide sensitivity bioassay, while the remaining material was stored at -80°C for DNA extraction and further analyses.

3.2.2 Genetic composition of P. viticola *population*

3.2.2.1 DNA extraction and microsatellite amplification

Single lesions were collected with a sterile 2 cm diameter cork borer. Each sample was placed in a wet chamber and incubated overnight at 24°C to promote *P. viticola* sporulation. The samples were transferred into a collection tube and then placed at -80 °C until DNA extraction. Total DNA was extracted from single sporulated oilspots using the DNeasy Plant Mini Kit (QIAGEN, Hilden, Germany), according to the manufacturer's instructions, slightly modified by adding PVP40 (1% w/v) to the extraction buffer AP1 (Neuhaus *et al.,* 2009). The DNA quantity and quality values were determined by a spectrophotometer (DeNovix Inc, Wilmington, Delaware) and the samples were brought to a final concentration of 10 ng μL^{-1} with Nuclease-free water (QIAGEN, Hilden, Germany). The *P. viticola* isolates were genotyped at 22 species-specific microsatellite *loci*: ISA (Gobbin *et al.*, 2003); PV7, PV13, Pv14, PV17, PV31, PV39 (Delmotte *et al.,* 2006); PV61, PV65, PV67, PV83, PV88, PV91, PV93, PV101, PV103, PV104, PV127, PV138, PV139, PV141, PV142 (Rouxel *et al.*, 2012). Primer sequences, amplicon size, melting temperature, fluorescent dye conjugated at the 5' end of the forward primers are reported in Table 2.

Table 2 Characteristics of the 22 microsatellite *loci* used in this study and grouped by Multiplex PCR: *locus* name, primer sequence, annealing temperature, number of alleles (**Na**) and size range of alleles (bp). Fluorophore used in this study.

Multiplex PCR	*Locus*	Primer sequence (5'−3')		Annealing temperature (°C)	Fluorophore	*Na*	Size range (bp)	Reference
		Forward	Reverse					
-	ISA	ATTAGCGGCATGGACGTT	GAGAAGTTCCGCCAAGTACA	60	6FAM	4	118 – 144	Gobbin *et al.*, 2003
1	PV14	CAGAAACGCACAAGGTCTGA	AATTGCATACTGCAGCAACG	65	ATTO565	5	120 – 128	Delmotte *et al.*, 2006
	PV17	CAGAGTCGAACAAGTACATTG	CTTTGTCGCCTTCTAACAAC	59	HEX	6	160 – 172	Delmotte *et al.*, 2006
	PV39	ACGCATGGCGAACACGTAAG	CAGACGGGAAGAAGTTGCTC	61	ATTO565	2	174 – 176	Delmotte *et al.*, 2006
	PV13	CGATGAAGTGGACCCTCATT	CCGGTAGTCAATTGCACCTT	61	6FAM	4	214 – 220	Delmotte *et al.*, 2006
	PV31	TCCCCATGCTGAAGAGTTTC	TTCTTTCTAAGGCCGTGTGG	60	HEX	4	241 – 247	Delmotte *et al.*, 2006
	PV7	TCTTCCGAAAAGGGACGTAA	GCGTCACTGCATCTACGAAA	60	6FAM	5	289 – 297	Delmotte *et al.*, 2006
2	PV139	GACCCGGACAATGGACTCTA	CCGCCATGTATTGAACAGTG	57	6FAM	3	126 – 133	Rouxel *et al.*, 2012
	PV141	ACGACGACATGAGCTGTACG	GAAGGTGGTGTCATGGGTTT	57	HEX	2	190 – 192	Rouxel *et al.*, 2012
	PV65	CTTTGGCCCACGTCATAGTT	CGCTTTCGGTAGGTCCATTA	57	ATTO565	2	196 – 202	Rouxel *et al.*, 2012
	PV142	TTATGCCACGCAAATCTCTG	AGGGCGAAATACGAGAGTGA	57	6FAM	2	209 – 219	Rouxel *et al.*, 2012

	PV138	CGTGGATCATGACGTTTGTC	CGACGAATCAGGGACAAGAT	57	HEX	5	225 - 235	Rouxel *et al.*, 2012
3	PV91	ACCAGCCTTTGCGAAGATAA	TGAAAGTTACGTGTCGCACC	54	6FAM	2	142 – 146	Rouxel *et al.*, 2012
	PV88	AATACCAAAAATGGCCGTCA	ACTCTCTTGCCAGCACCATC	54	HEX	2	202 – 208	Rouxel *et al.*, 2012
	PV127	TTGAAAACGCGGATAGGAAC	GAACGTCCAGTTCGGATTGT	54	6FAM	3	213 – 223	Rouxel *et al.*, 2012
	PV103	TGACCTACCACCCATTTACCA	ACGGTCAGGTCAAAAGCAGT	54	HEX	2	277 – 299	Rouxel *et al.*, 2012
	PV104	CTACGCTCGAGGATGACACA	GACATTGCCGCACCTAAGAT	54	6FAM	1	321 - 324	Rouxel *et al.*, 2012
4	PV93	TAGCACCGGACTAGGCGTAT	TGTACCCTGTTGCCCTCTTC	54	6FAM	3	147 – 151	Rouxel *et al.*, 2012
	PV61	TCTTCAGGTAGATGCGACCA	GGTGACTCCTCGGACGAATA	54	HEX	3	181 – 187	Rouxel *et al.*, 2012
	PV83	TGCAGCATTGTTTCATCCAT	ACACGGTACTTTGCGTTCCT	54	6FAM	2	238 – 242	Rouxel *et al.*, 2012
	PV101	AACACGGCGCCAAAGTATTA	GGGCATTAACGTGCAAATTC	54	HEX	2	263 – 266	Rouxel *et al.*, 2012
	PV67	GCATTGAGCAGACACCTTGA	GAGCGATAAGACCACAAATA GTGA	54	6FAM	2	348 – 368	Rouxel *et al.*, 2012

Considering the latter parameters, multiplex PCR was carried out by combining the primers pairs as reported in Table 2. The PCR mix contained 10 ng DNA template, 1 × PCRBIO HS Taq Mix (PCRBiosystem; London, UK), primers at the final concentration reported in Table 3 and Nuclease-free water to adjust the final volume to 15 μL.

The amplification protocol was: 5 min at 95.0 °C, 30 cycles of 30 s at 95.0 °C, 60 s at 55.0 °C, 45 s at 72.0 °C, and a final elongation step of 10 min at 72.0 °C. The amplification of the ISA *locus* was performed independently with the following conditions: 5 min at 96.0 °C, 30 cycles of 30 s at 96.0 °C, 30 s at 60.0 °C, 50 s at 72.0 °C, and a final elongation step of 10 min at 72.0 °C. The amplification products were diluted 20fold and fragment length analysis was performed by Microsynth Ecogenics GmbH (Balgach, Switzerland) in 96-well PCR plates by automated capillary gel electrophoresis on an ABI 3730 XL Genetic Analyzer system (Thermo Fisher Scientific, Merelbeke, Belgium) with the following working conditions: injection time, 10 s; injection voltage, 1.6 kV; run time, 2100 s; run voltage, 15 kV; Capillary length, 50 cm; Polymer, POP7; internal lane standard (ILS) 600.

Table 3 Details of Multiplex PCR protocol: final primer concentration (μM) for each forward and revers primer per *locus* per single amplification reaction and annealing temperature applied (°C).

Multiplex PCR	***Locus***	**Final primer concentration (μM)**	**Annealing temperature (°C)**
-	ISA	0.4	60
1	PV14		55
	PV17		
	PV39		
	PV13		
	PV31	0.2	
	PV7	0.4	
2	PV139	0.2	55
	PV141		
	PV65	0.1	
	PV142		
	PV138	0.4	
3	PV91		55
	PV104	0.2	
	PV88		
	PV103	0.1	
	PV127	0.4	
4	PV93	0.4	55
	PV61		
	PV101	0.1	
	PV83	0.2	
	PV67	0.8	

3.2.2.2 Data analysis

Fragment length analysis chromatograms were analyzed using the microsatellite plugin of Geneious Prime software (Biomatters Ltd, Auckland, New Zealand) and allele sizes were recorded in bp (ladder ILS 600). The GenAlEx 6.5 software (Peakall and Smouse, 2006, 2012) was used to calculate for each *locus*: number of alleles, allele frequencies, observed (H_o) and expected (H_e) heterozygosity, fixation index (F_{IS}) and deviation from Hardy-Weinberg Equilibrium (HWE). Moreover, GenAlEx 6.5 was applied to calculate the genetic differentiation (F_{ST}) via analysis of molecular variance AMOVA (Peakall, Smouse, & Huff, 1995) among *P. viticola* samples grouped according to sampling location, disease management strategy, grapevine cultivar and sampling vegetative season. Software STRUCTURE 2.3.4 (Pritchard *et al.,* 2000) was used to perform a Bayesian clustering analysis applying Markov Chain Monte Carlo (MCMC) estimation. The analysis was conducted by setting the admixture model and using the default parameters. The clusters ranged from K=1 to K=8, with a burn-in period of 100.000, followed by 100.000 MCMC replicates, and for each K 20 iterations were run. To infer the possible number of different clusters (K) represented by the data, the web-based software StructureSelector (Li and Liu, 2018) was used which allowed to visualize the output from Structure. The pairwise genetic dissimilarities among genotypes were calculated by using the software DARwin (Perrier and Jacquemoud-Collet, 2006), which can be used to determine the dissimilarity for allelic data. Based on the dissimilarities, a dendrogram was obtained using the Neighbor Joining method.

3.2.3 Fungicide sensitivity bioassay

3.2.3.1 Propagation of *P. viticola* isolates

After successful leaf sporulation, sporangia were suspended in 4°C distilled water and sprayed on healthy, susceptible Pinot noir plants, using a hand sprayer. Plants were kept in greenhouse conditions at 25°C for 6 days, as previously described by Puopolo *et al.* (2014). If necessary, the propagation protocol was repeated to obtain a sufficient amount of inoculum.

3.2.3.2 Active ingredient application

For efficacy tests against *P. viticola*, leaf discs (14 mm in diameter) were obtained from the third to fifth apical leaves from shoots of grapevine plants (*V. vinifera* cv. Pinot Noir, grafted onto Kober 5BB) grown in greenhouse conditions at 25°C with a photoperiod of 16 h of light and a relative humidity of 60% ± 10% for two months. Leaf discs were transferred in Petri dishes (lower surface uppermost) onto sterilized moist filter paper.

Eleven active ingredients were selected among those included in the Trentino South Tyrol integrated pest management protocol (Table 4). Pure active ingredients (PESTANAL®, Sigma Aldrich) were dissolved in 70% ethanol and applied on the leaf discs at different concentrations (100%, 10%, 3.3%, 1% and 0.1% of the maximum allowed field dose) using a Potter Tower (Burkard Scientific Co., Uxbridge, UK) with a volume of 4 mL per Petri dish, as described by Cabus *et al.* (2017). Leaf discs were treated with 70% ethanol as negative control. Finally, the leaf discs were dried and incubated at 25°C for 24 hours.

Table 4 Active ingredients evaluated in the present study. For each active ingredient, the mode of action, FRAC code and maximum dose of application recommended by the commercial product label are provided.

Active ingredient	Commercial name	Mode of action class	FRAC code	Application rate (mg L^{-1})
Ametoctradin	Enervin SC	QoSI	45	288
Amisulbron	Genkotsu	QiI	21	75
Azoxystrobin	Quadris	QoI	11	250
Cymoxanil	Curzate	Unknown	27	140
Dimetomorph	Forum 50 WP	CAA	40	250
Fluopicolide	R6 Albis	Benzamides	43	132
Mandipropamid	Pergado	CAA	40	150
Metalaxyl-m	Ridomil-gold	Phenylamides	4	100
Oxathiapiprolin	Zorvec Zelavin	OSBPI	49	40
Zoxamide	Zoxium 240	Benzamides	4	164

3.2.3.3 *Plasmopara viticola* artificial inoculation

After 6 days of incubation, plants infected with the *P. viticola* isolates collected from the vineyards were individually incubated overnight at 25°C and 95% ± 5% RH to induce sporulation. Afterwards, a sporangia suspension was obtained from each isolate by gently brushing the lower leaf page in 4°C distilled water and diluted to a concentration of 1×10^5 sporangia mL^{-1} by counting with a hemocytometer under a light microscope. Isolates sampled in the same vineyard were pooled. 24 hours after the active ingredient application, 4 mL of sporangia suspension were sprayed on every Petri dish. Finally, the leaf discs were moved to 24-wells Petri dishes, each well containing 1 mL of

1% water agar, and incubated for 7 to 9 days in greenhouse conditions at 25°C until sporulation. Four replicates per isolate were tested, with leaf discs within the replicate obtained from the same leaf.

3.2.3.4 Disease assessment and EC_{50} determination

The disease severity of each leaf disc was assessed visually as the percentage of abaxial leaf disc area covered by *P. viticola* sporulation, according to the standard guidelines of the European and Mediterranean Plant Protection Organization (EPPO). The disease reduction (efficacy) by the tested active ingredients on the isolates was calculated for each replicate according to the following formula:

$$Efficacy = \frac{\text{Severity Untreated Control} - \text{Severity sample}}{\text{Severity Untreated Control}} \times 100$$

The median effective concentration (EC_{50}), i.e. the fungicide concentration inhibiting sporulation of *P. viticola* by 50% compared to the untreated control, was calculated by PROBIT analysis of efficacy values on $\log_{10}$-transformed values of fungicide concentration.

3.3 Results

3.3.1 Microsatellite analysis

Out of 315 samples, 9 samples were excluded from the genotyping analysis due to no amplification (two samples of Mag_O) and high percentage of missing data (Vdl_ O+_F08, 48%; Vdl_ O+_C04, 69%; Vdl_I_B10, 38%; Vdl_ O+_E10, 77%; Vdl_ O+_E11, 77%; Rov_I_F12, 77%). Nine of the 22 microsatellite *loci* (PV7, PV14, PV39, PV65, PV138, PV142, PV61, PV67, PV93) were excluded from further analyses due to high percentage of missing data (>5%). Therefore, the final microsatellite DNA dataset comprised 307 samples and 13 microsatellites *loci* (Supplementary table 1). 201 *P. viticola* strains showed a unique allelic profile representing distinct multilocus genotypes, 106 strains distributed in 40 subgroups shared an allelic profile with at least another strain (26 couples, 8 triplets, 3 quadruplets and three subgroups of five, six and seven genotypes each). Identical genotypes (19 genotypes) were mainly reported in the same geographical area; nine of them were reported in localities close to each other (SaM-Mg, Mg-Tr, Ter-Tr) and ten shared the same locality (Mg, SaM, Ter, Tr) (Table 5**,** Supplementary table 3).

Table 5 Number of identical multilocus genotypes of *Plasmopara viticola* sheared per sampling location combination. Between graphs the No. of combinations per No. of samples recorded.

Two-way combination						
	Mg	**Ter**	**Tr**	**Rov**	**SaM**	**Vdl**
Mg	**2** (1*2; 1*3)					
Ter	2 (2*2)	**5** (4*2; 1*3)				
Tr	4 (2*2; 2*3)	3 (3*2)	**2** (2*2)			
Rov	3 (2*2; 1*3)	2 (2*2)	-	-		
SaM	2 (1*2; 1*3)	-	-	1 (1*2)	**1** (1*2)	
Vdl	-	3 (3*2)	1 (1*2)	-	1 (1*2)	-

Multiple combination	
Mag * SaM * Ter	1 (1*6)
Mag * SaM * Tr	2 (2*3)
Mag * SaM * Vdl	1 (1*7)
Mag * Ter * Tr	1 (1*4)
Mag * Rov * SaM * Vdl	1 (1*5)
Rov * Ter * Tr	1 (1*4)
Rov * SaM * Ter	1 (1*4)

Two more genotypes were recorded in three locations in the Adige Valley with the combination SaM-Mag-Tr. The three quadruplets were composed by samples collected in Rov(2)-

Ter(1)-Tr(1); Rov(1)-SaM(1)-Ter(2) and Mg(1)-Tr(2)-Ter(1), (the No. in parentheses indicate the nr. of identical genotypes per location). *Locus* PV104 resulted monomorphic, while the other 12 microsatellite *loci* showed between two and four alleles (Fehler! Verweisquelle konnte nicht gefunden werden.). The H_E ranged from 0.038 (PV83) to 0.471 (PV91) and was equal to 0.230 on average. Most of the *loci* showed F_{IS} values close or lower than zero, and only one *locus*, PV139, showed a positive value of 0.214. Five *loci* (PV13, PV91, PV101,103, PV139) showed significant deviation from the *HWE* ($p < 0.05$) (Table 6).

Table 6 - Missing data (MD%), number of alleles (Na), allele size range (bp), Observed Heterozygosity (H_O), Expected Heterozygosity (H_E), Fixation index (F_{IS}) and Hardy-Weinberg Equilibrium (HWE) per *locus*.

Locus	MD%	*Na*	Allele size range (bp)	H_O	H_E	F_{IS}	*HWE*[a]
ISA	0.7	3	127-135	0.348	0.342	-0.017	*ns*
PV13	0.7	2	217-219	0.230	0.203	-0.130	*
PV17	1.3	4	144-150	0.459	0.418	-0.097	*ns*
PV31	1.0	3	241-245	0.076	0.073	-0.031	*ns*
PV83	0.0	4	240-245	0.039	0.038	-0.016	*ns*
PV88	0.0	2	205-207	0.169	0.155	-0.093	*ns*
PV91	0.0	2	140-142	0.537	0.471	-0.141	*
PV101	0.3	3	261-266	0.503	0.433	-0.163	***
PV103	0.0	2	287-297	0.433	0.377	-0.148	**
PV104	0.0	1	324		monomorphic		
PV127	0.0	3	218-224	0.081	0.078	-0.038	*ns*
PV139	2.0	3	131-135	0.043	0.055	0.214	**
PV141	4.2	2	191-193	0.384	0.346	-0.110	*ns*
All *loci*	0.8	2.6	-	0.254	0.230	-0.064	-

[a] Level of significance: *ns*, not significant; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

The STRUCTURE analysis did not reveal evidence for more than one population of *P. viticola* in the Provinces of Trento and Bozen-Bolzano (Figure 1). Plots of LnP(K) (**Figure *1*** Results of the STRUCTURE analysis of the Plasmopara viticola population in the Autonomous Provinces of Trento and Bozen-Bolzano. Plots of LnP(K) (A) and Δ(K) (B) for values of K from 1 to 8 were obtained with StructureSelector. STRUCTURE bar plots generated from microsatellite data of 307 individuals considering K=2 (C) and K=6 (D). Figure 1A) and Δ*K* (Figure 1B) for values of K from 1 to 8 were obtained with

StructureSelector. STRUCTURE bar plots generated from microsatellite data of 307 individuals considering K=2 and K=6 show that there is no structure in the population despite the higher values of Δ*K*, while the higher value of LnP(K) indicates the most likely number of genetic clusters of K=1.

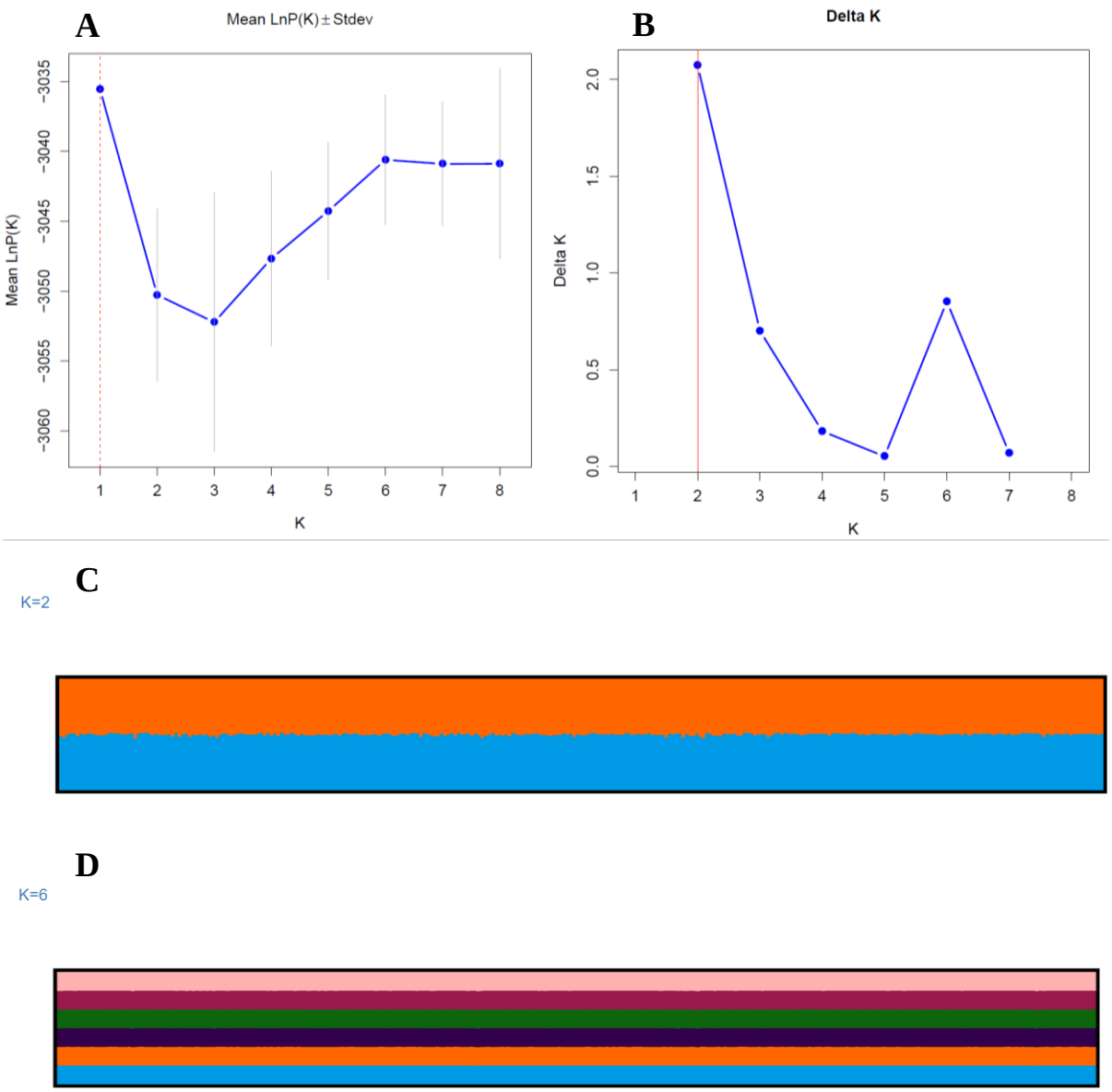

Figure 1 Results of the STRUCTURE analysis of the *Plasmopara viticola* population in the Autonomous Provinces of Trento and Bozen-Bolzano. Plots of LnP(K) (A) and Δ(K) (B) for values of K from 1 to 8 were obtained with StructureSelector. STRUCTURE bar plots generated from microsatellite data of 307 individuals considering K=2 (C) and K=6 (D). The y-axis in plots C and D shows the proportional assignment of each individual to each cluster.

Moreover, based on analysis of molecular variance (AMOVA), the isolates did not differ according to sampling area, disease management strategy, year of sampling (Table 7 to Table 9).

Table 7 Comparison of *Plasmopara viticola* population from different winegrowing areas using an analysis of molecular variance (AMOVA).

Source	df	SS	MS	Percentage of molecular variance %	*Fst*	*P*
Among areas	5	15.414	3.083	1		
Within areas	608	946.949	1.557	99	0.010	0.025
Total	613	962.363		100		

Table 8 Comparison *of Plasmopara viticola* population from different grapevine varieties using an analysis of molecular variance (AMOVA).

Source	df	SS	MS	Percentage of molecular variance %	*Fst*	*P*
Among varieties	1	8.653	8.653	2		
Within varieties	612	953.710	1.558	98	0.018	0.001
Total	613	962.363		100		

Table 9 Comparison *of Plasmopara viticola* population from different disease management strategies using an analysis of molecular variance (AMOVA).

Source	df	SS	MS	Percentage of molecular variance %	*Fst*	*P*
Among disease management strategies	2	4.925	2.463	0		
Within disease management strategies	611	957.438	1.567	100	0.003	0.077
Total	613	962.363		100		

3.3.2 Fungicide resistance in vivo bioassay

During the 2021 and 2022 growing seasons, 180 *P. viticola* isolates were collected and evaluated with 11 single-site chemical fungicides. EC_{50} and MIC were compared with literature references and interpolated to create four different adaptation classes: I) no adaptation, EC_{50} below

baseline value and MIC lower than maximum application rate; II) slight adaptation, MIC greater than maximum application rate; III) moderate adaptation, EC_{50} above baseline value; IV) full adaptation, EC_{50} above baseline and MIC greater than maximum application rate.

All the isolates were belonging solely to adaptation classes I and II for azoxystrobin, dimetomorph and zoxamide, with just one isolate per active ingredient belonging to Class II, where only the MIC exceeded the maximum field dose (Table 10). EC_{50} ranged from <0.01 mg L^{-1} to 6.60 mg L^{-1} for azoxystrobin, from <0.01 mg L^{-1} to 12.5 mg L^{-1} for dimetomorph and from <0.01 mg L^{-1} to 2.64 mg L^{-1} for zoxamide (full data in Supplementary Table 10 and 11). However, MIC was equal to the maximum field dose in 19% of the isolates for azoxystrobin, 28% for dimetomorph and 11% for zoxamide.

No adaptation was shown for oxathiapiprolin. In fact, a dose of 0.04 mg L^{-1}, equal to 0.01% of the field dose, was inhibiting completely the growth of 92% of *P. viticola* isolates. When EC_{50} could be determined, i.e. above detection limit, it did not exceed 0.05 mg L^{-1} and the MIC was equal to 0.4 mg L^{-1}.

19% of the isolates displayed a MIC equal or higher than the field dose for metalaxyl-m (more than 100 mg L^{-1}), 14% and 5%, respectively. EC_{50}, however, never exceeded 0.38 mg L^{-1}. In this case, since, at the time writing, no reference EC_{50} for *P. viticola* are present in literature, Class III was given to isolates presenting a MIC equal to the maximum field dose of 100 mg L^{-1}, while Class IV to those exceeding 100 mg L^{-1}.

On the other side, 28% and 25% of the isolates belonged to Class III for ametoctradin and amisulbrom. EC_{50} ranged between 0.01 mg L^{-1} and 0.89 mg L^{-1} for ametoctradin and between 0.01 mg L^{-1} and 2.74 mg L^{-1} for amisulbrom, where in only 2 isolates (5%) the maximum field rate did not

reach full growth inhibition. For both active ingredients, Isolates were considered adapted if EC_{50} was greater than 0.3 mg L^{-1}, as reported in previous studies (Bi and Ma, 2016; Fontaine *et al*., 2019).

Moderate adaptation to fluopicolide was observed, since 5% of the isolates belonged to classes III and IV, with EC_{50} reaching 5 mg L^{-1}, while the MIC was higher than the field dose in 20% of the cases.

A diffused adaptation was highlighted for cymoxanil: 22% of the isolates recorded an EC_{50} higher than 20 mg L^{-1}, which was the baseline value in a previous study (Toffolatti *et al*., 2015), while in 5% of the cases even beyond 100 mg L^{-1}. Moreover, cymoxanil did not guarantee full growth inhibition at maximum field dose (140 mg L^{-1}) in 21 out of 36 isolates (11 in 2021 and 10 in 2022).

Strong and diffuse adaptation to mandipropamid was recorded in both 2021 and 2022, especially in South Tyrol. Indeed, the 22%, 16% and 14% of the isolates were included in adaptation classes II, III and IV, respectively. EC_{50} ranged from 0.01 mg L^{-1} to 9.23 mg L^{-1} being 3 mg L^{-1} considered as threshold EC_{50} for adapted isolates (Delmas *et al*., 2017). One isolate recorded an EC_{50} greater than 500 mg L^{-1}. In addition to that, the MIC was greater or equal than the maximum field dose in 69% of the isolates (36% in 2021 and 33% in 2022).

Table 10 Fungicide resistance classification in samples collected in 18 vineyards in Trentino South Tyrol in 2021 and 2022. 1) no adaptation (grey): EC_{50} below baseline value and MIC lower than maximum application rate. 2) Slight, possible adaptation (light blue): MIC greater than maximum application rate. 3) Moderate adaptation (blue): EC_{50} above baseline value. 4) Certain adaptation (dark blue): EC_{50} above baseline and MIC greater than maximum application rate

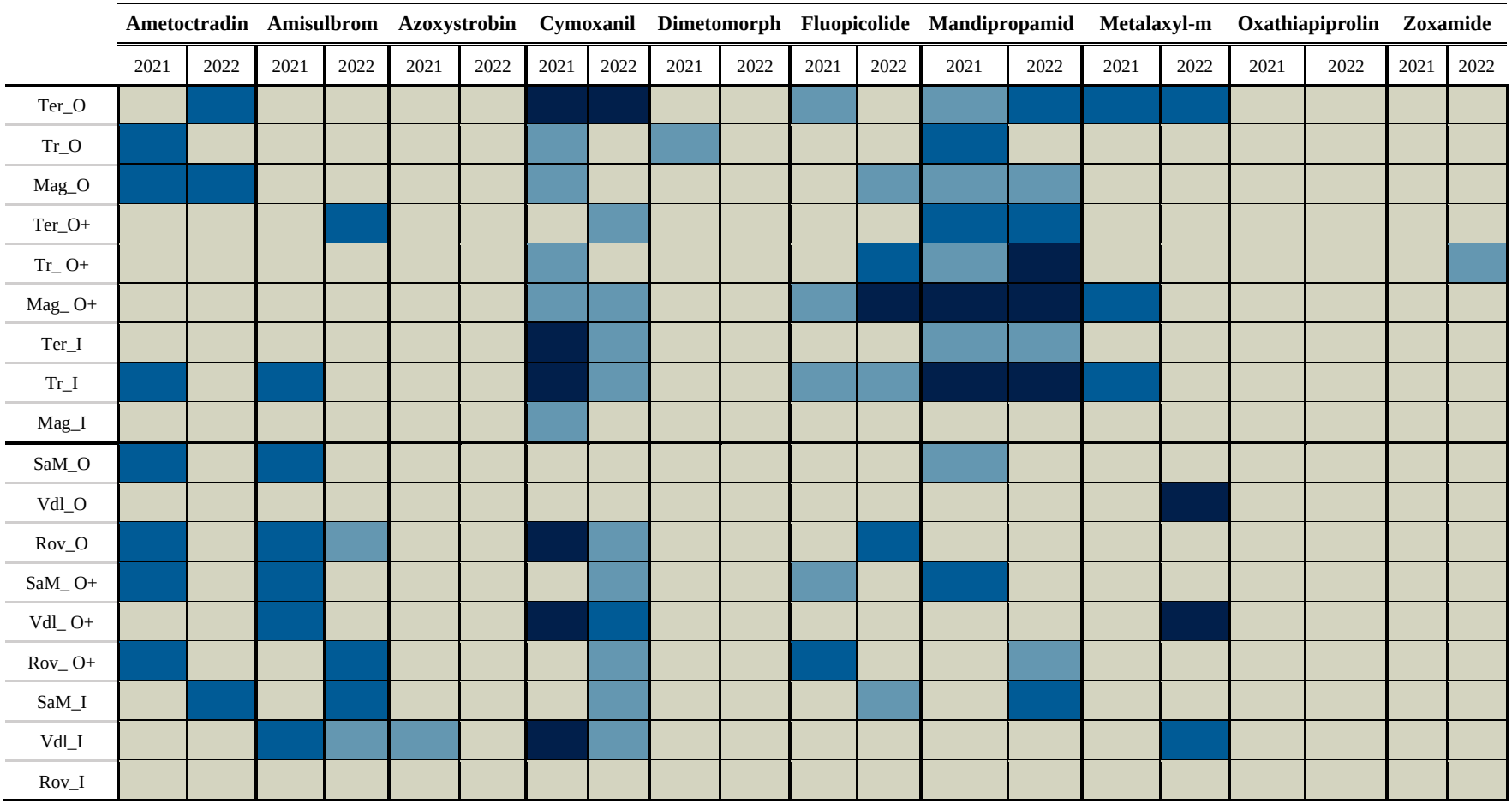

Table 11 Calculated EC_{50} for *Plasmopara viticola* bulk isolates sampled during 2021 and 2022. Bulk isolates consist in the merging of five isolates randomly sampled in the vineyard. *n.d.*: not detectable

	Ametoctradin		Amisulbrom		Azoxystrobin		Cymoxanil		Dimetomorph		Fluopicolide		Mandipropamid		Metalaxyl-m		Oxathiapiprolin		Zoxamide	
	EC_{50} (mg active ingredient L^{-1})																			
	2021	2022	2021	2022	2021	2022	2021	2022	2021	2022	2021	2022	2021	2022	2021	2022	2021	2022	2021	2022
Ter_O	0,22	0,36	0,02	0,29	0,24	<0.01	26,90	111	0,72	3,64	0,55	0,72	1,10	10,40	0,04	1,20	n.d.	n.d.	0,77	<0.01
Tr_O	0,36	<0.01	<0.01	0,03	0,07	<0.01	0,01	2,08	0,02	1,94	0,42	0,04	3,44	1,33	0,18	0,03	n.d.	n.d.	0,17	0,15
Mag_O	0,47	0,34	0,01	0,27	<0.01	0,33	2,10	0,97	0,74	2,03	0,01	0,23	1,28	1,12	0,26	0,09	n.d.	n.d.	0,01	0,16
Ter_O+	0,02	<0.01	0,03	0,50	0,01	0,04	4,41	9,17	0,83	0,83	0,21	0,31	3,62	9,23	0,05	1,06	<0.01	n.d.	0,34	0,02
Tr_ O+	0,05	0,22	0,14	0,19	2,39	<0.01	2,41	9,13	0,98	1,51	0,31	5,11	2,09	4,49	0,38	0,26	n.d.	<0.01	0,09	0,61
Mag_ O+	0,04	0,20	<0.01	0,25	<0.01	0,04	1,87	1,49	2,97	0,43	0,69	3,59	3,21	3,48	0,10	0,36	n.d.	n.d.	0,12	0,60
Ter_I	0,01	<0.01	0,05	0,08	0,12	<0.01	27,80	1,44	0,79	12,50	0,22	0,04	0,01	2,10	0,10	1,03	n.d.	n.d.	0,36	0,39
Tr_I	0,89	0,02	0,53	0,14	0,07	<0.01	832	4,05	<0.01	0,78	0,70	2,25	1583,00	5,20	0,31	0,10	n.d.	0,05	0,13	0,36
Mag_I	0,20	<0.01	<0.01	0,03	<0.01	0,03	3,63	0,36	0,01	0,62	<0.01	0,09	1,58	0,44	0,37	0,16	n.d.	n.d.	0,21	<0.01
SaM_O	0,37	<0.01	0,60	n.d.	<0.01	0,01	6,66	0,01	0,55	0,09	1,19	<0.01	1,31	0,16	n.d.	<0.01	n.d.	n.d.	0,02	<0.01
Vdl_O	0,07	0,01	<0.01	0,03	<0.01	0,32	<0.01	6,88	0,01	0,25	0,52	0,05	n.d.	<0.01	<0.01	0,05	n.d.	n.d.	0,35	n.d.
Rov_O	0,41	0,07	2,43	0,16	6,60	0,15	58,6	3,53	0,79	0,48	0,15	0,57	0,39	0,64	0,09	<0.01	n.d.	n.d.	2,64	0,13
SaM_ O+	0,40	0,03	1,12	0,05	n.d.	0,31	0,54	5,17	0,35	0,23	0,46	0,05	3,16	0,39	n.d.	0,01	n.d.	n.d.	0,05	<0.01
Vdl_ O+	<0.01	0,02	1,02	0,02	10,60	0,16	56029	1123	0,43	0,09	0,04	0,05	0,02	n.d.	0,12	0,15	n.d.	n.d.	2,63	<0.01
Rov_ O+	0,34	0,06	0,14	1,15	n.d.	<0.01	0,04	1,80	0,31	1,38	3,98	0,44	1,37	0,88	0,01	0,02	n.d.	n.d.	0,08	0,20
SaM_I	0,05	0,59	<0.01	1,50	<0.01	0,17	<0.01	5,13	0,14	0,73	0,03	0,02	0,04	5,20	<0.01	0,06	n.d.	n.d.	0,30	0,31
Vdl_I	0,03	0,04	2,74	0,06	2,98	0,05	55,10	10,50	0,93	0,12	0,45	0,20	0,16	0,20	0,35	0,26	n.d.	n.d.	0,97	<0.01
Rov_I	<0.01	<0.01	0,08	<0.01	0,02	<0.01	0,07	<0.01	<0.01	0,03	0,19	n.d.	<0.01	<0.01	<0.01	0,05	n.d.	n.d.	0,01	n.d.

Table 12 Estimated Minimum Inhibitory Concentration (MIC) for *Plasmopara viticola* bulk isolates sampled during 2021 and 2022. Bulk isolates consist in the merging of five isolates randomly sampled in the vineyard. *n.d.*: not detectable

	Ametoctradin		Amisulbrom		Azoxystrobin		Cymoxanil		Dimetomorph		Fluopicolide		Mandipropamid		Metalaxyl-m		Oxathiapiprolin		Zoxamide	
	MIC (mg active ingredient L^{-1})																			
	2021	2022	2021	2022	2021	2022	2021	2022	2021	2022	2021	2022	2021	2022	2021	2022	2021	2022	2021	2022
Ter_O	2,5	2,5	0,8	2,5	9,5	2,9	>140	>140	25	250	>132	132	>150	150	100	100	<0.04	<0.04	16,4	5,4
Tr_O	8,3	2,5	75	2,5	28,8	2,9	>140	140	>250	250	4,4	1,3	150,0	150	3,3	10	<0.04	<0.04	5,4	16,4
Mag_O	8,3	8,3	2,5	7,5	0,3	0,3	>140	140	250	250	132,0	>132	>150	>150	10	10	<0.04	<0.04	163	16,4
Ter_O+	2,5	2,5	0,8	7,5	9,5	288	14	>140	250	25	13,2	132	150,0	150	10	10	0,4	<0.04	16,4	16,4
Tr_ O+	2,5	8,3	7,5	0,8	288	2,9	>140	140	250	250	13,2	132	>150	>150	10	10	<0.04	0,4	5,4	>163
Mag_ O+	2,5	2,5	2,5	7,5	2,9	9,5	>140	>140	25	250	>132	>132	>150	>150	100	10	<0.04	<0.04	5,4	163
Ter_I	2,5	2,5	0,8	2,5	2,9	2,9	>140	>140	250	250	4,4	132	>150	>150	10	10	<0.04	<0.04	5,4	5,4
Tr_I	8,3	2,5	7,5	2,5	2,9	28,8	>140	>140	250	250	>132	>132	>150	>150	100	10	<0.04	0,4	16,4	163,0
Mag_I	2,5	2,5	2,5	75	2,9	2,9	>140	14	8,3	250	4,4	1,3	150	150	1	3,3	<0.04	<0.04	5,4	1,6
SaM_O	2,5	2,5	2,5	0,8	2,9	0,3	140	1,4	250	2,5	132	1,3	>150	5	0,1	1	<0.04	<0.04	5,4	1,6
Vdl_O	2,5	2,5	0,8	7,5	2,9	2,9	1,4	140	2,5	2,5	132	13,2	<0.15	0,2	1	>100	<0.04	<0.04	5,4	1,6
Rov_O	25	2,5	7,5	>75	288	288	>140	>140	8,3	250	1,3	>132	1,5	150	10	1	<0.04	<0.04	16,4	5,4
SaM_ O+	2,5	2,5	2,5	0,1	0,3	2,9	14	>140	8,3	8,3	>132	4,4	50	150	<0.1	1	<0.04	<0.04	1,6	1,6
Vdl_ O+	2,5	2,5	7,5	7,5	288	2,9	>140	140	8,3	2,5	4,4	13,2	1,5	0,2	10	>100	<0.04	<0.04	163	1,6
Rov_ O+	2,5	25	0,8	0,1	0,3	9,5	14	>140	8,3	25,0	132,0	132	150	>150	10	1	<0.04	<0.04	1,6	5,4
SaM_I	2,5	25	0,8	7,5	288	288	1,4	>140	8,3	250,0	1,3	>132	5	150	1	1	<0.04	<0.04	1,6	16,4
Vdl_I	250	2,5	7,5	>75	>288	9,5	>140	>140	25	25	132	4,4	15	150	10	100	<0.04	<0.04	163	1,6
Rov_I	2,5	2,5	0,8	0,8	2,9	2,9	1,4	1,4	2,5	2,5	13,2	<0.1	1,5	1,5	1	1	<0.04	<0.04	1,6	<0.2

3.4 Discussion

In this paper, the genetic structure of *P. viticola* populations in Trentino South Tyrol was investigated analyzing 307 isolates collected during 2021 and 2022 from vineyards undergoing different disease management strategies.

Genotyping was performed with 22 microsatellite *loci*, however, 9 were excluded due to a percentage of missing data > 5%. Compared to a previous study conducted in Italy (Maddalena *et al*., 2020), among the 11 commonly shared *loci*, 8 *loci* exhibited the same number of alleles (PV17, PV31, PV88, PV91, PV101, PV127, PV 139 and PV141), while only two a lower number (ISA, 3 alleles instead of 6, while PV104 in the present study was monomorphic). Only at one *locus* the number of alleles was recorded higher in PV83, with 3 alleles instead of 2.

As expected, our results display that, the collected *P. viticola* populations highlighted an extremely low genetic variability, with H_E = 0.23, even lower than those reported previously in two European population clusters in West and East Europe (H_E = 0.44 and H_E = 0.39, respectively) clear consequence of the bottleneck originated by the pathogen introduction from North America to Europe, as already reported in other studies on European (Rouxel *et al*., 2012, 2013; Fontaine *et al*., 2013, 2021) and National scale (Maddalena *et al*., 2020) and even accentuated at the Regional level. In fact, among *P. viticola* populations, significantly low to moderate genetic differentiation was observed, especially between geographically close populations (Gobbin *et al*., 2006).

In the case of Trentino South Tyrol, alongside with the limited distance within the sampling sites, the main viticulture areas in the Region are confined principally to the Adige Valley and its surrounding valleys, therefore it is extremely unlikely that within such a limited areal, genetic

diversity could have been originated, since already on the European level only slight differentiation was observable (Fontaine *et al*., 2013).

Moreover, in this study, the same genotypic profiles have been found not only within the same vineyard, but also among spatially distant sampling spots, even overcoming geomorphological barriers, and in vineyards undergoing different disease management strategies. In this regard, it has been already demonstrated that sporangia can be transported more than 100 meters from the source of inoculum in one single infection cycles (Gobbin *et al*., 2006). The climatic situation of the region, characterised by frequent, high intensity precipitations during the summer period, may significantly favour the occurrence of repeated secondary infections and hence the transport of sporangia across the territory.

In some extent, the geomorphological conformation of the Region may be a factor in creating a further selection bottleneck, contributing to lower the genetic diversity of *P. viticola* populations. In fact, the results support the hypothesis that at the small-scale secondary infections contribute substantially to damage, implying that generalized vineyard-wide epidemics are most probably the results of a large number of randomly distributed primary infections followed by uncontrolled secondary multiplication at the small scale (Gessler *et al*. 2006). All the examined *loci* presented negative F_{IS}, clear sign of excess of heterozygotes, since negative F_{IS} are typical of partially asexual populations, reproducing through sexual and asexual reproduction. In addition to that, the majority of the *loci* (8 out of 13) did not present significant deviation from HWE, suggesting that the sampled populations are predominantly panmictic, as reported by previous studies (Gobbin *et al*., 2003,2006; Fontaine *et al*., 2013; Delmas *et al*., 2016, 2017)

However, asexual reproduction can maintain and increase heterozygosity through the accumulation of mutations through generations (Maddalena *et al*., 2020). Obviously, this aspect

represents a concrete risk towards the occurrence and the outburst of *P. viticola* populations resistant to single site fungicides.

For this reason, a sensitivity leaf discs bioassay was conducted in both sampling years on the *P. viticola* populations collected, including a wide range of different mode of action classes such as CAA (mandipropamid and dimetomorph), benzamides (fluopicolide and zoxamide), phenylamides (metalaxyl-m), quinone inhibitors (azoxystrobin, ametoctradin and amisulbrom), cymoxanil and oxathiapiprolin (OSBPI).

The bioassay showed a heterogeneous situation: on one side, 10 out of 18 bulk isolates collected in 2021 were included in class III and IV for two or more active ingredients, while 6 out of 18 in 2022, suggesting cross-adaptation towards multiple mode of action fungicides. In 2021, one bulk isolate (Tr_I), showed moderate to certain adaptation to 5 different active ingredients (ametoctradin, amisulbrom, cymoxanil, mandipropamid and metalaxyl-m), while Rov_O and SaM_O+ to 3 active ingredients (cymoxanil and mandipropamid, respectively, while both towards ametoctradin and amisulbrom). However, in these vineyards, the adaptation profile was not repeated, with maximum one active ingredient belonging to classes III and IV in both years. Similarly, one bulk isolate presenting adaptations to multiple active ingredients in 2022 (SaM_I, towards ametoctradin, amisulbrom and mandipropamid) was belonging solely to class I in 2021. Only one vineyard presented bulk isolates adapted towards cymoxanil and metalaxyl-m, Ter_O in both 2021 and 2022.

All active ingredients, with the exception of oxathiapiprolin, zoxamide and azoxystrobin presented at least one record of EC_{50} beyond the adaptation threshold in previous studies.

The most critical scenario regarded mandipropamid, for which the recorded EC_{50} exceeded the 3 mg L^{-1} presented as baseline value by Delmas *et al.*, (2017), in 50% of the bulk isolates in South

Tyrol, considering both 2021 and 2022, suggesting a diffused adaptation to this active ingredient in this viticultural area.

Similarly, the calculated EC_{50} for the complex III inhibitors ametoctradin and amisulbrom frequently exceeded the 0.3 mg L^{-1} baseline (Bi and Ma, 2016; Fontaine *et al*., 2019) in 28% and 25% of the isolates, respectively, especially in Trentino province, where the majority the adapted isolates was recorded. Moreover, 25% of the isolates classified as adapted to ametoctradin, were resistant to amisulbrom as well. In fact, it has been reported that the S34L substitution is responsible not only for ametoctradin resistance but may also involve in adaptation to amisulbrom (Delmas *et al*., 2017).

On the other hand, sporulation in leaf discs treated with oxathiapiprolin was inhibited in 95% of the tested isolates at 0.04 mg L^{-1} equal to 0.1% of the maximum allowed field dose, suggesting an extreme sensitivity of the isolates towards this active ingredient. However, in another study, the presence of isolates carrying the N837I substitution, known to confer resistance to oxathiapiprolin, has been reported in Trentino South Tyrol and the neighbouring regions in the Northeast of Italy.

In the same way, isolates collected for the present study were almost always belonging to Class I, whereas in Veneto and Friuli Venezia Giulia, the C239S substitution was found in isolates resistant to zoxamide (Cherrad *et al*., 2024). Despite the excellent efficacy in controlling *P. viticola* displayed by both active ingredients in the present study, extreme caution and appropriate fungicide resistance mitigation strategies are required in the use of these molecules, in order to preserve their plant protection activity in the vineyards, in the likely case of selection of resistant genotypes in the region.

Surprisingly, then, the occurrence of adaptations was almost evenly distributed among the three management strategies: moderate and certain adaptation was recorded 17 times in organic 4 kg ha^{-1} $year^{-1}$ vineyards (O), 19 times in organic 3 kg ha^{-1} $year^{-1}$ vineyards (O+) and 13 times in vineyards

undergoing integrated pest management (I). However, these findings may find an explanation in the intrinsic nature of viticulture in Trentino South Tyrol. In fact, the average size of wine growing farm barely exceeds 1.5 ha in Trentino (Consorzio Vini del Trentino, 2021) and 1 ha in South Tyrol (Südtirol Wein, 2024). This implies that the vineyards undergoing different disease management strategies are extremely exposed to the contamination *P. viticola* populations that were selected in neighbouring vineyards, explaining the fact that adapted populations were found even where single-site fungicides have not been used for multiple seasons.

On the other hand, it is reassuring that, even if with few exceptions, as discussed before, bulk isolates presenting adaptations towards the same mode of action on multiple sampling years were almost never found in the same vineyards. The competition among *P. viticola* isolates may influence the alternate prevalence of adapted over sensitive genotypes (Gessler *et al*., 2011), while it may also be an indicator of the effect of disease management strategies implemented with the aim of minimizing the long-term outburst of adapted isolates, negatively impacting the efficacy of fungicide applications.

3.5 Conclusions

This study provides an in-depth analysis of the genetic structure and fungicide sensitivity of *Plasmopara viticola* populations in Trentino South Tyrol, a key wine-producing region in Italy. The findings reveal low genetic diversity among the pathogen population, with no significant differentiation based on location, management strategy, or sampling year. The predominantly panmictic structure, driven by sexual and asexual reproduction, highlights the pathogen's adaptability and the potential for widespread dissemination.

A critical outcome is the identification of fungicide resistance, particularly to mandipropamid and cymoxanil, with several isolates displaying EC_{50} values above baseline thresholds. Resistance

was observed across all disease management strategies, indicating cross-contamination and the necessity for integrated disease control approaches. However, fungicides like oxathiapiprolin and zoxamide remain highly effective, though vigilance is required to prevent resistance development.

This study is the first to comprehensively examine *P. viticola* populations in this region, offering novel insights into its epidemiology and resistance patterns. These findings provide essential guidance for sustainable disease management and highlight the need for data-driven policies to preserve the efficacy of fungicides and protect viticulture in Trentino South Tyrol.

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CHAPTER 4.

BIOLOGICAL CONTROL OF *PLASMOPARA VITICOLA*: WHERE ARE WE NOW?

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Abstract

Grapevine downy mildew, caused by *Plasmopara viticola*, is one of the most critical diseases on grapevine worldwide causing relevant economic losses. Nowadays, the management of grapevine downy mildew relies mainly on the application of copper and synthetic fungicides. However, due to the occurrence of *P. viticola* populations resistant to fungicides and the impact of chemicals on humans and the environment, the development of eco-friendly plant protection products is highly requested.

Biocontrol agents effective in controlling *P. viticola* on grapevine represent an important alternative to the use of synthetic fungicides and copper in viticulture. Several bacterial and fungal biocontrol agents have been evaluated for their ability to control directly *P. viticola* or by stimulating defense mechanisms in grapevine plants. In future, the progressive reduction of the chemical input in viticulture and the current lack of commercial microbial biopesticides active against *P. viticola* will make the management of grapevine downy mildew a compelling challenge in the field of biological control of plant diseases

***Key words**: Plasmopara viticola*, Biocontrol, Plant extracts, Microbial Biocontrol Agents, Disease management

4.1 Agricultural relevance of *Plasmopara viticola*

Grapevine (*Vitis vinifera* L.) is one of the most relevant crops worldwide, taking into consideration both table grape and wine production. In 2021, the world grape production accounted for a total of 64 million tons over 7.3 million hectares of cultivated land, with 33.9 million tons of grapes produced just for wine making. 261 million hL of wine is produced on a yearly basis (OIV database, 2022).

However, grapevines are highly susceptible to several plant pathogenic microorganisms. Among them, there is the plant pathogenic oomycete *Plasmopara viticola* (Berk. & Curt) Berl. & de Toni, the causal agent of grapevine downy mildew, which is one of the most devastating diseases affecting *V. vinifera*, causing severe losses in terms of yield and quality.

Plasmopara viticola is endemic in American *Vitis* spp. and was introduced in Europe during the 19th century with infected nursery plant material for rootstock grafting following the destructive advent of phylloxera (Gobbin *et al.*, 2006).

In Europe, the first reports of the disease date back to 1878 in France, but within a few years, the disease was present in the entire continent. The first real demonstration of its destructive potential was in 1893, when 50% of French production was lost in an epidemic attack. From the beginning of the following century, the disease occurred regularly, regardless of weather conditions, causing very serious and strong damage to vineyards (Gessler *et al.*, 2011). Therefore, repeated and precise fungicide applications are required to control the disease, with relevant costs for wine growers.

4.2 Life cycle of *Plasmopara viticola*

Plasmopara viticola is an obligate biotroph, meaning that it can only grow in association with its host plant. Similarly to most oomycetes, *P. viticola* passes through both sexual and asexual stages during its life cycle.

During the sexual stage, the fusion of antheridia (male reproductive structures) and oogonia (female reproductive structures) inside the host tissues generates outcrossed couplings. Parental gametangia undergo meiosis forming an oospore, which is then dispersed to the ground in dead leaf tissues and litter. After a dormancy period, the oospores germinate to produce sporangia during rainy periods in the spring.

Germination was found to occur within a temperature range of 13°C-33°C, with optimal germination observed at 25°C. Low temperatures shorten the dormant period, whereas dry conditions prolong it and may, if protracted, injure the oospores (Gessler *et al.*, 2011).

Once the sporangia get in contact with the lower grapevine leaf surface in warm and humid conditions, zoosporogenesis occurs, by which biflagellate zoospores are released. In optimal conditions, zoospores can remain vital for several hours until they encyst in the proximity of the stomata (Figure 2A). However, zoospores are not able to survive in the absence of water since they do not possess cell walls (Koledenkova *et al.*, 2022). Within 30 minutes, the encysted zoospores germinate and produce germ tubes, which enter the plant tissue via stomata openings and develop an intercellular mycelial network inside the mesophyll tissue (Figure 2B).

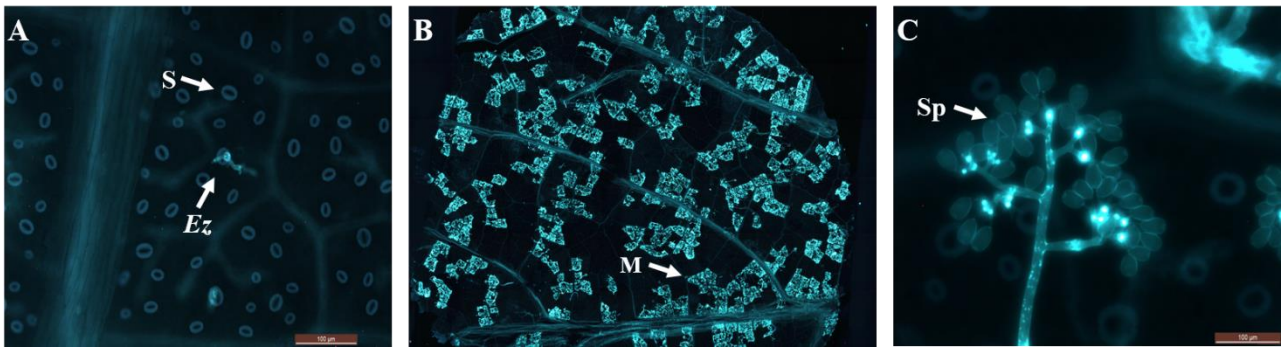

Figure 2 *Plasmopara viticola* structures observed under epifluorescence microscope after aniline blue staining. (**A**) Encysted zoospore (Ez) penetrating a stoma (s) 48 hours after the infection, (**B**) Wide-spread mycelium (M) on the leaf surface five days after the infection. (**C**) Sporangiophore carrying fully developed sporangia (Sp), seven days after the infection.

In a coordinated process lasting approximately seven hours in the dark, sporangiophores emerge through stomatal openings approximately 6-10 days after infection and for sporangia (Figure 2C), which are released through wind or raindrops, starting secondary infections (Rumbolz *et al.*, 2002). Sporulation becomes visible as a dense, raised, white-cottony mildew on the abaxial surface of grapevine leaves, green shoots and young berries.

Similarly, *P. viticola* can penetrate inflorescences, young berries and green stems since these organs, even if in less considerably amounts, possess stomata openings (Palliotti *et al.*, 2010).

On grapevine leaves, *P. viticola* causes the typical chlorotic oil spot symptom visible on the adaxial side of the leaf (Figure 3A).

As the disease progresses, the infected tissue necrotizes and merges, forming large necrotic areas on the leaf, ultimately bringing premature defoliation in severe infections (Agrios, 2005).

On grapevine inflorescences, the pathogen causes the death of the infected tissues, leading to rachis distortion and eventually to partial or even total necrosis and detachment of the inflorescence (Figure 3B-C).

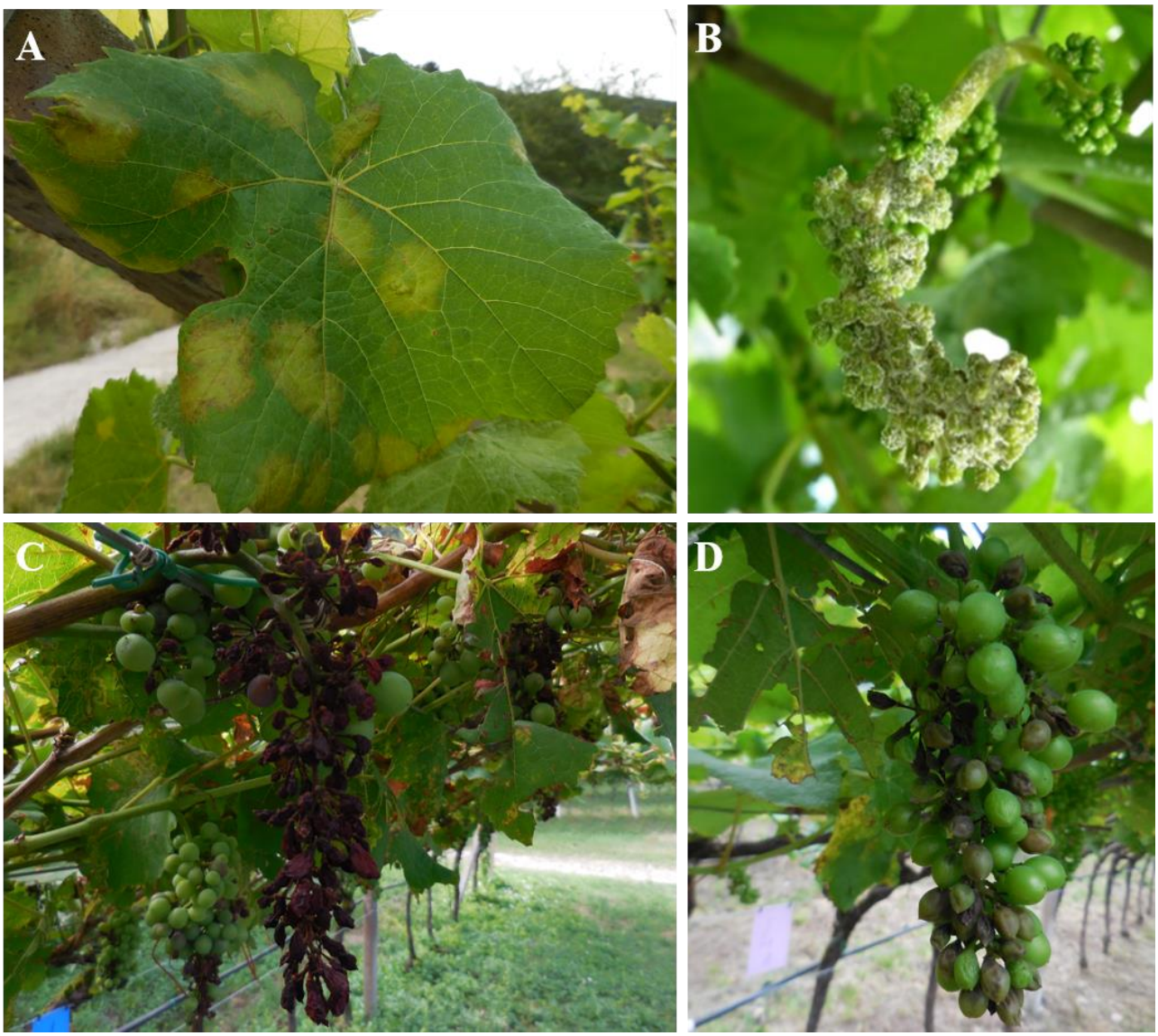

Figure 3 *Plasmopara viticola* symptoms on grapevine tissues. (**A**) Oil spots on grapevine leaf, (**B**) evident sporulation on the inflorescence before blooming, (**C**) necrosis on berries after fruit set and (**D**) before veraison.

On adult grapevine berries, the fruit skin thickens, and its shape distorts. A color change to a violet-brown can be noted as well as a mosaic pattern when grape bunches are infected (Figure 3D).

In shoots, enlargement of the infected cells and the large volume of mycelium in intercellular areas triggers distortion and hypertrophy, which leads to their death and collapse, generating brown and sunken shoots (Koledenkova *et al.*, 2022).

4.3 Grapevine downy mildew management

4.3.1 Breeding approach to select new varieties resistant to Plasmopara viticola

In the last decades, improving plant defense mechanisms by genetic engineering constitutes an attractive alternative for the control of grapevine downy mildew (Robert *et al.,* 2001).

In fact, although European *V. vinifera* cultivars are highly susceptible to *P. viticola*, several American (*V. rotundifolia, V. rupestris*, *V. labrusca*, *V. riparia*, *V. cinerea*) and Asian (*V. amurensis, V. piasezkii, V. coignetiae*) *Vitis* species show varying levels and different strategies of resistance to the plant pathogenic oomycete.

Thus, plant breeders started crossing susceptible *V. vinifera* varieties with other resistant *Vitis* spp. since the 19th century (Sargolzaei *et al.,* 2020). Nowadays, a relevant number of grapevine varieties combining resistance traits from American and Asian species and the quality traits of *V. vinifera* are available on the market (Reynolds, 2015; VIVC, 2022).

At the time of writing, 31 Quantitative Trait *Loci* (QTL), conferring moderate to high levels of resistance, have been identified in other *Vitis* spp. (Table 13).

However, resistant grapevine varieties mostly rely on the resistance conferred by the *loci Rpv1, Rpv3, Rpv10* and *Rpv12,* although the great amount of resistance *loci* identified.

The use of downy mildew-resistant varieties may bring significant benefits for growers, such as reducing the number of treatments per growing season and labor costs, while increasing grape yield. However, resistant varieties still have to face the scarce awareness by the common consumer, since wines produced from resistant varieties still represent a tiny part of the wines present on the market (Pedneault and Provost, 2016). Moreover, even though in some studies wines coming resistant

varieties were noted as equivalent or even superior to the reference wines, there is still the common perception of lower quality wines (Espinoza *et al.*, 2018). The need to educate consumers about these novel wines is therefore significant.

Besides difficulties in market acceptance, there are often legal problems in many countries, where growing resistant varieties for wine production is still partially not allowed. Moreover, resistant varieties are not included in the production procedural guidelines of the majority of the protected designations of origin (Dagostin *et al.*, 2011; Pertot *et al.*, 2017).

It is also worth noting that some *P. viticola* isolates, able to overcome the protection provided by some of these QTLs, have already been identified (Wingerter *et al.*, 2021). The occurrence of new *P. viticola* isolates able to infect resistant grapevine varieties might represent a serious risk for the implementation of resistant varieties in the future.

Table 13 List of Quantitative Trait *Loci* (QTLs) conferring resistance to *Plasmopara viticola*. Information on the *Vitis* spp. of origin and the chromosome where the QTLs were located.

QTL	Origin of resistance	Chromosome	Reference
Rpv1	*Vitis rotundifolia*	12	Merdinoglu *et al.*, 2003
Rpv2	*Vitis rotundifolia*	18	Wiedemann-Merdinoglu *et al.*, 2006
Rpv3	*Vitis rupestris*	18	Welter *et al.*, 2007; Bellin *et al.*, 2009; Di Gaspero *et al.*, 2012; van Heerden *et al.*, 2014; Zyprian *et al.*, 2016; Vezzulli *et al.*, 2019
Rpv4	*Vitis vinifera* cv. "Regent"	4	Welter *et al.*, 2007
Rpv5	*Vitis riparia*	9	Marguerit *et al.*, 2009
Rpv6	*Vitis riparia*	12	Marguerit *et al.*, 2009
Rpv7	*Vitis vinifera* cv. "Bianca"	7	Bellin *et al.*, 2009
Rpv8	*Vitis amurensis*	14	Blasi *et al.*, 2011
Rpv9	*Vitis riparia*	7	Moreira *et al.*, 2011
Rpv10	*Vitis amurensis*	9	Schwander *et al.*, 2012
Rpv11	*Vitis vinifera* cv. "Regent"	5	Fischer *et al.*, 2004
Rpv12	*Vitis amurensis*	14	Venuti *et al.*, 2013
Rpv13	*Vitis riparia*	12	Moreira *et al.*, 2011
Rpv14	*Vitis cinerea*	5	Ochssner *et al.*, 2016
Rpv17	*Vitis vinifera* cv. "Horizon"	8	
Rpv18	*Vitis vinifera* cv. "Horizon"	11	
Rpv19	*Vitis* rupestris	14	Divilov *et al.*, 2018
Rpv20	*Vitis vinifera* cv. "Horizon"	6	
Rpv21	*Vitis vinifera* cv. "Horizon"	7	
Rpv22		2	
Rpv23	*Vitis amurensis*	15	Fu *et al.*, 2020
Rpv24		18	
Rpv25	*Vitis amurensis*		Lin *et al.*, 2019
Rpv26		15	
Rpv27	*Vitis aestivalis*	18	Sapkota *et al.*, 2019
Rpv28	*Vitis rupestris*	10	Bhattarai *et al.*, 2021
Rpv29		14	
Rpv30	*Vitis vinifera* cv. Mgaloblishvili	3	Sargolzaei *et al.*, 2020
Rpv31		16	

4.3.2 Prevention of Plasmopara viticola *infections using chemicals*

Nowadays, the control of *P. viticola* relies exclusively on multiple and repeated fungicide applications to achieve a sustainable yield, under conditions favorable for disease development. Growers typically apply fungicides on a host phenology-based schedule starting at grapevine bud break and continuing until veraison. Almost 70% of the total fungicide applications in Europe are performed in vineyards (Wingerter *et al.,* 2021).

Copper-based fungicides are the oldest plant protection products used against *P. viticola,* and they still play a crucial role in disease management, especially in organic viticulture. Copper compounds, such as copper hydroxide, oxychloride and sulphate, are multi-site fungicides used for preventive applications, since they form a protectant layer on the plant tissue (Koledenkova *et al.,* 2022).

However, copper has a negative impact on the environment and on human health. Being a heavy metal, long-term application and runoff from treated plants, caused extensive copper accumulation in soil and groundwater (Cabús *et al.* 2017). In fact, among all the crops, vineyards have the highest mean soil concentration, potentially leading to phytotoxicity. Moreover, copper excess in the human body may lead to serious liver diseases, neurological effects and Alzheimer disease. (Ballabio *et al.,* 2018) Although the use of copper is still tolerated because of its unique properties as a wide-spectrum fungicide and bactericide, the reduction of copper inputs in agriculture is one of the main goals for researchers in plant protection (Thuerig *et al.,* 2018).

Similarly, other chemical multi-site contact fungicides are used in pre-infection applications. This group includes dithiocarbamates (ie. mancozeb, metiram and propineb), quinone (ie. Dithianon)

and phthalimides, ie. captan and folpet (Falk *et al.*, 1996; Gullino *et al.*, 2010; Corio-Costet 2012; Nanni *et al.*, 2016,).

However, these products mostly accumulate in the wax of the outer cuticle layer of the leaf and are not transported to the inner leaf tissues, where actually the majority of the disease development occurs. Moreover, due to low mobility, newly organs will not be protected and thus exposed to infections. Therefore, preventive applications with contact fungicides have to be performed as close as possible to the possible infection. In this regard, Decision Support Systems (DSS) can significantly increase the efficacy of the applications, since they assess the likelihood of infection from canopy microclimate data and predict the occurrence of primary and secondary infections, favoring precise and efficient treatments (Bove *et al.* 2020; Brischetto *et al.* 2021).

The discovery of cytotropic and systemic fungicides greatly facilitated disease management. These products possess a long-lasting control activity (from nine to 18 days) as they are not washed off by precipitations and can protect even newly formed organs, which is extremely important especially at the beginning of bloom when grapevine plant susceptibility to *P. viticola* is in the peak phase. Moreover, these products have curative activity as well, since sporulation may be severely inhibited with applications on *P. viticola* lesions. (Wicks, 1982)

Nowadays, the main fungicide groups used to manage grapevine downy mildew are carboxylic acid amides like mandipropamid and dimetomorph (Blum *et al.*, 2010), phenylamides such as metalaxyl-m and benalaxyl (Koledenkova *et al.*, 2022), complex III inhibitors, such as stobilurins, cyazofamid, amisulbrom and ametoctradin (Fontaine *et al.*, 2019; Mounkoro *et al.*, 2019) and the newly discovered OxySterol Binding Protein Inhibitor (OSBPI) oxathiapiprolin (Massi *et al.*, 2021).

However, the majority of these products possess a single-site mode of action, which exposes to the risk of fungicide resistance (reported below). For this reason, these active ingredients are often used in combination or commercialized in formulations with multi-site fungicides and consecutive, repeated treatments have to be avoided.

4.4 Occurrence of fungicide resistance in *Plasmopara viticola*

In general, fungicide resistance can be defined as the acquired and heritable reduction in the sensitivity of a fungus and/or oomycete to a specific anti-fungal agent (Background Information, www.frac.info).

Fungicide resistance can be reached in five main different mechanisms: i) alterations in the target site that inhibits binding to the fungicide active ingredient; ii) overproduction of the target protein; iii) presence of an alternative metabolic pathway able to bypass the process inhibited by the fungicide active ingredient; iv) metabolic breakdown of the fungicide active ingredient; v) active export or exclusion of the fungicide active ingredients (Deising *et al.*, 2008).

Mutations naturally occur during the evolution of a plant pathogen, some of which may lead to the increase of tolerance towards a fungicide active ingredient. Few resistant plant pathogen individuals do not represent a concrete threat in the field. However, if the resistant plant pathogen population becomes predominant, the fungicide may fail in controlling the plant pathogen. The evolution of fungicide resistance in a population is determined by the interaction of several factors, such as the fungicide mode of action and utilization, the plant pathogen biology and epidemiology, and the agronomic practices adopted in the field (Massi *et al.*, 2021).

Oomycetes, such as *P. viticola*, are ranked as high-risk plant pathogens, since they possess a relevant evolutionary capacity due to the high reproduction frequency and the complex life cycle,

which includes sexual and asexual reproduction and polycyclic behavior (Poeydebat *et al.*, 2022). Moreover, the repeated use of a single-site fungicide significantly increases the selection pressure on the plant pathogen, favoring the survival of tolerant *P. viticola* populations (Ma and Michailides 2005). In fact, already after a short time on the market, the majority of the most commonly used single-site fungicides have been reported for fungicide resistance (Table 14).

Complex III inhibitors represent a crucial fungicide class. According to the FRAC classification, they are divided into three groups: Quinone outside Inhibitors (QoI, including strobilurins and famoxadone), Quinone inside Inhibitors (QiI, among which cyazofamid and amisulbrom) and Quinone inside outside stigmatellin binding site Inhibitors (QoSIs, ametoctradin) (Cherrad *et al.*, 2018).

The QoI fungicides act as inhibitors of mitochondrial respiration at the Qo site of the cytochrome *bc1* complex (complex III), blocking electron transfer and disrupting ATP synthesis (Bartlett *et al.*, 2002). Shortly after their introduction on the market, *P. viticola* isolates resistant to QoIs have been detected worldwide (Gullino *et al.*, 2004; Gisi and Sierotzki 2015; Chen *et al.*, 2007). Sequence analysis of the complete cytochrome *b* gene showed that all resistant *P. viticola* isolates carried a mutation resulting in the replacement of glycine by alanine at codon 143 (G143A) (Chen *et al.* 2007).

Among the QoSIs, two studies in France highlighted an increasing resistance to ametoctradin, both involving a single point mutation in the cytochrome *b*, by S34L substitution, and an alternative oxidase activity (Mounkoro *et al.* 2019; Fontaine *et al.* 2019). Nevertheless, the S34L substitution has been found in *P. viticola* isolates resistant to cyazofamid as well (Cherrad *et al.*, 2018).

Table 14 List of the main single- and oligo-site active ingredients for the control of *Plasmopara viticola*, with their mode of action according to FRAC and resistance reference. '-': not reported

Group name	Active Ingredient	Mode of Action	FRAC Group	Report of Fungicide Resistance	
				Country	Reference
Benzamides	Fluopicolide	Delocalization of spectrin-like proteins	U9	-	-
	Zoxamide	Cellular division	22	-	-
Cyanoacetamide -oxime	Cymoxanil	Unkwnown	27	Italy	Gullino *et al.*, 1997 Toffolatti *et al.*, 2014
CAA	Dimetomorph	Cell wall biosynthesis	40	United States	Siegenthaler & Hansen 2021
	Mandipropamid	Cell wall biosynthesis	40	Switzerland	Blum *et al.*, 2010
				Japan	Aoki *et al.*, 2015
				Italy	Nanni *et al.*, 2016
				India	Sawant *et al.*, 2017
OSBPI	Oxathiapiprolin	Oxysterol binding protein	U15	France	Mboup *et al.*, 2021
				Italy	Massi *et al.*, 2022
Phenylamides	Metalaxyl, Metalaxy-m	rRNA synthesis	4	France	Staub and Sozzi. 1981
QoI	Azoxystrobin Famoxadone	Complex III of fungal respiration: ubiquinol oxidase, Qo site	11	Italy	Gullino *et al.*, 2004
				France	Chen *et al.*, 2007
				Japan	Furuya *et al.*, 2010
				Brasil	Santos *et al.*, 2020
				United States	Colcol and Baudoin, 2016 Campbell *et al.*, 2021
QiI	Amisulbrom Cyazofamide	Mitochondrial respiration, Complex III, Qi site	21	France	Cherrad *et al.*, 2018
QoSI	Amectotradin	Mitochondrial respiration, Complex III, Qi and Qo site	45	France	Mounkoro *et al.*, 2018; Fontaine *et al.*, 2019

The carboxylic acid amide (CAA) fungicides, including dimethomorph and mandipropamid, are single-site fungicides inhibiting cell wall biosynthesis of oomycetes (Corio-Costet, 2012).

Recently, several resistant *P. viticola* isolates have been reported worldwide (Aoki *et al.*, 2015; Nanni *et al.*, 2016; Ghule and Sawant 2018; Toffolatti *et al.*, 2018). A mutation in the *PvCesA3* gene (G1105S) may be the cause of the reported resistance (Blum *et al.*, 2010). However, several other

recessive genes are involved in resistant *P. viticola* isolates, decreasing the risk of resistance in the field (Corio-Costet, 2012).

Between 1977 and 2007, seven molecules belonging to the phenylamides group have been released, among which metalaxyl, metalaxyl-m and mefenoxam (Gisi and Sierotzki, 2015). Recently, a single point mutation was identified as Y382F in the RNA *polI* gene, which showed an association with resistance to mefenoxam in *P. viticola* isolates (Randall *et al.*, 2014).

Finally, *P. viticola* isolates resistant to oxathiapiprolin have been detected in France and Italy. In this case the resistance was conferred by amino acids substitutions G770V, N837L and L863W on the *OSBP* gene (Mboup *et al.*, 2022) and N837I at *PvORP1* gene (Massi *et al.*, 2022).

4.5 New Frontiers in the Biological Control of *Plasmopara viticola*

In this critical scenario, research is aiming at developing new, innovative and environmentally sustainable methods to control *P. viticola*, especially for organic disease management, which relies primarily on the use of copper-based fungicides. For this reason, potential fungicides based on plant extracts or microorganisms attracted the attention of the scientific community. In this chapter, the most promising candidates are presented.

4.5.1 Plant extracts

Plant extracts have been tested intensively as potential bio-fungicides (Table 15). In fact, plant extracts generally possess significant disease suppression activity against several plant pathogens, including *P. viticola*, in controlled conditions. However, their use is limited by their high production costs, their limited availability in large quantity but more importantly by their low persistence and rain-fastness (Dagostin *et al.*, 2011; Gessler *et al.*, 2011; Pertot *et al.*, 2017).

Table 15 List of plant extracts and essential oils successfully tested for the control of *Plasmopara viticola*.

Species	Main Metabolite	Efficacy (%)	Trial	Reference
Abies sibirica	Unknown	66	Leaf disc	La Torre *et al.*, 2019
Artemisia absinthium	O-methylated flavonols and hydroxycinnamic acids	100	Leaf disc	Andreu *et al.*, 2018
Artemisia vulgaris	Unknown	90	Leaf disc	Andreu *et al.*, 2018
Chloris virgata	Unknown	84,7	Leaf disc	Chen *et al.*, 2002
Cinnamomum zeylanicum Blume	Essential oil	70	Germination	De Oliveira Fialho *et al.*, 2016
Cymbopogon citratus	Essential oil	48	Field	Maia *et al.*, 2014
Dalbergia hupeana	Unknown	87,5	Leaf disc	Chen *et al.*, 2002
Equisetum arvense	Unknown	90	Leaf disc	Andreu *et al.*, 2018
Frangula alnus	Rhein, frangulin A, emodin, aloe-emodin, chrysophanol, and physcion	91	Leaf disc	Godard *et al.*, 2009
Glycyrrhiza glabra	Glycyrrhizin	90 100	Germination Leaf disc	Troester *et al.*, 2016 La Torre *et al.*, 2019
Inga sapindoides	Ingadoside A-C and saponins	97	Plant	Heng *et al.*, 2021
Inula viscosa	Tomentosin, costic acid	90 98	Leaf disc Leaves	Islam *et al.*, 2015 Cohen *et al.*, 2006
Juncus effusus	Dehydroeffusol	96	Plant	Thuerig *et al.*, 2015
Larix decidua	Larixol and larixyl acetate	68	Field	Thuerig *et al.*, 2018
Larix sibirica	Larixol and larixyl acetate	95	Plant	Mulholland *et al.*, 2017
Magnolia officinalis	Magnolol and honokiol	71	Field	Thuerig *et al.*, 2018b
Melaleuca alternifolia	Essential oil	100	Leaf disc	La Torre *et al.*, 2014

Species	Main Metabolite	Efficacy (%)	Trial	Reference
Origanum vulgare	Essential oil	95	Plant	Rienth *et al.*, 2019
Paeonia suffuticosa	Unknown	84	Leaf disc	Chen *et al.*, 2002
Picea abies	Stilbenes	100	Leaf disc	Gabaston *et al.*, 2017a
Pinus massoniana	Unknown	88,5	Leaf disc	Chen *et al.*, 2002
Pinus pinaster	Nortrachelogenin, pinoresinol, matairesinol, isolariciresinol, secoisolariciresinol, pinocembrin, pinobanksin, dihydrokaempferol, taxifolin, pinosylvin, pinosylvin monomethyl ether, pterostilbene	100	Germination	Gabaston *et al.*, 2017b
Pinus sylvestris	7α,15-dihydroxydehydroabietic acid	80	Plant	Mulholland *et al.*, 2017
Potentilla erecta	Unknown	90	Leaf disc	Blaeser *et al et al.*, 2002
Quillaia saponaria	Unknown	98	Leaf disc	Dagostin *et al.*, 2011
Rheum palmatum	Rhein, frangulin A, emodin, aloe-emodin, chrysophanol, and physcion	95,2	Leaf disc	Godard *et al.*, 2009
Robinia pseudoacacia	Unknown	83,2	Leaf disc	Chen *et al.*, 2002
Salix alba	Eriodyctiol, catechins and derivates	100	Leaf disc	Andreu *et al.*, 2018
Salvia officinalis	Unknown	94	Field	Dagostin *et al.*, 2010
Solidago canadensis	Unknown	90	Plant	Harm *et al.*, 2011

Species	Main Metabolite	Efficacy (%)	Trial	Reference
Verbesina lanata	6β-cinnamoyloxy-4β,9β,15 trihydroxyeudesmane 6β-cinnamoyloxy-1 β,15-dihydroxyeudesm-4-en-3-one	82	Plant	Ramseyer *et al.*, 2017
Vitis vinifera	ampelopsin A, hopeaphenol, trans-resveratrol, ampelopsin H, ε-viniferin, and E-*Vitis*in B		Germination	Schnee *et al.*, 2013
Vitis vinifera	r and ε viniferin, Resveratrol	100	Leaf disc	Taillis *et al.*, 2022
Yucca schidigera	Saponins	99	Leaf disc	La Torre *et al.*, 2019

In a screening involving 58 plant extracts candidates, extracts from *Chloris vigata*, *Dalbergia hupeana*, *Pinus massoniana*, *Paeonia suffruticosa*, *Robinia pseudoacacia* had inhibitory effect against *P. viticola* sporangia germination by 85%, 88%, 88%, 84%, 83%, respectively. Leaf discs treated with these extracts did not display any symptoms (Chen *et al.*, 2002).

Ethanol extracts from *Artemisia absinthium*, *Artemisia vulgaris*, *Equisetum arvense* and *Salix alba* (leaves and bark) completely inhibited *P. viticola* sporangial germination and sporulation on leaf discs was barely visible when applied after the infection. *Salix alba* extract contained flavanones (eriodyctiol and derivates) and flavanols (catechins and derivates), as major compounds, whereas *A. absinthium* extract was rich in O-methylated flavanols and hydroxycinnamic acids. However, the three candidates did not display any plant protection activity in the vineyard, probably due to UV light degradation (Andreu *et al.*, 2018).

Frangula alnus bark extract and *Rheum palmatum* root extract showed an excellent disease suppression activity on leaf discs, with 91 and 95% efficacy, respectively. These two extracts contained many phenolic compounds belonging to the anthraquinone family, such as rhein, frangulin A, aloe-emodin, chrysophanol and physion. In particular, emodin alone was able to partially control *P. viticola* (Godard *et al.*, 2009).

The extract of *Glycyrrhiza glabra* leaves could prevent *P. viticola* infection due to its inhibiting effect on the activity of the contractile vacuole, leading to the disintegration of the zoospores. Moreover, the main compound, glycyrrhizin, could enhance immune response in grapevine cells (Tröster, 2016). Another study highlighted a complete plant protection efficacy on leaf disc by a formulated *G. glabra* extract. Furthermore, the same study revealed an excellent protection of *Yucca schidigera* extract and a moderate activity by *Abies sibirica* with 99% and 66% of efficacy, respectively (La Torre *et al.*, 2019).

A 96% ethanol extract from *Inga sapindoides* leaves showed potent inhibitory activity against *P. viticola* zoospores, reducing the disease severity by 97% in plantlets. HPLC analysis identified ingadosides A-C and several saponins, which were able to inhibit completely zoospore formation at 3µg mL^{-1} (Heng *et al.*, 2022).

Species belonging to the family *Pinaceae* attracted the interest of the scientific community as potential candidates for bio-fungicides. Larixol and larixyl acetate obtained from *Larix decidua* bark extract were able to reach a 68% of plant protection efficacy in the field, when applied as stand-alone, while 84% in a low-copper input strategy. Larixol and larixyl acetate displayed a Minimal Inhibitory Concentration of 6-23µg mL^{-1} in vitro and an EC_{50} of 0.2-0.4 mg mL^{-1} *in planta* (Thuerig *et al.*, 2018). These two compounds, alongside lariciresinol and lariciresinol acetate, were found in *Larix sibirica* dichlorometane extract as well. *Larix decidua* and *L. sibirica* extracts accounted for more than 90%

of plant protection efficacy. In the same study, dichlorometane extract from *Pinus sylvestris* bark obtained almost 80% of disease suppression. The main compound was identified as 7α,15-dihydroxydehydroabietic acid (Mulholland *et al.*, 2017).

Picea abies extract guaranteed a complete *P. viticola* inhibition on leaf discs, due to the high concentration of glycosylated monomeric forms such as piceatannol, astringin, resveratrol, piceid, isorhapontigenin, isorhapontin and several stilbene glycoside dimers were also identified such as piceasides I-N (Gabaston *et al.*, 2017a). Finally, *Pinus pinaster* knot extract was effective against *P. viticola,* by inhibiting zoospore mobility and development. Fourteen polyphenols divided in four classes were isolated from Pinus pinaster knot extract and identified as: i) lignans (nortrachelogenin, pinoresinol, matairesinol, isolariciresinol, secoisolariciresinol); ii) flavonoids (pinocembrin, pinobanksin, dihydrokaempferol, taxifolin); iii) stilbenes (pinosylvin, pinosylvin monomethyl ether, pterostilbene); and iv) phenolic acids (caffeic acid, ferulic acid) (Gabaston *et al.*, 2017b).

The *Juncus effusus* medulla extract showed satisfying grapevine downy mildew control in planta, reaching 98% efficacy. Dehydrodiffusol was identified as the main active compound. Moreover, this candidate resulted active in controlling apple scab, caused by *Venturia inaequalis* (Thuerig *et al.*, 2016). Similarly, a suspension containing 1 mg mL^{-1} of the ethyl acetate extract from *Verbesina lanata* inflorescences lowered *P. viticola* infection of leaves on grapevine seedlings by 82%. The two major active compounds were 6*β*-cinnamoyloxy-4*β*,9*β*,15-trihydroxyeudesmane and 6*β*-cinnamoyloxy-1*β*,15-dihydroxyeudesm-4-en-3-one (Ramseyer *et al.*, 2017).

Two independent studies highlighted the potential of oily paste extract of *Inula viscosa* leaves as a valid alternative to control *P. viticola*. In fact, this extract rich in tomentosin and costic acid provided 90% plant protection efficacy on leaf discs (Islam, 2015) and 98% on detached leaves (Cohen *et al.*, 2006).

Under controlled conditions, *Magnolia officinalis* bark extract showed a mean efficacy of 97% against *P. viticola* at 1 mg mL^{-1}, and an EC_{50} between 0.14 and 0.20 mg mL^{-1}. Magnolol and honokiol were identified as the main active compounds, both with $EC_{50} \leq 0.08$ mg mL^{-1}. Under field conditions, preliminary formulations reached efficacies up to 71% at 1–2 mg plant extract mL^{-1} (Thuerig *et al.*, 2018b).

Furthermore, *Potentilla erecta* and *Solidago canadiensis* extracts showed a relevant grapevine downy control capacity, both reaching 90% of plant protection efficacy on leaf discs (Blaeser *et al.*, 2002).

Quillaia saponaria and *Salvia officinalis* extracts were able to reduce *P. viticola* infection by 98% on leaf discs and by 94% in the field, respectively (Dagostin *et al.*, 2010, 2011).

Extracts coming from *Vitis vinifera* have been tested as well. Methanolic and ethanol extract from Pinot noir, Gamaret and Divico canes showed acute fungitoxic activity inhibiting *P. viticola* sporulation and zoospore mobility at 1 mg mL^{-1}. Six main active compounds were identified as ampelopsin A, hopeaphenol, trans-resveratrol, ampelopsin H, ε-viniferin, and E-*Vitis*in B. (Schnee *et al.*, 2013). Moreover, grapevine root and trunk extract containing considerable amounts of ε- and r-viniferins displayed toxic activity towards *P. viticola* zoospore mobility, sporulation and by stimulating plant defenses. However, in plants under greenhouse conditions, the extract reached a moderate efficacy, reducing the disease severity by 35 to 50%. (Taillis *et al.*, 2022)

Essential oils (EO) are natural plant extracts containing volatile compounds, typically extracted by steam distillation or by pressing and they can be used as fungicides, especially in organic agriculture.

In a wide screening of EO coming from different plant species, *Cynnamomum zeylanicum* EO showed high fungitoxic activity, inhibiting *P. viticola* zoospores germination by 70% (De Oliveira Fialho *et al.*, 2017) and reducing infection in the field by 64% in a comparable manner to Bordeaux mixture (Da Silva *et al.*, 2014). However, both studies highlighted a significant reduction in the fungitoxic activity by increasing the incubation period between the product application and the infection, probably due to the chemical instability of the EO.

Tea tree oil, obtained by steam distillation of leaves and terminal branches of *Malaleuca alternifolia*, provided 100% disease suppression on leaf discs (La Torre *et al.*, 2014).

Oregano vulgare EO, reduced *P. viticola* development by 95% on grapevine cuttings, after a post-infection 24 hours vapor treatment. Transcriptomic analysis revealed that the EO triggered the overexpression of genes involved in salicylic, jasmonic acid and ethylene synthesis and signaling, activating Pathogenesis-Related-proteins as well as phytoalexin synthesis (Rienth *et al.*, 2019).

A field trial over two consecutive growing seasons displayed the potential of lemon grass essential oil (*Cymbopogon citratus*). In fact, the treatments not only significantly decreased the Area Under the Disease Progress Curve (AUDPC) but had a positive effect on the number and mass of the bunches as well chemical characteristics of the grape. (Maia *et al.*, 2014).

Overall, the data obtained on the efficacy of plant extracts against *P. viticola* indicate that they may be considered as valuable source of new active ingredients that may developed as an alternative to chemical-based fungicide. However, more work is needed to achieve satisfying results in the vineyard, with plant extracts that can provide sufficient plant protection but at reasonable costs.

4.5.2 Microbial Biocontrol Agents

Biofungicides based on microbial biocontrol agents represent a valid solution to decrease the negative impact of conventional fungicides on the environment and on human health, since they possess a wide range of advantages. In fact, biofungicides do not leave residues on berries, can be applied close to harvest without interfering with fermentation, are safer for workers and, since their mechanisms of action is complex, can potentially be used successfully in anti-resistance strategies.

On the other hand, biofungicides possess a lower persistency, shelf-life and efficacy in case of severe infections. Moreover, they require precise applications in terms of timing and environmental conditions (Pertot *et al.*, 2017).

4.5.2.1 Fungal Biocontrol Agents

Fungal biocontrol agents represent an interesting frontier towards the use of more sustainable fungicides to control *P. viticola*, since they can act by releasing lytic enzymes, by hyperparasistism and inducing plant defence mechamisms. In the present paragraph, the most promising candidates are shown. (Table 16)

The endophytic fungi *Acremonium persicinum* and *Acremonium sclerotigenum*, isolated from the leaves of grapevine cv. Regina Bianca and Inzolia, showed a significant disease suppression activity against *P. viticola* (Lo Piccolo *et al.*, 2015). *Acremonium sclerotigenum* actively colonized grapevine tissues, especially buds during winter and leaves during the growing season and hyperparasitized *P. viticola*. Culture filtrates and the crude extract of *A. sclerotigenum* completely inhibited *P. viticola* sporangia germination after 2 hours of exposure. In particular, sporangia appeared thinner and deformed compared to the untreated control (Burruano *et al.*, 2008).

In other studies, germination assays highlighted the inhibition of *P. viticola* sporangia germination after the treatment with *Acremonium* sp. A20 and *Acremonium persicinum*. In particular, six metabolites (acremines A-F) were isolated from *Acremonium* sp. A20 agar cultures and characterized. These secondary metabolites were individually tested to assess their effect on *P. viticola* sporangia germination. Acremine D showed complete inhibition at 1 mM, while the other acremines had a more moderate effect, showing almost 60% of inhibition (Assante *et al.,* 2005; Lo Piccolo *et al.*, 2015).

Following a broad screening on endophytic microorganisms, five isolates able to inhibit *P. viticola* sporulation were isolated and identified as *Alternaria alternata*. Transmission electron microscope observations highlighted several severe structural alterations in *P. viticola* mycelium upon exposure to *A. alternata*, such as necrotic and irregularly shaped haustoria. Via Thin Layer Chromatography, three main low-molecular-weight metabolites were identified as three diketopiperazines, namely *cyclo*(L-phenylalanine-*trans*-4-hydroxy-L-proline), *cyclo*(L-leucine-*trans*-4-hydroxy-L-proline) and *cyclo*(L-alanine-*trans*-4-hydroxy-L-proline). A combination of these diketopiperazines was able to completely control *P. viticola* on grapevine leaf disc and in plants, applied both before and after the infection at a concentration of 0.33 mM (Musetti *et al.*, 2006).

Epicoccum nigrum, a saprophyte fungus isolated from *P. viticola* infected leaves, was efficient in controlling several plant pathogenic fungi based on the production of antifungal compounds like flavipin. However, its mode of action against *P. viticola* was not completely defined, since it is still unknown whether *E. nigrum* acts as a hyperparasite or a saprophyte growing on *P. viticola* cells (Kortekamp, 1997).

A recent study showed the potential of several *Fusarium* species as biocontrol agents against *P. viticola*. Five isolate, belonging to *F. delphinoides*, *F. brachygibbosum*, *F. pseudonygamai* and a

Fusarium sp., collected from grapevine leaves were tested on leaf disc and in field conditions. When applied before the *P. viticola* infection, all five *Fusarium* isolates strongly reduced sporangia production via sporangia hyperparasitism and the release of lytic enzymes such as glucanase, chitinase and protease. In a field experiment, the isolates significantly reduced *P. viticola* symptoms on leaves and bunches (Ghule and Sawant, 2018).

Previously, Falk *et al.* (1996) highlighted the excellent ability of *F. proliferatum* G6 to control grapevine downy mildew. On grapevine leaf discs, post-infection application of a conidial suspension of *F. proliferatum* G6 reduced the sporulation by 97%, due to its ability to parasitize *P. viticola* mycelium. This biocontrol agent showed promising performances in the field over a three-year trial, with disease reduction ranging from 53% to 99% on grapevine bunches. In following studies, the cold-tolerant mycoparasitic strain *F. proliferatum* 1505 showed significant relevant levels of extracellular β-glucosidase and endo-1,4-β-glucanase activity in the culture filtrate, leading to 85% grapevine downy mildew suppression (Bakshi *et al.,* 2001).

The endophytic fungus *Phomopsis* sp. CAFT69, isolated from *Endodesmia calophylloides*, remarkably impaired motility of *P. viticola* zoospores followed by lysis already at 1 to 10 µg mL^{-1}. The active ingredients in the extracts were isolated and identified as excelsional, alternariol-5-O-methyl ether and altenusin (Talontsi *et al.,* 2012). Another fungal biocontrol agent effective in controlling *P. viticola* is represented by *Penicillium crysogenum* that showed an efficacy comparable to copper-based fungicides, with better performances compared to well-known defense inducers asβ-aminobutyric acid (BABA) and Acyl benzolar s-methyl (Tamm *et al.,* 2011).

Arbuscular mycorrhizal fungi, such as Rhizophagus irregularis, are obligate plant symbionts, which can significantly alter plant physiology, promoting nutrient uptake, plant growth and stimulating plant defenses. (Liu *et al.* 2007; Parniske 2008). In fact, *R. irregularis*-mycorrhized

grapevine plants showed an up-regulation of genes involved in the stilbenoid biosynthesis pathway and a modulation of pathogenicity effectors expression 48 hours after *P. viticola* inoculation (Cruz-Silva *et al.,* 2021).

Trichoderma harzanium T39 is widely used as biocontrol agent and commercialized as THRICODEX on grapevine against *Botrytis cinerea* (Elad, 1994). However, THRICODEX was also tested on potted grapevine plants against *P. viticola* with excellent disease suppression capacity (98%, Dagostin *et al.,* 2011), suggesting the potential of *T. harzanium* T39 biocontrol activity against this plant pathogen. In fact, *T. harzanium* T39 was able to reduce *P. viticola* infection by 83% in greenhouse conditions, triggering an induced systemic response in grapevine plant (Perazzolli *et al.,* 2008). The induction of systemic resistance was linked to the priming and modulation of defense-related genes after *P. viticola* infection, especially genes belonging to jasmonic acid and ethylene pathways (Perazzolli *et al.,* 2011). Moreover, *T. harzanium* T39, *T. asperellum* T34 and *T. atroviride* SC1 produce several volatile organic compounds (VOCs), which were able to reduce significantly *P. viticola* disease severity on grapevine leaf discs. VOCs were detected by Head Space-Solid Phase Microextraction Gas Chromatography–Mass Spectrometry and identified as α-farnesene, cadinene, 1,3-octadiene, 2-pentylfuran, and 6-pentyl-2H-pyran-2-one. In particular, the exposure of grapevine leaf discs to these VOCs induced the production of callose and enhanced the modulation of defense-related genes after *P. viticola* inoculation. In addition, 6-pentyl-2H-pyran-2-one activated the hypersensitive response in inoculated leaf discs (Lazazzara *et al.,* 2021).

Table 16 List of the main fungi tested as microbial biocontrol agents against *Plasmopara viticola*, their putative mode of action, the main active metabolite and testing conditions.

Microorganism	Mode of action	Main metabolites	Efficacy (%)	Trial	Reference
Acremonium byssoides A21	Unknown	Unknown	100	Germination	Burruano *et al.*, 2008
Acremonium persicinum	Anti germinative metabolites	Acremines	100	Germination	Lo Piccolo *et al.*, 2015
Acremonium sp. A20	Anti germinative metabolites	Acremine C	100	Germination	Assante *et al.*, 2005
Alternaria alternata	Antifungal metabolites	Cyclic aminoacids	100	Leaf disc	Musetti *et al.*, 2006
Epicoccum nigrum	Hyperparasitism	Unknown	None	Germination	Kortekamp *et al.*, 1997
Fusarium brachygibbosum	Hyperparasitism and lytic enzymes	Unknown	67	Leaf disc	Ghule and Sawant 2017
Fusarium delphinoides	Hyperparasitism and lytic enzymes	Unknown	65	Leaf disc	Ghule and Sawant 2017
Fusarium proliferatum	Lytic enzymes	β-glucosidase and endo-1,4-β-glucanase	85	Leaf disc	Falk *et al.*, 1996, Bakshi *et al.*, 2001
Fusarium pseudonygamai MCC 1345	Hyperparasitism and lytic enzymes	Unknown	71	Leaf disc	Ghule and Sawant 2017
Fusarium pseudonygamai MCC 1346	Hyperparasitism and lytic enzymes	Unknown	76	Leaf disc	Ghule and Sawant 2018
Fusarium sp. MCC134	Hyperparasitism and lytic enzymes	Unknown	63	Leaf disc	Ghule and Sawant 2017
Penicillum chrysogenum	Induced resistance	Aqueous micelium extract	99	Field	Tamm *et al.*, 2011
Phomopsis sp. CAFT69	Lytic enzymes and motility inhibition	Excelsional, alternariol-5-O-methyl ether, altenusin	None	Germination	Talontsi *et al.*, 2012
Rhizophagus irregularis	Induced resistance	Unknown	None	Plant	Bruisson *et al.*, 2016, Cruz-Silva *et al.*, 2021
Trichoderma arzanium HL1	Induced resistance	Unknown	67	Field	El-Sharkawy *et al.*, 2018
Trichoderma arzanium T39	Induced resistance	α-farnesene, cadinene, 1,3-octadiene, 2-pentylfuran, and 6-pentyl-2H-pyran-2-one	98	Leaf disc	Perazzolli *et al.*, 2008, Perazzolli *et al.*, 2011, Dagostin *et al.*, 2011, Lazazzara *et al.*, 2021
Trichoderma viride HL5	Induced resistance	Unknown	56	Field	El-Sharkawy *et al.*, 2018

Recently, *T. harzianum* HL1 and *T. viride* HL5 were able to reduce *P. viticola* disease severity by 67% and 56%, respectively, under field conditions. Furthermore, treatments with *T. harzianum*

HL1 and *T. viride* HL5 promoted plant growth and increased quality trait parameters in the berries (El-Sharkawy *et al.,* 2018).

4.5.2.2 Bacterial Biocontrol Agents

Bacteria have always attracted scientific interest as biocontrol agents against many plant pathogens, including *P. viticola*. In particular, the release of antifungal metabolites and lytic enzymes and the induction of plant defense mechanisms provide interesting perspectives towards the development of biofungicides (Afoshin *et al.,* 2020). The main bacterial biocontrol agents, able to control successfully *P. viticola,* are presented in Table 17.

Bacillus subtilis GLB191 and *B. pumilus* GLB197 showed a remarkable inhibition of *P. viticola*. In fact, the disease control efficacy of *B.subtilis* GLB191 and *B. pumilus* GLB197 treated grapevine leaves was 69% and 58% respectively, suggesting that these two bacterial strains could significantly inhibit the pathogen development. Moreover, after repeated treatments under field conditions on Muscat Hamburg and Cabernet Sauvignon plants, both candidates presented a disease suppression comparable to the chemical fungicide, with a significant effect even after 25 days from infection (Zhang *et al.,* 2017). Further studies evidenced that *B. subtilis* GLB191 supernatant contains the cyclic lipopeptides fengycin and surfactin, which were highly active against *P. viticola* via a direct toxic effect and an indirect effect, by modulating defense gene expression and inducing callose production and deposition (Li *et al.,* 2019). Furthermore, *B. subtilis* KS1 possesses the *ituD* and *lpa-14* genes, which both play a role in iturin A production, resulting in the control of grapevine downy mildew suppression on leaves and berries when repeatedly applied in the vineyard (Furuya *et al.,* 2011).

When applied at 1×10^8 CFU mL^{-1}, *B. velezensis* KOF112 at completely controlled *P. viticola* by inhibiting zoospore release and drastically upregulating the expression of genes encoding class IV chitinase and β-1,3-glucanase in grapevine leaves, suggesting an elicitor activity in grapevine plants (Hamaoka *et al.*, 2021).

Table 17 List of the main fungi tested as microbial biocontrol agents against *Plasmopara viticola*, their putative mode of action, the main active metabolite and testing conditions.

Microorganism	Mode of action	Main metabolites	Efficacy (%)	Trial	Reference
Bacillus pumilus GLB197	Antifungal metabolites	Unknown	57	Leaf disc	Zhang *et al.*, 2017
Bacillus subtilis GLB191	Antifungal metabolites and induced resistance	fengycin and surfactin	69	Leaf disc	Zhang *et al.*, 2017
Bacillus subtilis GLB191			98	Plant	Li *et al.*, 2019
Bacillus subtilis KS1	Antifungal metabolites	Iturin A	None	-	Furuya *et al.*, 2011
Bacillus velezensis KOF112	Zoospore release inhibition and induced resistance	Unknown	100	Leaf disc	Hamaoka *et al.*, 2021
Lysobacter capsici AZ78	Lytic enzymes, antifungal metabolites	Cyclo(L-Pro-L-Tyr)	93	Plant	Puopolo *et al.*, 2014a, 2014b, Cimmino *et al.*, 2020
Ochrobacterium sp. *SY286*	Cell wall degradation	Unknown	93	Leaf disc	Zang *et al.*, 2020
Paenibacillus sp. B2	Lytic enzymes and induced resistance	paenimyxin	None	Germination	Hao *et al.*, 2017
Penicillium chrysogenum	Induced resistance	Unknown	99	Field	Tamm *et al.*, 2011
Pseudomonas fluorescens	Zoosporicidal activity	diacetylphloroglucinol	None	Germination	Islam & Von Tiedemann 2011
	Induced resistance				Archana *et al.*, 2011
Pseudomonas fluorescens PTA-CT2	Induced resistance	Unknown	54	Leaf disc	Lakkis *et al.*, 2019
Streptomyces atratus PY-1	Zoosporicidal activity	5-acetoxycycloheximide and cycloheximide	92	Leaf disc	Liang *et al.*, 2016
			83	Field	
Streptomyces sp. ANK 313	Zoosporicidal activity	khatmiamycin	None	Leaf disc	Abdalla *et al.*, 2011
Streptomyces sp. B5136	Unknown	Staurosporine	None	Leave	Islam *et al.*, 2015
Streptomyces violatus HH5	Induced resistance	Unknown	58	Field	El-Sharkawy *et al.*, 2018
Streptomyces viridosporus HH1	Induced resistance	Unknown	67	Field	El-Sharkawy *et al.*, 2018

The fermentation liquor of *Ochrobacterium* sp. SY286 reduced the disease severity on detached grapevine leaves by 93% by damaging *P. viticola* cell walls. Moreover, this bacterial strain showed

an antagonistic activity against some other plant pathogens such as *Botrytis cinerea*, *Fusarium oxysporum* and *Phytophthora infestans* (Zang *et al.,* 2020).

Pseudomonas P. fluorescens treated grapevine seedlings showed an enhanced defense-related enzymatic activity, such as peroxidase, polyphenol oxidase and phenylalanine ammonia lyase (Archana *et al.,* 2011), while *P. fluorescens* PTA-CT2 reduced sporulation density of *P. viticola* by about 54% (Lakkis *et al.,* 2019). *P. fluorescens* produces 2,4-Diacetylphloroglucinol (DAPG) and some DAPG derivatives, among which dipropylphloroglucinol (DPPG). When applied at 3 and 10 µg ml^{-1}, DPPG and DAPG inhibited respectively zoosporogenesis and the motility of *P. viticola* zoospores (Islam and von Tiedemann 2011).

Streptomyces atratus PY-1 reduced grapevine downy mildew severity by 92% in leaf assay, and by 83% in a field assay. Scanning electron microscope observation highlighted that major damage occurred to the *P. viticola* sporangia and sporangiophores. Two imide compounds were identified as 5-acetoxycycloheximide and cycloheximide, both determining a reduction in disease severity by 65% and 84%, respectively (Liang *et al.,* 2016). Khatmiamycin, isolated from the culture broth of *Streptomyces* sp. ANK313, showed a significant motility inhibition and a lytic activity against *P. viticola* zoospores at a concentration of 10 µg mL^{-1} (Abdalla *et al.,* 2011). Moreover, El-Sharkawy *et al.,* (2018) highlighted not only the capacity of *Streptomyces viridosporus* HH1 and *S. violates* HH5 to reduce *P. viticola* disease severity in the field, (by 67% and 58%, respectively) but also their ability to improve grapevine plant growth, yield, and berry quality. Levels of polyphenol oxidase, total phenols and berry quality traits parameters were significantly higher in *S. viridosporus* HH1 and *S. violates* HH5 treated grapevine plants. The protein kinases inhibitor staurosporine, present in ethyl acetate extracts of the marine *Streptomyces* sp. strain B5136, rapidly impaired the motility *P. viticola* zoospores already 0.1 µg mL^{-1}. At a concentration of 2 µM, staurosporine completely suppressed *P.*

viticola on grapevine leaves, while at 5 μM zoospore release was inhibited in dose-related manner (Islam *et al.,* 2011).

4.6 *Lysobacter capsici* AZ78, a promising candidate for the biocontrol of *Plasmopara viticola*

The bacterial genus *Lysobacter* includes several species that may be potentially developed as biocontrol agents. In fact, some *Lysobacter* strains showed satisfying control activity against soil-borne plant pathogenic oomycetes such as *Phytophthora capsici* and *Pythium ultimum* and tomato root rot caused by *Fusarium oxysporum* (Puopolo *et al.,* 2010; Puopolo *et al.,* 2014b).

Among the genus *Lysobacter*, *L. capsici* AZ78 stands out as a potential candidate for the development of a new microbial biopesticide. Indeed, this strain showed a remarkable antimicrobial activity against Gram-positive bacteria, soil-borne plant pathogens and plant pathogenic oomycetes (Puopolo *et al.*, 2014b; Puopolo *et al.*, 2016).

Genome mining revealed that *L. capsici* AZ78 has genes involved in the production of antibiotics, lytic enzymes and siderophores (Puopolo *et al.*, 2016). In addition, *L. capsici* AZ78 tolerates UV light irradiation, mild heat shock, freezing and starvation (Puopolo *et al.*, 2016).

In particular, further studies evidenced the presence of genes involved in the production of polycyclic tetramate lactames such as the Heat-Stable Antifungal Factor (HSAF) and the cyclic depsipeptide WAP-8294A2, similarly to other *Lysobacter* spp. (Puopolo *et al.*, 2014a; Puopolo *et al.*, 2016; Bejarano *et al.*, 2021; Yue *et al.*, 2021; Liu *et al.*, 2022).

Moreover, recently the production of secondary metabolites with inhibitory activity against *P. ultimum*, *Rhizoctonia solani* and *Sclerotinia minor* was assessed. In particular, these metabolites were identified as the 2,5-diketopiperazine cyclo-(l-Pro-l-Tyr), toxic for sporangia in oomycete, cyclic

lipodepsipeptides, macrolides and several VOCs such as pyrazines derivatives (Puopolo *et al.,* 2014a; Vlassi *et al.,* 2020; Brescia *et al.,* 2021).

Interestingly, the use of *L. capsici* AZ78 to control *P. viticola* and *Phytophthora infestans* was patented in combination with low amounts of copper. In fact, *L. capsici* AZ78 has genes encoding copper oxidase (*copA*) and copper exporting PIB-type ATPases (*ctpA*), which confer copper tolerance. The ability to resist copper might be at the basis for the development of pest management strategies in combination with copper-based fungicide, increasing plant protection activity, rain fastness and persistence.

A liquid formulation of *L. capsici* AZ78 granted plant protection in the vineyard, comparable with copper-based fungicides. Moreover, this formulation improved *L. capsici* AZ78 persistence on grapevine leaves, protecting the bacterial cells from environmental factors (Segarra *et al.,* 2016). A large-scale field trial confirmed the protectant activity by *L. capsici* AZ78 against *P. viticola*. In addition, toxicological studies evidenced the absence of negative effects against beneficial arthropods and other non-target organisms such as the crustacean *Daphnia magna*, the algae *Selenastrum capricornutum* and earthworms. Moreover, the yeast population dynamics were not affected, thus not compromising wine quality (Markellou *et al.,* 2022).

The potential of this bacterium as biocontrol agent is remarkable, further optimization in the formulation may lead to the development of a commercial biopesticide, hence offering a valid alternative in order to implement sustainable pest management strategies.

4.7 Conclusions and Future Perspectives

In the next years, grapevine downy mildew management in viticulture will face a critical scenario. The constant reduction of copper inputs due to its impact on human health and environment, the increasing number of reported fungicide resistances and the withdrawal from the market of several active ingredients used so far urgently pushes the research community towards the development of new, sustainable plant protection products.

In this regard, scientific interest focused on screening a plethora of plant extracts and microorganisms with potential toxic activity against *P. viticola,* with interesting results in controlled conditions. However, so far, a small amount of candidates showed satisfying performances in the vineyards and very few of them were included in a commercial biopesticide. So far, no commercial microbial biopesticides are available in the market.

Nowadays, the biggest challenge for the research community and for companies investing in the field of microbial biopesticides is to design final formulations able to efficiently control *P. viticola* in the vineyard, with satisfying persistency, rain fastness and shelf life. At the same time, such products might also have sustainable costs for the farmers.

In this regard, the development of formulations that will help in reaching these ambitious goals may lead towards a more sustainable, environmentally friendly disease management.

4.8 References

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CHAPTER 5.

SUPPRESSIVE ACTIVITY OF *GLECHOMA HEDERACEA* EXTRACTS AGAINST THE PHYTOPATHOGENIC OOMYCETE *PLASMOPARA VITICOLA*, AND FIRST SCREENING OF THE ACTIVE METABOLITES

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Abstract

Plasmopara viticola is a destructive oomycete that affects grapevines, causing significant economic losses worldwide. This study highlights how the plant Glechoma hederacea might be at the basis for the development of biofungicides to control *P. viticola*. The aqueous extract obtained from *G. hederacea* aerial parts showed strong inhibition activity against *P. viticola*, comparable to that of copper hydroxide. The bio guided purification of the extract by chromatographic techniques led to the isolation of six pure metabolites, identified as the aromatic compounds carvacrol, caffeic acid and methyl caffeate, the flavonoids cirsimaritin and apigenin and the polyphenolic acid rosmarinic acid by spectroscopic methods. This is the first report about the isolation of methyl caffeate and cirsimaritin from *G. hederacea*. Caffeic acid and methyl caffeate showed the highest disease severity reduction, while carvacrol, cirsimaritin and apigenin also showed moderate activity against *P. viticola*. The inhibitory activity of the aqueous extract could suggest synergetic or additive action of caffeic acid and methyl caffeate together with other compounds contained in the extract. This study provides insights into the potential of *G. hederacea* as an allelopathic tool for developing control methods against *P. viticola*, revealing the combined action of different metabolites involved in the mechanism of action of the active compounds.

5.1 Introduction

Plasmopara viticola, the causal agent of grapevine (*Vitis vinifera L.*) downy mildew, is a plant pathogenic oomycete that affects grapevines, causing significant losses in grapevine production worldwide, and is considered as one of the most devastating diseases in viticulture. *P. viticola* infections lead to severe yield losses and decrease of the quality of grapes and wines while having an indirect negative impact on the environment caused by massive use of fungicides for its control (Jermini *et al.*, 2010a; Fontaine *et al.*, 2021; Gava *et al.*, 2021). The pathogen can spread rapidly under favorable environmental conditions, such as high humidity and moderate temperatures, through repeated infection cycles (Burruano *et al.*, 2006). Infections occur in the leaves, showing distinctive yellow discoloration lesions (oil spots) on the upper side and a white visible sporulation on the undersides, impairing the photosynthetic potential of the plant (Massi *et al.*, 2021). Moreover, *P. viticola* can infect inflorescences before and during blooming, while clusters and berries in the early stages after fruit set, leading to tissue necrosis and hence to potentially heavy yield losses (Jermini *et al.*, 2010b; Gessler *et al.*, 2011; Fröbel *et al.*, 2019). Thus, effective disease management is crucial to protect grapevine plants and ensure the production of high-quality grapes and wines.

In organic viticulture, copper-based fungicides are the oldest plant protection products used against *P. viticola*, and they still play a crucial role in disease management. However, copper, being a heavy metal, has a negative impact on the environment and on human health, since it accumulates in agricultural soils and has been linked to liver and neurological diseases (Cabús *et al.*, 2017; Ballabio *et al.*, 2018). On the other hand, growers have to face the outburst of *P. viticola* populations resistant to single-site chemical fungicides, which have been used widely in integrated pest management (Massi *et al.*, 2021).

For these reasons, the scientific community is focusing on research for alternative plant protection products, based on plant extracts or biocontrol agents, in order to reduce copper inputs in agriculture and to develop sustainable disease management strategies (Thuerig *et al.,* 2018a). In past decades, several studies have investigated the potential anti-oomycete activity of different plant extracts in germination tests, leaf disc bioassays and on greenhouse cultivated grapevine seedlings, obtaining significant inhibitory activity against *P. viticola*.

For instance, *Pinus pinaster* knot and *V. vinifera* canes extracts guarantee complete zoospore mobility and development inhibition (Schnee *et al.,* 2013; Gabaston *et al.,* 2017a). Other studies highlighted disease severity reduction above 90% on leaf discs with plant extracts and essential oils obtained from different species such as *Artemisia absinthium*, *Equisetum arvense*, *Frangula alnus*, *Glycyrrhiza glabra*, *Melaleuca alternifolia*, *Picea abies*, *Quillaia saponaria*, *Rheum palmatum*, *Salix alba* and *Yucca schidigera* (Godard *et al.*, 2009; Dagostin *et al.*, 2011; Thuerig *et al.*, 2016; Gabaston *et al.,* 2017b; Andreu *et al.,* 2018; La Torre *et al.*, 2019; Taillis *et al.,* 2022). Moreover, *Inga sapindoides*, *Juncus effusus*, *Larix sibirica*, *Oreganum vulgare*, *Pinus sylvestris*, *Solidago canadensis* and *Verbesina lanata* plant extracts have been successfully tested in greenhouse-cultivated seedlings, with plant protection efficacy above 80% (Harm *et al.*, 2011; Mulholland *et al.,* 2017; Ramseyer *et al.* 2017; Rienth *et al.,* 2019; Heng *et al.,* 2022).

However, only a limited number of candidates have displayed satisfying results during field trials, with plant protection efficacies ranging from around 70%, after the application of *Larix decidua* and *Magnolia officinalis* plant extracts (Thuerig *et al.,* 2018a, 2018b), to 94% on grapevine plants treated with a *Salvia officinalis* extract (Dagostin *et al.*, 2010). *Glechoma* spp., flowering plants of the Lamiaceae family with some traditional uses in herbal medicine and culinary applications (Pereira *et al.*, 2020; Gwiazdowska *et al.*, 2022), stand as a potential source to be explored for bioactive extracts

or metabolites of interest for the control of *P. viticola*. Recently, ground ivy (*G. hederacea*) extracts showed relevant antifungal activity against different fungi (i.e., *Candida albicans* and *Sclerotinia sclerotiorum*) (Gwiazdowska *et al.*, 2022). Thus, further studies on *G. hederacea* could provide novel results regarding its applicability. As applied in classical allelopathic studies, the evaluation of extracts and their metabolites in bioassays could provide new sources to be proposed for the development of new tools for more sustainable disease management (Endo *et al.*, 2010; Jmii *et al.*, 2011). This study covers the first evaluation of *G. hederacea* extracts against *P. viticola*, followed by a screening of the metabolites responsible for the biological activity.

5.2 Materials and Methods

5.2.1 Experimental Procedures

Proton nuclear magnetic resonance (^{1}H NMR) spectra were recorded at 400 MHz on a Bruker 400 Anova Advance (Bruker, Karlsruhe, Germany) spectrometer. The spectra were recorded using deuterated chloroform ($CDCl_3$) or deuterated methanol (CD_3OD), and the same solvents were used as internal standards. Electrospray ionization mass spectra (ESIMS) were performed using a liquid chromatography/mass spectrometry time of flight (LC/MS TOF) system Agilent 6230B (Agilent Technologies, Milan, Italy), high-performance liquid chromatography (HPLC) 1260 Infinity in positive mode. Column chromatography (CC) was performed using a silica gel (Kieselgel 60, 0.063–0.200 mm, Merck, Darmstadt, Germany). Thin-layer chromatography (TLC) was performed on analytical and preparative silica-gel plates (Kieselgel 60, F_{254}, 0.25 and 0.5 mm, respectively, Merck, Darmstadt, Germany). Spots were visualized by exposure to ultraviolet (UV) light (254 nm) and/or iodine vapors, and/or by spraying with 10% sulphuric acid (H_2SO_4) in methanol (MeOH) and 5% phosphomolybdic acid in ethanol (EtOH), followed by heating at 110° C for 10 min. Sigma-Aldrich Co. (Milan, Italy) supplied all the solvents. C log*P* values of the isolated compounds were calculated using ChemOffice v20.1 (Perkin Elmer, Waltham, MA, USA) by means of the appropriate tool in ChemDraw Professional (Mejías *et al.*, 2021)

5.2.2 Plant Material and Microorganisms

Glechoma hederacea plants were collected in May 2022 from a field at San Michele all'Adige (Italy) and maintained under controlled conditions (25 ± 0.5 °C; 70 ± 10% relative humidity, RH) in 0.7 L pots containing a mixture of peat and pumice (3:1) for six months. The aerial part of the plants, consisting of stems and leaves, was collected each month to have sufficient material for the evaluation

of aqueous extracts and the subsequent chemical analysis. *P. viticola* was isolated from an untreated vineyard in San Michele all'Adige (Italy) in 2022 and maintained on grapevine plants (*V. vinifera* cv. Pinot Noir, grafted onto Kober 5BB.) by subsequent weekly inoculations using a hand sprayer according to Puopolo *et al.* (2014). Briefly, grapevine plants showing oil spot symptoms were kept overnight in the dark at 20–21°C and 100% RH to induce the sporulation of *P. viticola*. The sporangia of *P. viticola* were collected by washing the abaxial leaf surface of grapevine leaves covered with sporulating lesions with cold (4–5 °C) distilled water. The final concentration of the sporangia suspension was adjusted to 2.5×10^4 sporangia/mL by counting with a hemocytometer under a light microscope.

5.2.3 Production of G. hederacea *Aqueous Extracts and Activity Evaluation against* P. viticola

Using scissors, 40 g of the aerial parts (stems and leaves) of *G. hederacea* was cut into small pieces and macerated in a 1 L box with 200 mL of sterile distilled water at 20 °C in the dark. After 14 days, the aqueous extract was collected into sterile 50 mL tubes, centrifuged (4000 rpm, 20 min) to remove plant debris and filter-sterilized (0.22 μm; Sigma-Aldrich, Milan, Italy). A 1 L box containing only sterile distilled water (200 mL) was treated similarly and used as the untreated control. For efficacy tests against *P. viticola*, leaf discs (18 mm in diameter) were obtained from the third and fourth apical leaves from the shoots of grapevine plants (*V. vinifera* cv. Pinot Noir, grafted onto Kober 5BB) grown in greenhouse conditions at 25 °C with relative humidity of 60 ± 10% and a photoperiod of 16 h of light for two months, resulting in a light intensity of 1050 mmol m^{-2} s^{-1}. Leaf discs were transferred (lower surface uppermost) onto sterilized moist filter paper (three foils) contained in Petri dishes (five grapevine leaf discs for each dish). The aqueous extract of *G. hederacea* was sprayed on grapevine leaf discs using a hand sprayer (7 mL for each Petri dish). Sterile distilled water and copper hydroxide (Coprantol Hi Bio, Syngenta, Basel, Switzerland; 2 g L^{-1}) were used as controls. Once

treated, Petri dishes were stored at 25 °C in the dark. After 24 h, a *P. viticola* sporangia suspension (2.5×10^4 sporangia mL^{-1}) was sprayed on the grapevine leaf discs (7 mL for each Petri dish) using a hand sprayer. Petri dishes were incubated in the dark at 25 °C overnight and then maintained under controlled greenhouse conditions (25 °C, 60–80% RH with a 16/8-h day/nightlight regime). After seven days, the severity of downy mildew disease was assessed visually as the percentage of abaxial leaf disc area covered by white sporulation of *P. viticola*, according to the standard guidelines of the European and Mediterranean Plant Protection Organization (EPPO, 2001). Five replicates (Petri dishes) were used for each treatment, and the experiment was repeated.

5.2.4 Obtaining G. hederacea *Extracts, and Isolation and Identification of Secondary Metabolites*

In 300 mL of distilled H_2O/MeOH (1/1, *v*/*v*), 40 g of dried aerial parts (leaves and stems) of *G. hederacea* were blended and macerated at room temperature for 48 h. The resulting hydroalcoholic suspension (225 mL) was centrifuged (7000 rpm; 25 min) and extracted with *n*-hexane (3 × 150 mL) and methylene chloride (CH_2Cl_2, 3 × 150 mL). The residue aqueous phase was concentrated under reduced pressure to remove the methanol and was then extracted with ethyl acetate (EtOAc, 3 × 150 mL). Each extract was dried over anhydrous sodium sulphate (Na_2SO_4), filtered and concentrated to dryness under reduced pressure. The extraction process was performed for a second time. The total amount obtained for each extract was 98.6 mg (*n*-hexane), 105.5 mg (CH_2Cl_2) and 170.3 mg (EtOAc).

The *n*-hexane extract (98.6 mg) was purified by column chromatography on Si-gel eluted with $CHCl_3$/*i*-propanol (9/1, *v*/*v*), yielding seven homogeneous fractions (FH1-FH7). The residue of FH4 (27.7 mg) was purified by preparative TLC eluted with CHCl3/*i*-propanol (99/1, *v*/*v*), yielding a pure compound identified as carvacrol (26.2 mg). The CH_2Cl_2 extract (105.5 mg) was purified by column chromatography on Si-gel eluted with $CHCl_3$/*i*-propanol (gradient from 9/1 to 7/3, *v*/*v*), yielding nine homogeneous fractions (FC1-FC9). The residues of FC2 (8.2 mg), FC3 (7.9 mg) and FC4 (16.1) were

individually purified by two successive steps of analytical TLC eluted with $CHCl_3$/*i*-propanol (95/5, *v/v*) and with n-hexane/EtOAc (1/1, *v/v*), obtaining three pure compounds identified as cirsimaritin (6.4 mg), apigenin (3.6 mg) and methyl caffeate (5.9 mg). The EtOAc extract (170.3 mg) was purified by column chromatography on Si-gel eluted with EtOAc/MeOH/H_2O (85/10/5, *v/v*), yielding seven homogeneous fractions (FE1-FE7). The residue of FE2 (8.7 mg) was purified by reversed-phase TLC eluted with H_2O/MeOH (1/1, *v/v*), yielding a pure compound identified as caffeic acid (3.3 mg). The residue of FE5 (31.7 mg) was purified by preparative TLC eluted with EtOAc/MeOH/H_2O (85/10/5, *v/v*), yielding a pure compound identified as rosmarinic acid (4.9 mg). The structural characterization of the six compounds isolated was performed by comparison of their spectroscopic nuclear magnetic resonance (NMR) and mass data with those previously reported for the identified compounds as summarized below. The NMR spectra are provided below.

Carvacrol: ^{1}H NMR spectrum (Figure 4) in agreement with data previously reported (Trindade *et al.,* 2019); ESIMS (+) m/z: 151 [M + H]+, consistent with the molecular formula $C_{10}H_{14}O$.

Cirsimaritin: ^{1}H NMR spectrum (Figure 5) in agreement with data previously reported Suleimenov *et al*., 2008); ESIMS (+) m/z: 315 $[M + H]^+$, consistent with the molecular formula $C_{17}H_{14}O_6$.

Apigenin: ^{1}H NMR spectrum (Figure 6) in agreement with data previously reported (Osigwe *et al.*, 2017); ESIMS (+) *m/z*: 271 $[M + H]^+$, consistent with the molecular formula $C_{15}H_{10}O_5$.

Methyl caffeate: ^{1}H NMR spectrum (Figure 7) in agreement with data previously reported (Xiang *et al.*, 2011); ESIMS (+) *m/z*: 195 $[M + H]^+$, consistent with the molecular formula $C_{10}H_{10}O_4$

Caffeic acid: ^{1}H NMR spectrum (Figure 8) in agreement with data previously reported (Swisłocka *et al.*, 2013); ESIMS (+) *m/z*: 181 $[M + H]^{+}$, consistent with the molecular formula $C_9H_8O_4$.

Rosmarinic acid: ^{1}H NMR spectrum (Figure 9) in agreement with data previously reported (Lu *et al.*, 1999); ESIMS (+) *m/z*: 361 $[M + H]^{+}$, consistent with the molecular formula $C_{18}H_{16}O_8$. [α]20 D + 61° (c 0.2, MeOH); [α]20 $_D$ + 68° (c 0.2, MeOH) lit. (Le Claire *et al.*, 2005).

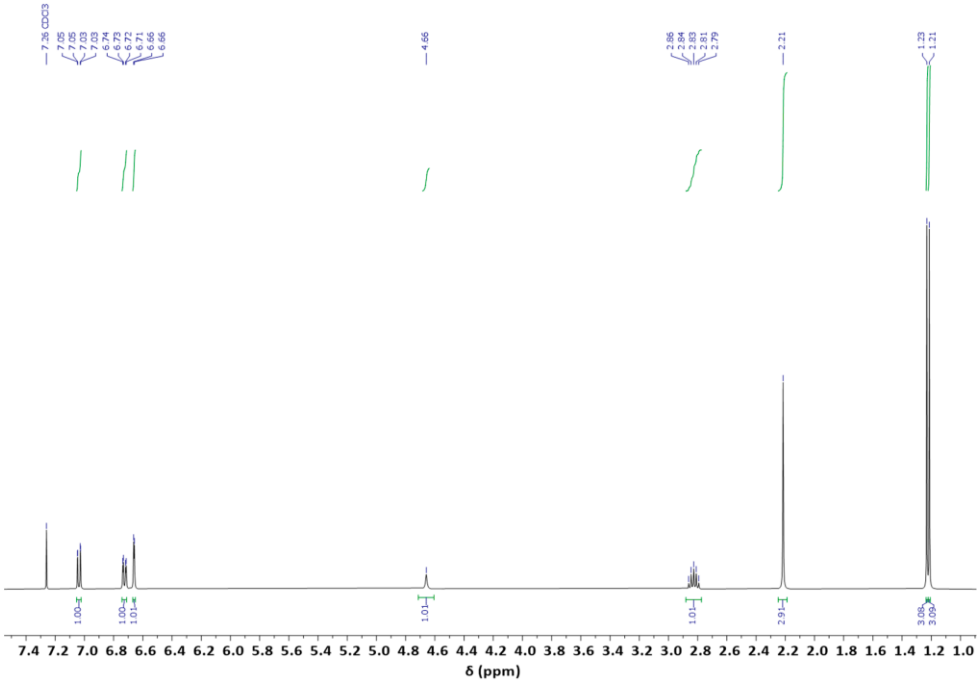

Figure 4 Carvacrol ^{1}H NMR spectrum.

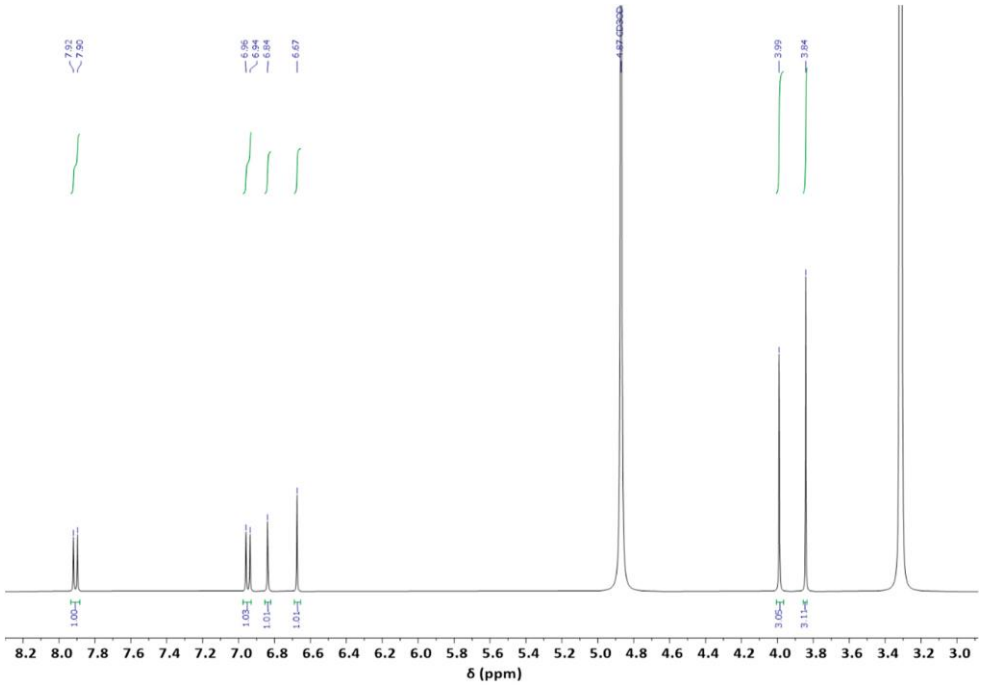

Figure 5 Cirsimaritin ^{1}H NMR spectrum.

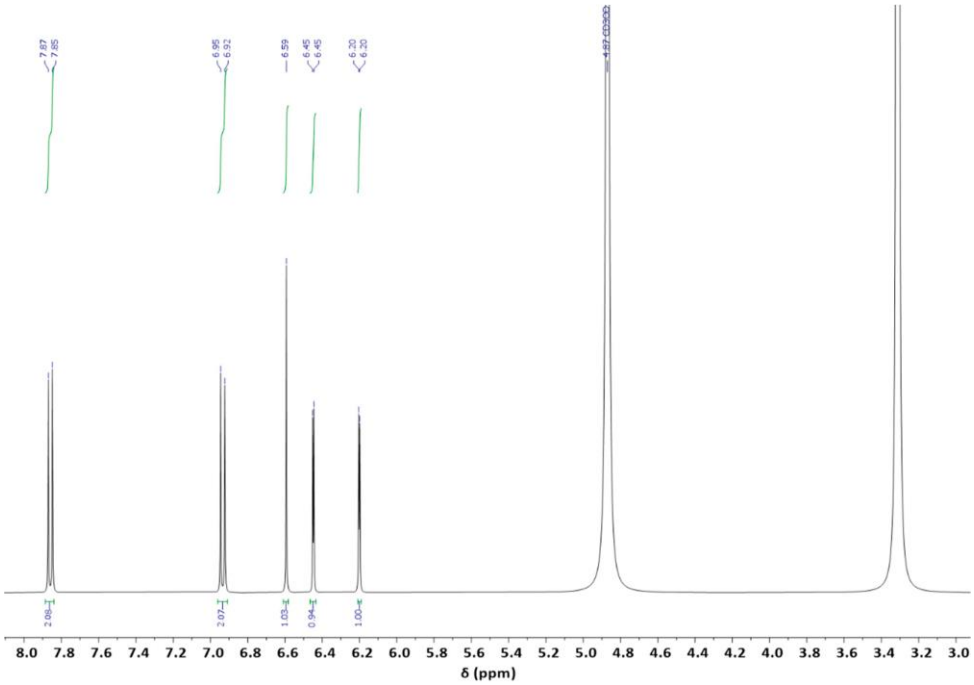

Figure 6 Apigenin ^{1}H NMR spectrum.

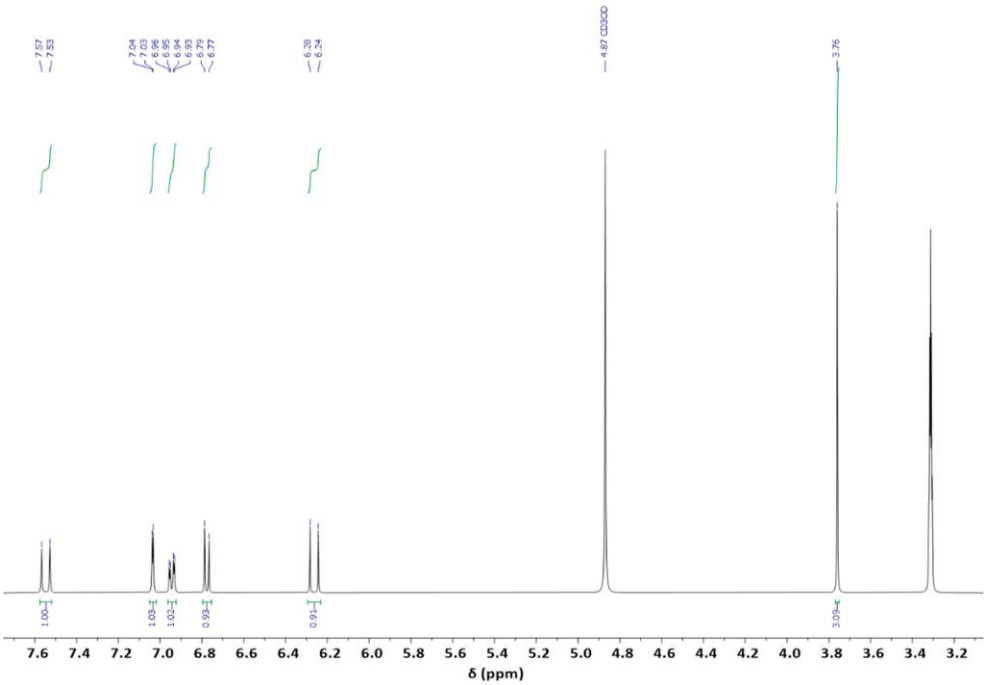

Figure 7 Methyl caffeate ^{1}H NMR spectrum.

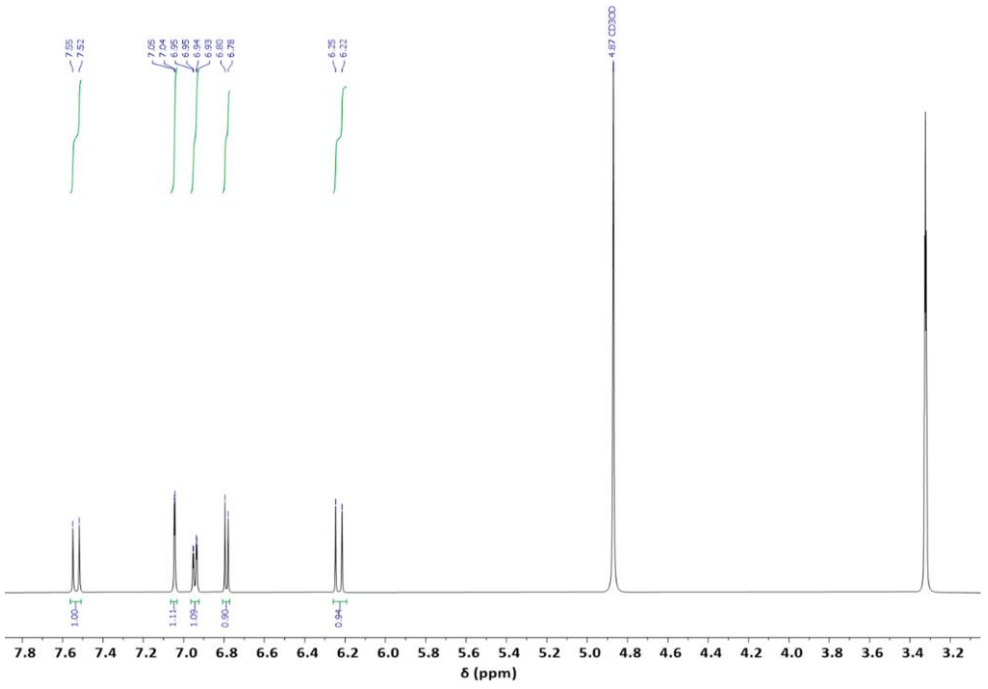

Figure 8 Caffeic acid ^{1}H NMR spectrum.

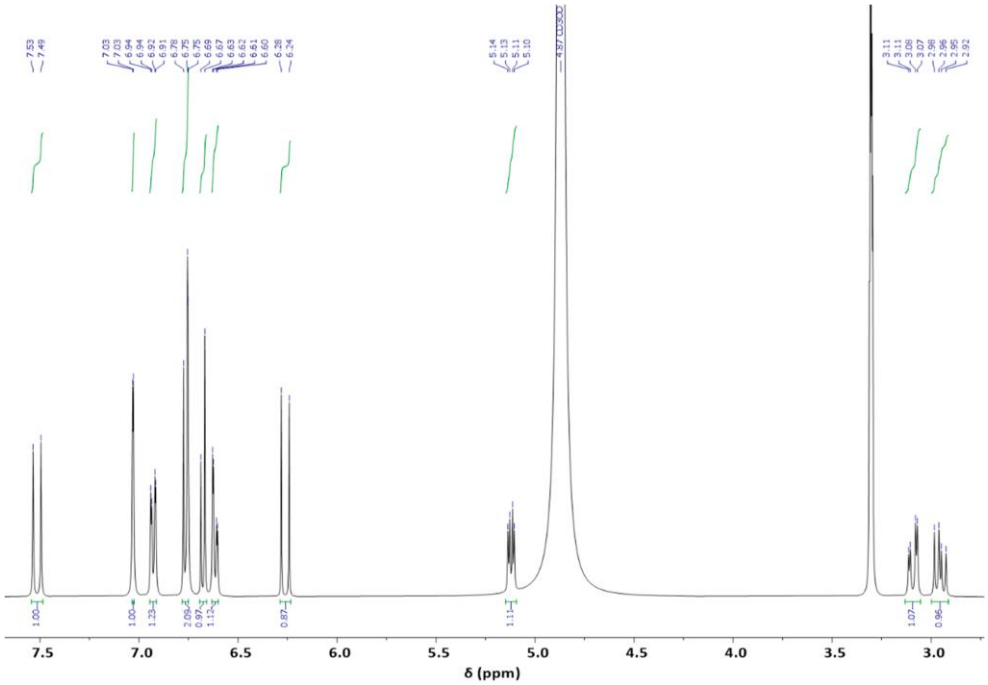

Figure 9 Rosmarinic acid ^{1}H NMR spectrum.

5.2.5 Evaluation of G. hederacea *Extracts and Secondary Metabolites against* P. viticola

Residues of the organic extracts (obtained in *n*-hexane, CH_2Cl_2 and EtOAc) and the secondary metabolites (namely carvacrol, cirsimaritin, apigenin, methyl caffeate, caffeic acid and rosmarinic acid) were resuspended in dimethyl sulfoxide (DMSO, 0.5% *v/v*) in order to obtain a final concentration of 500 μg/mL. These solutions were evaluated for the protection of grapevine leaf discs against *P. viticola* following the procedure reported above. A DMSO solution (0.5% *v/v*), water and copper hydroxide (Coprantol Hi Bio, Syngenta; 2 g/L) were used as controls. Five replicates (Petri dishes) containing five grapevine leaf discs each were used for each treatment, and the experiment was repeated.

5.2.6 Statistical Analysis

The data of efficacy tests on grapevine leaf discs were analyzed using Statistica 9 software (StatSoft, Tulsa, OK, USA). After validation of the normal distribution (K-S test, $p > 0.05$) and variance homogeneity (Levene's test, $p > 0.05$) of the data, an analysis of variance (ANOVA) was carried out using Tukey's HSD test ($\alpha = 0.05$) to detect significant differences among treatments.

5.3 Results and Discussion

5.3.1 Activity of G. hederacea *Aqueous Extracts against* P. viticola *on Grapevine Leaf Discs*

P. viticola was evaluated in a leaf disc bioassay (Figure 10 and Figure 11). The application of *G. hederacea* aqueous extracts determined a remarkable reduction of grapevine downy mildew severity (11.4%) compared to that in grapevine leaf discs treated with distilled water (78.0%).

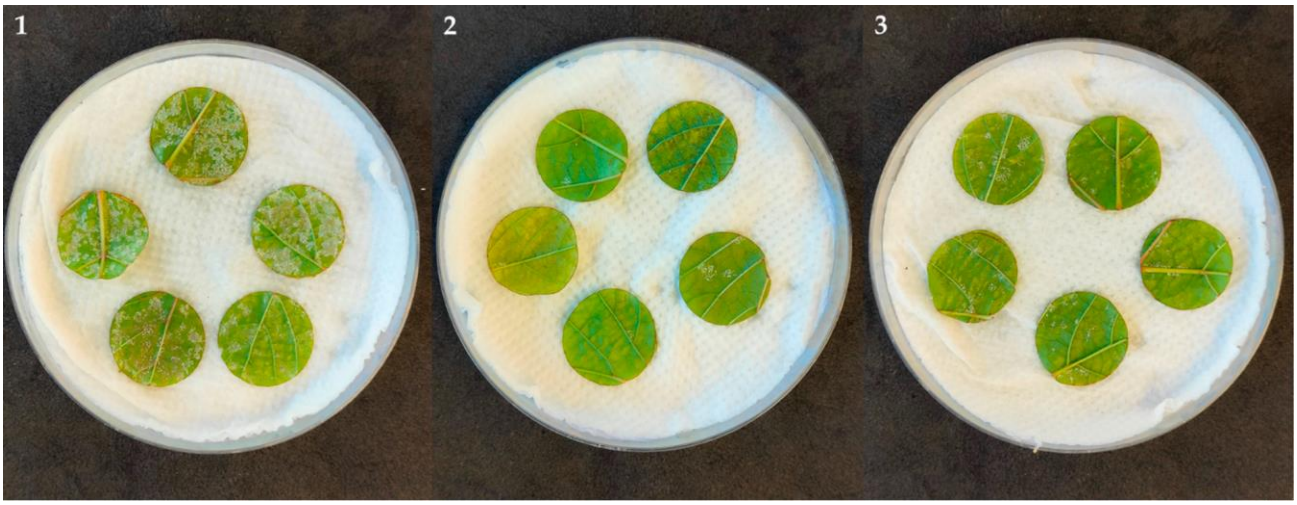

Figure 10 Control of *Plasmopara viticola* on grapevine leaf discs through the application of *Glechoma hederacea* aqueous extracts. Representative photographs showing the effect of the application of treatments in controlling *P. viticola* on grapevine leaf discs. The treatments evaluated were: (**1**) grapevine leaf discs treated with distilled water; (**2**) grapevine leaf discs treated with copper hydroxide (2 g L^{-1}) and (**3**) grapevine leaf discs treated with *G. hederacea* aqueous extracts.

Although significantly different, the ability of *G. hederacea* aqueous extracts to reduce grapevine downy mildew severity was similar to the protection achieved by the application of the copper-based fungicide (2.3%). To the best of our knowledge, this is the first evidence of anti-oomycete activity by *G. hederacea* aqueous extracts. These results showed that *G. hederacea* produces compounds active against *P. viticola*. Thus, the isolation and identification of those compounds is essential to get insights into their characterization.

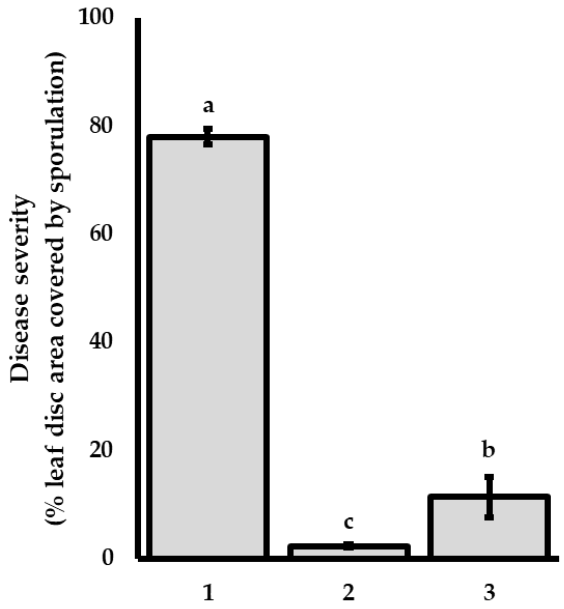

Figure 11 Efficacy of *Glechoma hederacea* aqueous extracts in controlling *Plasmopara viticola* on grapevine leaf discs. The treatments evaluated were: (**1**) grapevine leaf discs treated with distilled water; (**2**) Grapevine leaf discs treated with copper hydroxide (2 g L^{-1}) and (**3**) grapevine leaf discs treated with *G. hederacea* aqueous extracts. Columns represent mean ± standard error of five replicates (Petri dishes) containing five grapevine leaf discs each. Columns with different letters are significantly different according to the Tukey's HSD test ($p < 0.05$).

5.3.2 Bio guided Isolation and Suppressive Activity of Pure Compounds

Dried aerial parts of *G. hederacea* were macerated in a H2O/MeOH (1/1, *v/v*) solution and extracted with solvents of increasing polarity (namely *n*-hexane, CH_2Cl_2 and EtOAc) as described in *Section 3.3.4*. The weight of the collected extracts increased with the polarity of the solvent (98.6 mg for *n*-hexane, 105.5 mg for CH_2Cl_2 and 170.3 mg for EtOAc). The anti-oomycete activity of these extracts against *P. viticola* was subsequently evaluated (Figure 12).

The CH_2Cl_2 extract showed significantly lower disease severity (20.4%) compared to that of the untreated control (63.6%), followed by that of the *n*-hexane extract (38.4%). On the other hand, the EtOAc extract did not provide a significant reduction of disease severity (59.6%).

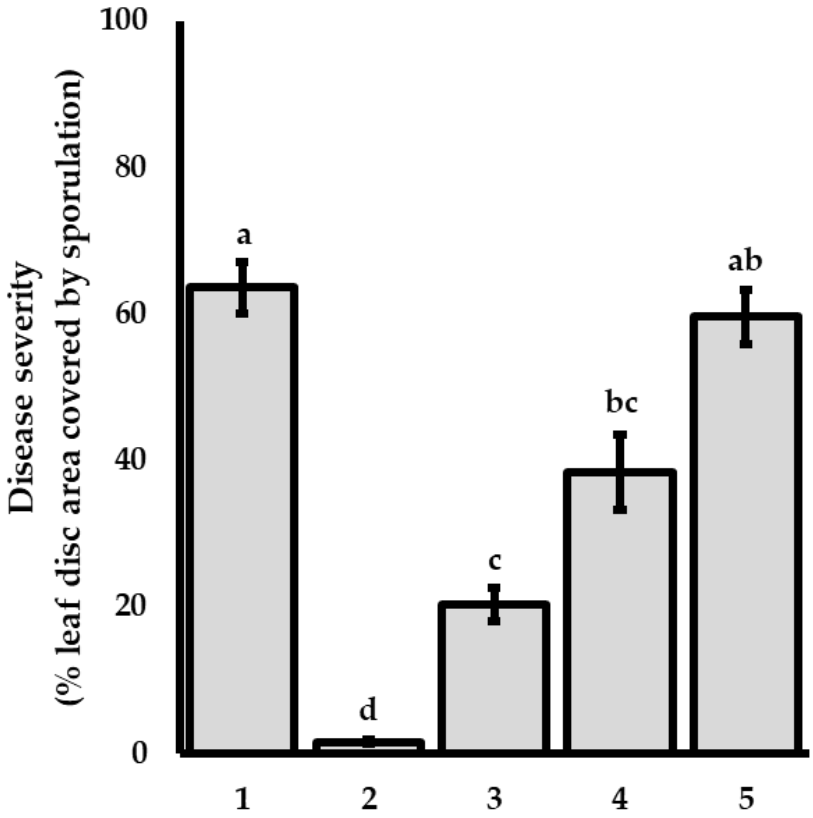

Figure 12 Efficacy of organic extracts of *Glechoma hederacea* in controlling *Plasmopara viticola* on grapevine leaf discs. The treatments evaluated were: (**1**) grapevine leaf discs treated with DMSO solution (0.5% *v/v*); (**2**) grapevine leaf discs treated with copper hydroxide (2 g L^{-1}); (**3**) grapevine leaf discs treated with *G. hederacea* organic extract in CH_2Cl_2; (**4**) grapevine leaf discs treated with *G. hederacea* organic extract in *n*-hexane; and (**5**) grapevine leaf discs treated with *G. hederacea* organic extract in ethyl acetate. Organic extracts of *G. hederacea* were evaluated at a final concentration of 500 μg mL^{-1}. Data presented as mean ± standard error of five replicates (Petri dishes) containing five grapevine leaf discs each. Columns with different letters are significantly different according to the Tukey's HSD test ($\alpha = 0.05$).

These results suggested the purification of the organic extracts in order to identify the low-molecular-weight metabolites produced by *G. hederacea* and evaluate their standalone anti-oomycete activity against *P. viticola*. The *n*-hexane, CH_2Cl_2 and EtOAc extracts were purified by chromatographic means (mainly column and TLC chromatography) following the procedures described in Section 3.3.4. Six pure metabolites (Figure 13) were isolated and identified as three aromatic compounds (carvacrol, caffeic acid and methyl caffeate), two flavonoids (cirsimaritin and apigenin) and a polyphenolic acid (rosmarinic acid). Specifically, carvacrol was collected from the n-hexane extract, cirsimaritin, apigenin and methyl caffeate from the CH_2Cl_2 extract and caffeic acid

and rosmarinic acid from the EtOAc extract. All the compounds contain an aromatic system in their structure. Carvacrol is a monoterpenic phenol found in essential oils from different plant species. It has been reported as a volatile constituent of the essential oil of *G. hederacea* (Radulovic *et al.*, 2010), the unique Glechoma species in which this metabolite has been described so far. Structurally related compounds are common metabolites described in the literature, such as the recently discovered natural carvacrol dimer Marinelli *et al.,* 2018; Cala *et al.,* 2021).

The flavonoids cirsimaritin and apigenin were also isolated, with cirsimaritin being reported for the first time from *G. hederacea* (it was previously found in *Glechoma herbaceae* (Nikolova and Asenov, 2006). Apigenin is often isolated in glycosylated forms and has already been identified in *G. hederacea* (Silva *et al.,* 2019; El-Aasr *et al.,* 2023). Herein, methyl caffeate is reported for the first time for a *Glechoma* species, while caffeic acid and rosmarinic acid, a water-soluble ester of caffeic acid, are among the most abundant phytochemicals detected in hot water extracts of *G. hederacea* (Topal *et al.,* 2014; Chao *et al.,* 2022; El-Aasr *et al.,* 2023). *P. viticola* disease severity was evaluated after the application of the six isolated compounds at 500 μg mL^{-1} (Figure 14).

The metabolites showing the highest anti-oomycete activity were caffeic acid and methyl caffeate (18.8% and 21.2% disease severity, respectively), followed by carvacrol, cirsimaritin and apigenin (48.0–41.0%), while rosmarinic acid did not display any disease reduction, with disease severity (66.0%) comparable to that of the negative control (65.6%). Very few reports have been found regarding the biological activity of the six isolated compounds against *P. viticola*, while antifungal activity has been described for all of them (Aziz *et al.,* 1998; Alcerito *et al.,* 2002; Boubaker *et al.,* 2016; Lee *et al.,* 2018; Lima *et al.,* 2018; Zhang *et al.,* 2019; Ivanov *et al.,* 2022; Saghrouchni *et al.,* 2023).

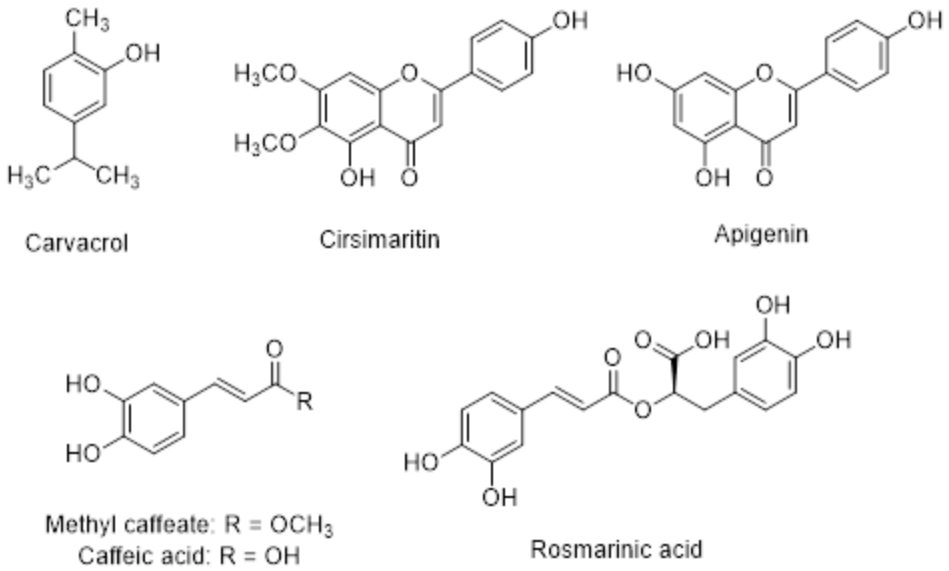

Figure 13 Structure of the metabolites isolated from the *Glechoma hederacea* extracts.

Caffeic acid is included among the metabolites responsible for significant *P. viticola* disease reduction in a *Pinus pinaster* knot extract (Gabaston *et al.,* 2017b) and is reported to be related to oxidative mechanisms in response to *P. viticola* infections (Mattivi *et al.,* 2011) and to constitutive resistance in the grapevine cultivar 'Regent' (Figueiredo *et al.,* 2015). On the other hand, caffeic acid has been found in considerable amounts in susceptible *V. vinifera* varieties (Maia *et al.,* 2020). Thus, it is probable that the contribution of caffeic acid to the resistance of grapevine varieties might be linked to the phenological phase when this secondary metabolite is produced. Methyl caffeate, isolated from a methanolic extract from *Solanum torvum* Swartz., displays antimicrobial and antimycobacterial activity against several *Mycobacterium* strains (Balachandran *et al.,* 2012).

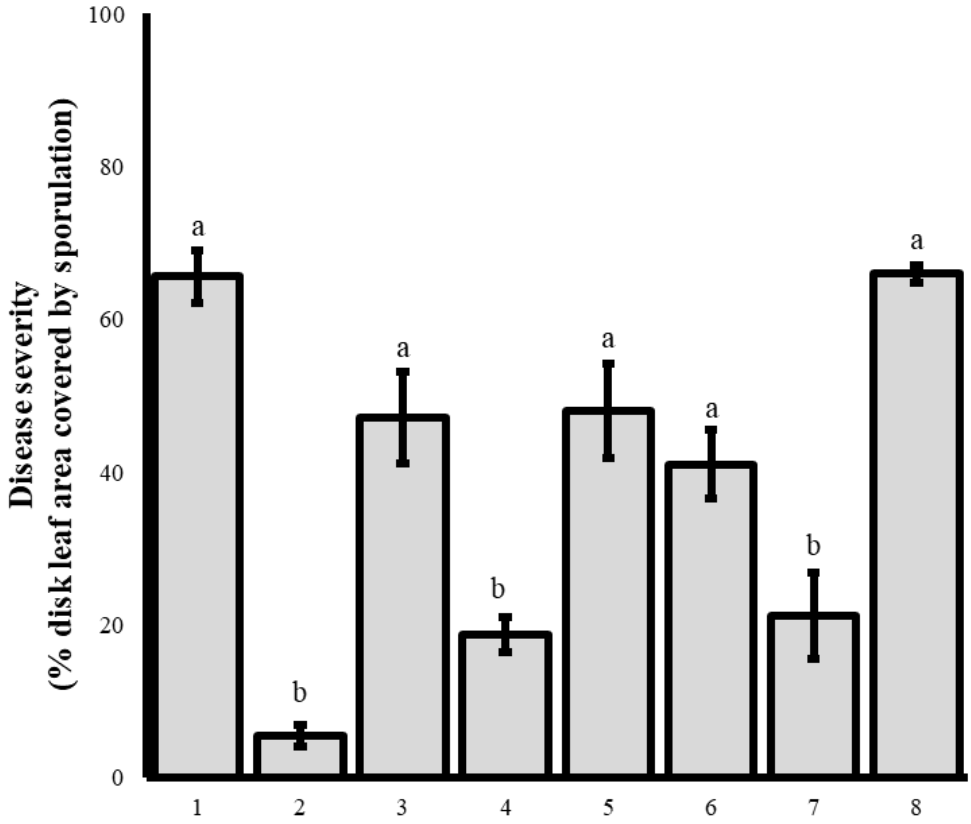

Figure 14 Efficacy of secondary metabolites extracted from *Glechoma hederacea* in controlling *Plasmopara viticola* on grapevine leaf discs. The treatments evaluated were: (**1**) grapevine leaf discs treated with DMSO solution (0.5% *v/v*); (**2**) grapevine leaf discs treated with copper hydroxide (2 g L^{-1}); (**3**) grapevine leaf discs treated with apigenin; (**4**) grapevine leaf discs treated with caffeic acid; (**5**) grapevine leaf discs treated with carvacrol; (**6**) grapevine leaf discs treated with cirsimaritin; (**7**) grapevine leaf discs treated with methyl caffeate and (**8**) grapevine leaf discs treated with rosmarinic acid. Secondary metabolites isolated from *G. hederacea* plant tissues and aqueous extracts were evaluated at a final concentration of 500 μg mL^{-1}. Data presented as mean ± standard error of five replicates (Petri dishes) containing five grapevine leaf discs each. Columns with different letters are significantly different according to the Tukey's HSD test ($\alpha = 0.05$).

Moderate disease severity reduction by carvacrol partially confirms the results displayed in a recent study on *O. vulgare* essential oils against *P. viticola*, where the anti-oomycete activity was attributed to a cell-membrane-damaging effect after interaction with membrane sterols (Rienth *et al.*, 2019). Moreover, among the wide array of biological activities of pharmacological and agronomical interest (Baser, 2008; Abd-El Gawad *et al.*, 2020), carvacrol has been reported for its antifungal activity against different fungal species and for its use for the development of formulations against the oomycete *Pythium insidiosum* (Pina-Vaz *et al.*, 2003; Chami *et al.*, 2004, 2005; Kordali *et al.*,

2008; Lima *et al.*, 2013; Jesus *et al.*, 2015). It is interesting to note that in the case of flavonoids (in this study, cirsimaritin and apigenin), a key role has been suggested for this family of compounds in the resistance of grapevine callus tissue to *P. viticola* (Dai *et al.*, 1995).

While the aqueous extract showed remarkable disease severity reduction (11.4%, Figure 10), among the six isolated metabolites, only caffeic acid and methyl caffeate showed comparable levels of activity (18.8% and 21.2%, respectively, Figure 14). From a structural point of view, both compounds display the same main structure, with the difference of the functionalization of the carboxylic group of caffeic acid.

Nevertheless, rosmarinic acid, which is an ester of two caffeic acid units, resulted in the less active metabolite (66.0%). This accentuated decrease could be explained by a minor action of the aromatic ring in the activity, and consequently the lateral chain would play a direct action. Alternatively, the loss of activity of rosmarinic acid could be explained in terms of hindrance. The analysis of the Clog *P* values theoretically calculated for the six compounds (Table 18) could provide insights in this regard. Specifically, the lipophilicity expressed in this way shows how the value for rosmarinic acid (1.10) is between those of methyl caffeate (1.20) and caffeic acid (0.98). Thus, the different level of activity among caffeic acid, methyl caffeate and rosmarinic acid would not be related to a different level of solubility, and the potential hindrance of rosmarinic acid would be related to other features like molecular size and specificity with the target sites.

Table 18 Lipophilicity values of the six compounds isolated calculated by the Clog *P* algorithm

Compound	**Carvacrol**	**Cirsimaritin**	**Apigenin**	**Methyl Caffeate**	**Caffeic Acid**	**Rosmarinic Acid**
Clog *P*	3.35	2.86	2.91	1.20	0.98	1.10

It is interesting to note that two flavonoids were evaluated in this study (cirsimaritin and apigenin), obtaining similar disease severity (41.0% vs. 47.2%). The slightly improved activity for cirsimaritin might be correlated with the methoxy groups contained in its structure. Given the close Clog *P* values of these compounds (2.86 vs. 2.91), the methoxy groups would play a direct role in the reactivity of the compound with the target sites.

Another point to highlight regarding the lipophilicity of the six tested metabolites could be concluded by a global view of their Clog *P* values (Table 18). Specifically, a general decrease of activity was observed when the lipophilicity was higher, as carvacrol, cirsimaritin and apigenin showed the highest Clog *P* values (2.86–3.35), apart from rosmarinic acid, as previously discussed.

The higher disease reduction of the aqueous extract compared to that of the most active standalone compounds (such as caffeic acid and methyl caffeate) may be correlated to additive or synergetic action of the different compounds taken into consideration. Indeed, a previous study proved the synergistic effects of caffeic acid in relation to its antifungal activity (Sánchez-Maldonado *et al* ., 2016). The activity evaluation of the organic extracts (Figure 12) and the isolated compounds (Figure 14) can provide additional information. The CH_2Cl_2 extract was the most active organic extract (20.4%), and this activity must be strongly related with the action of methyl caffeate (18.8%). Cirsimaritin or apigenin, with disease severities of 41.0% and 47.2%, respectively, did not seem to negatively affect the activity of the CH_2Cl_2 extract.

The *n*-hexane extract, which should contain the less polar compounds contained in the aqueous extract, also showed moderate activity (38.4%), which slightly improved that of carvacrol (48.0%), the only compound isolated from it. Thus, other potential minor compounds in the *n*-hexane extract could play a key role in the activity. Finally, the EtOAc extract showed poor activity, similarly to

rosmarinic acid. Since caffeic acid showed high activity (18.8%), it could be concluded that this compound is a minor component of the EtOAC extract showing relevant anti-oomycete activity.

Research on the six isolated compounds may be useful to define these potential correlations, which would provide insights into the applicability of *G. hederacea* extracts for the control of *P. viticola* or into the development of new pesticides based on natural products.

5.4 Conclusions

This study explored the potential of *Glechoma hederacea* extracts and isolated compounds active against grapevine downy mildew, *Plasmopara viticola*. The aqueous extract caused significant *P. viticola* growth inhibition, close to that of the positive control. Subsequent isolation of compounds from organic extracts revealed six metabolites: three aromatic compounds (carvacrol, caffeic acid and methyl caffeate), two flavonoids (cirsimaritin and apigenin) and a polyphenolic acid (rosmarinic acid). Among these, caffeic acid and methyl caffeate exhibited the highest activity, while carvacrol, cirsimaritin and apigenin displayed moderate activity. Notably, rosmarinic acid was inactive. The study suggests that the efficacy obtained by the aqueous extract may have resulted from synergistic or additive activity of methyl caffeate and caffeic acid with other extract components. This study highlights the potential of *G. hederacea* extracts to control *P. viticola* and the complexity of interactions among its active compounds, offering prospects for developing natural pesticides.

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CHAPTER 6.

12110 AND 12035: TWO NOVEL CANDIDATES AS POTENTIAL BIOFUNGICIDES TO CONTROL *PLASMOPARA VITICOLA*

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6.1 Extended abstract

The plant pathogenic oomycete *Plasmopara viticola*, the causal agent of grapevine downy mildew, is one of the major concerns in viticulture. In fact, if not properly controlled, it can cause severe damage to the crops, leading to consistent losses in terms of yield and quality. To control this plant pathogen, frequent and repeated fungicide applications in the vineyard are required. Nowadays, disease management relies primarily on synthetic and copper-based fungicides, which have been facing several issues in the last decades. In fact, the occurrence of *P. viticola* populations resistant to synthetic, single-site fungicides and the impact of chemicals on human health and on environment have pushed the scientific interest towards the development of eco-friendly plant protection products.

In this context, biofungicides based on plant extracts may potentially play a relevant role, since several studies highlighted their efficacy in laboratory, greenhouse and open field trials, with promising results. However, few active ingredients have been commercialized for the control of *P. viticola*, at the moment. In the present study, the plant protection activity and the mode of action of two potential plant extract-based biofungicides provided by a commercial partner, namely 12035 and 12110, have been assessed and characterized.

To assess the plant protection activity and determine the optimal application dosages, the candidates have been sprayed on five leaf discs placed in Petri dishes 24h before the inoculum with a suspension containing 2.5×10^5 *P. viticola* sporangia mL^{-1}. The disease severity was assessed days after the inoculum as the percentage of the leaf disc surface covered by *P. viticola* sporulation and Abbott's efficacy was then calculated. 12035 and 12110 showed an excellent plant protection activity, recording an efficacy of 93% and 95% at a dose of 3.75 mL $L^{-1}$and 4.4 mL L^{-1} (*v*/*v*), respectively, with performances comparable to the chemical reference copper hydroxide (Champ 20DF, 2 g L^{-1}).

Afterwards, the mode of action of the candidates has been investigated. Firstly, a suspension containing 2.5×10^5 *P. viticola* sporangia/ml has been exposed to the pure 12035 and 12110 active ingredients and then applied on leaf discs, in order to determine the direct toxic activity of the candidates on sporangia. Six days after the inoculum, 12035 was able to inhibit completely *P. viticola* sporulation, while 12110 reduced it by 77% compared to the untreated control.

Moreover, these findings have been verified via staining and fluorescence microscopy. In particular, leaf discs presenting evident sporulation, have been treated with 12035 and 12110 pure active ingredients and then stained with 5'6'-carboxyfluoresceindiacetate (CFDA) and propidium iodide as proposed by Sergeeva *et al*. (2002), with slight modifications. CFDA binds to viable sporangia, while propidium iodide to dead sporangia, resulting respectively in light green and red

fluorescence when exposed to fluorescent light. At least 500 sporangia per treatment were observed. 12110 reduced sporangia viability to 25 %, while 12035 had a lethal effect on 99 % of the sporangia, confirming the previous results.

To determine the induction of callose production, leaf discs were treated with the active ingredients and after 24 h inoculated with a *P. viticola* sporangial suspension, at a concentration of 2.5×10^5 sporangia mL^{-1}. Aniline blue staining was performed at 0, 1, 5 and 7 days after the inoculum according to Palmieri *et al*. (2012) and leaf discs were observed at the microscope under fluorescent light. Callose production was recognized as turquoise and intense fluorescence on the stomata guard cells, while *P. viticola* structures as bright blue fluorescence. No callose production was observed on untreated leaf discs. The application of 12110 induced a diffuse callose production on the guard cells, already 1 day after the inoculum, with a strongly reduced development of *P. viticola* structures. On the other hand, callose was not observed on 12035-treated leaf discs.

Moreover, the production of reactive oxygen species (ROS) was assessed by 3'3'-diaminobenzidine (DAB) staining as described by Trouvelot *et al*. (2008) and Khiook *et al*. (2013), with minor modifications. 48 hours after the treatment with the active ingredients, leaf discs were incubated for 5 hours in 1 mg mL^{-1} DAB staining solution in the dark. H_2O_2 production was then observed at the microscope as a dark brown precipitate on the leaf tissues. No H_2O_2 production was observed on untreated leaf discs. 12035 showed a diffused, dotted H_2O_2 induction, in particular surrounding the stomata and on the guard cells, while 12110 caused a slightly moderate and localized induction.

Finally, both candidates have been tested on greenhouse cultivated *Vitis vinifera* cv. 'Pinot noir' plants, presenting approximately 25-35 leaves each. Products have been applied at a rate of 3.75 mL L^{-1} and 4.4 mL L^{-1} (*v*/*v*) 24 h before the inoculum with a 2.5×10^5 *P. viticola* sporangia mL^{-1}

suspension. Disease severity and plant protection efficacy were assessed as described above 6 days after the inoculum. Both candidates provided excellent plant protection efficacy, since 12035 reduced *P. viticola* symptoms by 91%, while 12110 by 81%, statistically comparable to the chemical reference copper hydroxide.

This study highlights the promising potential of the proposed candidates as biofungicides to control *P. viticola*, not only because of their high plant protection efficacy, but for their multiple level mode of action as well, by a direct toxicity against *P. viticola* sporangia and by inducing plant defenses. Further characterization may lead in future to the proposal on the market of the candidates, as valid alternatives in eco-friendly disease management strategies.

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CHAPTER 7.

CHARACTERIZATION OF THE BIOCONTROL ACTIVITY OF *LYSOBACTER CAPSICI* AZ78 AGAINST *PLASMOPARA VITICOLA*

Abstract

The grapevine downy mildew, caused by *Plasmopara viticola*, poses a significant threat to viticulture, necessitating the development of eco-friendly alternatives to traditional fungicides. This study investigates the biocontrol potential of *Lysobacter capsici* AZ78, focusing on its direct toxic activity against P. viticola sporangia and its ability to prime plant defenses. The results demonstrate that *L. capsici* AZ78 achieves a plant protection efficacy exceeding 90% under greenhouse conditions, even following simulated rain, comparable to copper-based fungicides. AZ78 exhibits direct toxicity against *P. viticola*, significantly reducing sporangia viability and induces key plant defense responses, including callose deposition and reactive oxygen species (ROS) production.

Two secondary metabolites were isolated and identified, namely dihydromaltophylin (also known as HSAF) and maltophylin, being responsible for complete growth inhibition at 2.5 mg L^{-1}. Moreover, this was the first report of the role of these metabolites in the induction of plant defenses, such callose deposition. These findings highlight the multi-level mode of action of AZ78, encompassing both direct antimicrobial activity and the induction of plant defense mechanisms. The combination of AZ78 with copper hydroxide further enhances its efficacy, suggesting its potential for integration into sustainable viticulture practices.

7.1 Introduction

The grapevine downy mildew, *Plasmopara viticola*, is one of the most significant threats to viticulture worldwide, causing severe yield losses if not adequately controlled. Current management strategies heavily rely on single site fungicides and copper-based compounds. However, the long-term use of these chemicals raises environmental concerns and contributes to copper accumulation in soils, necessitating the development of sustainable alternatives.

Biological control agents (BCAs) are gaining attention as eco-friendly partners in integrated pest management, with promising results. Among these, the bacterial genus *Lysobacter* is recognized for its biocontrol potential, with several species demonstrating efficacy against soil-borne plant pathogenic oomycetes. For example, strains of *Lysobacter* have shown promising activity against pathogens such as *Phytophthora capsici* and *Pythium ultimum* and against *Fusarium oxysporum*, which causes tomato root rot (Puopolo *et al.*, 2010; Puopolo *et al.*, 2014a).

Within this genus, *Lysobacter capsici* AZ78 has emerged as a noteworthy candidate for biopesticide development due to its broad-spectrum antimicrobial activity. This strain has exhibited significant effectiveness against Gram-positive bacteria, soil-borne pathogens, and plant pathogenic oomycetes (Puopolo *et al.*, 2014a; Puopolo *et al.*, 2016). Genome analysis of *L. capsici* AZ78 has revealed genes encoding antibiotics, lytic enzymes, and siderophores, underscoring its potential as a biocontrol agent (Puopolo *et al.*, 2016). Additionally, the strain demonstrates remarkable resilience to environmental stresses such as UV light, mild heat shock, freezing, and starvation (Puopolo *et al.*, 2016).

Secondary metabolites produced by *L. capsici* AZ78 contribute to its biocontrol efficacy. Studies have identified metabolites such as polycyclic tetramate lactams, including Heat-Stable

Antifungal Factor (HSAF) and cyclic depsipeptide WAP-8294A2, along with other inhibitory compounds like 2,5-diketopiperazines, cyclic lipodepsipeptides, macrolides, and volatile organic compounds (VOCs) (Puopolo *et al.*, 2014b; Vlassi *et al.*, 2020; Bejarano *et al.*, 2021; Yue *et al.*, 2021; Liu *et al.*, 2022). These metabolites exhibit inhibitory activity against several plant pathogens, including *P. ultimum*, *Rhizoctonia solani*, and *Sclerotinia minor* (Vlassi *et al.*, 2020; Brescia *et al.*, 2021).

Recent advancements have highlighted the compatibility of *L. capsici* AZ78 with copper-based fungicides. This strain possesses genes for copper oxidase (*copA*) and copper-exporting P-type ATPases (*ctpA*), conferring copper tolerance. A patented application of *L. capsici* AZ78 involves its combined use with low copper concentrations to enhance pest management efficacy while mitigating copper's environmental impact (Puopolo *et al.*, 2014b; Segarra *et al.*, 2016). Field studies have demonstrated the effectiveness of a liquid formulation of *L. capsici* AZ78 in protecting grapevines against *P. viticola*, showing comparable results to traditional copper-based fungicides. Additionally, this formulation improved the strain's persistence on grapevine leaves, enhancing its protective activity under field conditions (Segarra *et al.*, 2016).

Importantly, toxicological assessments have confirmed the safety of *L. capsici* AZ78 for non-target organisms, including beneficial arthropods, earthworms, crustaceans such as *Daphnia magna*, and algae like *Selenastrum capricornutum*. Furthermore, its use does not interfere with yeast populations, ensuring wine quality remains unaffected (Markellou *et al.*, 2022).

Given its promising characteristics, *L. capsici* AZ78 represents a potential sustainable alternative for managing *P. viticola*. This study investigates its biocontrol efficacy, emphasizing its role in integrated pest management strategies aimed at reducing chemical inputs in viticulture.

In the present chapter, the biocontrol activity against *P. viticola* of *L. capsici* AZ78 will be further investigated. In particular, not only the plant protection activity but especially the characterization of the mode of action will constitute the central focus of this study, from the direct activity towards *P. viticola* sporangia as well as the induction of priming effect of plant´s defense response. The acquired knowledge will be crucial for understanding the relationship between BCA, plant and pathogen, providing fundamental information towards the development of a sustainable disease management strategy in which *L. capsici* AZ78 could play a relevant role for viticulture.

7.2 Materials and Methods

7.2.1 Lysobacter capsici *AZ78 cell suspension*

Lysobacter capsici AZ78 (AZ78), was stored in 40% glycerol stocks at -80 °C. For routine culture, it was grown on Nutrient Agar (NA) in 90 mm Petri dishes for 48 h at 27 °C. To prepare cell suspensions, the Petri dishes were flooded with 5 mL of sterile distilled water and the cells were gently scraped off using sterile L-shaped loops, followed by resuspension in sterile distilled water. The optical density at 600 nm was adjusted to 0.1, corresponding to approximately 1×10^8 cells mL^{-1} using spectrophotometer (UV-2450, Shimadzu, Kyoto, Japan) (Puopolo *et al.*, 2014a).

7.2.2 Plasmopara viticola *spore suspension*

Plasmopara viticola was isolated from an untreated vineyard in San Michele all'Adige (Italy) in 2022 and maintained on grapevine plants (*V. vinifera* cv. Pinot Noir, grafted onto Kober 5BB.) by subsequent weekly inoculations using a hand sprayer according to Puopolo *et al*. (2014a). Briefly, grapevine plants showing oil spot symptoms were kept overnight in the dark at 25°C and 100% RH to induce the sporulation of *P. viticola*. The sporangia of *P. viticola* were collected by washing the abaxial leaf surface of grapevine leaves covered with sporulating lesions with cold (4–5 °C) distilled water. The final concentration of the sporangia suspension was adjusted to 2.5×10^5 sporangia mL^{-1} by counting with a hemocytometer under a light microscope.

7.2.3 Plant protection efficacy and rain fastness of L capsici *AZ78.*

An AZ78 cell suspension in a liquid formulation (Segarra *et al.,* 2016) was applied until runoff on healthy greenhouse cultivated *Vitis vinifera* cv. 'Pinot noir' plants (grafted onto Kober 5BB), presenting at least 15 leaves. AZ78 was applied as a stand-alone and in mixture with copper hydroxide

(Champ 20 DF) at 1 and 0.5 g L^{-1}. Copper hydroxide applied as stand-alone at 2 g L^{-1}, 1 g L^{-1} and 0.5 g L^{-1} was used as positive control.

After complete drying of the spore suspension, or at least 4 hours after the application, plants were moved into a rain simulator and exposed to a simulated rain equivalent to 10mm. 24 hours after the application, plants were inoculated with a *P. viticola* sporangia suspension (2.5×10^5 sporangia mL^{-1}) using an air sprayer and incubated overnight in the dark at 25°C and 100% RH. After 6 days plants were incubated again in the dark at 25°C and 100% RH to induce *P. viticola* sporulation. The diseases severity, expressed as percentage of leaf surface covered by sporulation, was visually assessed for every leaf and the plant protection efficacy was calculated as follows:

$$Plant\ protection\ efficacy = \frac{\text{Severity Untreated Control} - \text{Severity sample}}{\text{Severity Untreated Control}} \times 100$$

Five plants per treatment were used, the experiment has been repeated.

7.2.4 Characterization of L. capsici *AZ78 mode of action*

7.2.4.1 Direct toxic activity against *P. viticola* sporangia

Leaf discs were obtained from sporulated leaves and treated on the abaxial side with AZ78 cell suspension (1×10^8 CFU mL^{-1}), copper hydroxide (Champ 20 DF, 2 g L^{-1}) while distilled water was used as untreated control. After leaf disc drying, 30 µL of propidium iodide (0.1 mg mL^{-1}) and 30 µL of 5,6-carboxyfluorescein diacetate (0.5 mg mL^{-1}, prepared in 0,01% DMSO) were poured on the leaf disc. After 5 minutes of incubation, sporangia have been gently scraped with a loop to suspend the sporangia (adapted from Sergeeva *et al.*, 2002). The suspension has been collected and observed with a Leica LMD7000 microscope (Germany) under fluorescent light using a LMD filter (BP 550-

590 nm, 590 nm dichroic mirror and BP 600-680nm emission). Viable sporangia emitted intense green, fluorescent light, while devitalized sporangia dark red light.

Five leaf discs per treatment were examined, at least 500 sporangia were observed for each leaf disc. The experiment has been repeated.

7.2.4.2 Induction of callose deposition

In order to observe *P. viticola* structures and callose deposition in plant cells, leaf discs were collected before *P. viticola* inoculation and at 24 hours post inoculation and then stained with aniline blue (Sigma-Aldrich) according to Díez-Navajas *et al.,* (2007). In detail, discs were incubated for 15 minutes in 1M KOH at 95°C, then for 15 minutes in 1M KOH at room temperature and washed three times in distilled water for 15 minutes. Finally, the discs were stained for 15 minutes in 0.05% aniline blue in 0.067M K_2HPO_4 at pH 8. Microscope observations were carried out with a Leica LMD7000 microscope (Germany) using an A4 excitation filter (BP 320–400nm excitation, 400nm dichroic mirror, and BP 470nm emission).

Callose deposits were recognized by a turquoise fluorescence, while encysted zoospores and *P. viticola* structures by an intense, bright-blue fluorescence.

7.2.4.3 Induction of Reactive Oxygen Species (ROS) production

Leaf discs were treated with a bacterial suspension containing AZ78 cells (1×10^8 CFU mL^{-1}). After 24 h, leaf discs were inoculated with *P. viticola* suspension (2.5×10^5 sporangia mL^{-1}). DAB staining (3,3'-diaminobenzidine) was performed 48 h after AZ78 application. Briefly, leaf discs were incubated for 5 h in 1 mg mL^{-1} DAB staining solution in HCl H_2O at 3.8 pH as described by Trouvelot *et al*. (2008) and Khiook *et al*. (2013), with minor modifications.

Afterwards, leaf discs were incubated for 15 minutes at 90°C in a destaining solution containing ethanol, acetic acid and glycerol (3:1:1) to remove chlorophylls, carotenoids and other plant pigments. ROS were visualized under bright field microscope as dark reddish-brown spots, caused by the absorption and polymerization of DAB. Five biological replicates per treatment were analyzed.

7.2.4.4 Extraction, identification and characterization of *L. capsici* AZ78 secondary metabolites

AZ78 cultures were incubated for 72 h at 27 °C to prepare a cell suspension in a sterile 0.85% (w/v) NaCl solution, reaching a concentration of 1×10^8 CFU mL^{-1}. 50 µL of this suspension was evenly distributed on the surface of 90 mm Petri dishes containing 15 mL of solid LBA 1:10 medium using a sterile cell spreader and incubated for 72 h at 27°C. Subsequently, the medium was cut into pieces with a sterile spatula and placed into sterile flasks with 30 mL of an extraction solution consisting of methanol (MeOH), acetonitrile (ACN), and water (1.5:1.5:1) plus 0.1% formic acid (*v*/*v*). The flasks were stirred for 1 h, and the extracts were then transferred to sterile 15 mL centrifuge tubes. Cell-free extracts were obtained by centrifugation at 35,000×g for 30 min. The samples were frozen at -80 °C and lyophilized using a freeze dryer (FreeZone 6 Plus, Labconco, Kansas City, MO, USA) for 94 hours. The dried samples were reconstituted in 3 mL of extraction solution. 500 mL of the

concentrated extracts was centrifuged at 30,000×g for 20 min at -4 °C, and the supernatant was filtered through a Millex-GV PVDF syringe filter (0.22 µm, Merck, Darmstadt, Germany) and transferred to 2 mL HPLC vials, which were stored at −80 °C until analysis (Brescia *et al.*, 2021).

The *L. capsici* AZ78 cell-free extract was concentrated under vacuum and ammonium sulfate $(NH_4)_2SO_4$ was added (final concentration of 0.5 mg mL^{-1}). The solution was stirred for 1 hour, left overnight at 4 °C, and then centrifuged at 3600 rpm for 50 min. The supernatant was discarded, and the pellet was washed with methanol (3 mL x 2) and left 1 h at room temperature. The sample was centrifuged at 10000 rpm for 30 min and the supernatants applied to a C18 SPE. The SPE column was previously conditioned with 6 mL of acetonitrile (CH_3CN) and equilibrated with 12 mL distilled water. The purification yielded five fractions (F1-5) obtained by stepwise elution with H_2O (6 mL), CH_3CN/H_2O 3:7, *v*/*v* (12 mL), CH_3CN/H_2O 1:1, *v*/*v* (12 mL), CH_3CN/H_2O 7:3, *v*/*v* (12 mL), acetonitrile (CH_3CN) (12 mL), respectively. Successively, the organic solvents were removed under reduced pressure and the eventual residual water phases were lyophilized.

The residue of F3 (8.1 mg), showing high anti-oomycete activity against *P. ultimum*, was analyzed by ^1H NMR (Figure 15), and further purified by Thin Layer Chromatography (TLC) using as eluent the mixture CH_2Cl_2:MeOH/conc.NH_4OH=60:36:4, *v*/*v*/*v*. The main fractions obtained, F3.1 C (1.7 mg, Rf 0.4) and F3.2 E (1.0 mg, Rf 0.6), were further purified by HPLC using multiple injections on a reverse phase column. The mobile phase used to elute the samples was H_2O, 0.1 % formic acid:CH_3CN, 0.1 % formic acid at a flow rate of 0.5 mL min^{-1}. The analysis was performed using a gradient starting from 60% CH_3CN, 0.1 % formic acid increased linearly to 80% in 20 min, and finally re-balanced to the initial rate for 10 min. Detection was performed at 220 nm and samples were injected using a 20-µL loop and monitored for 35 min.

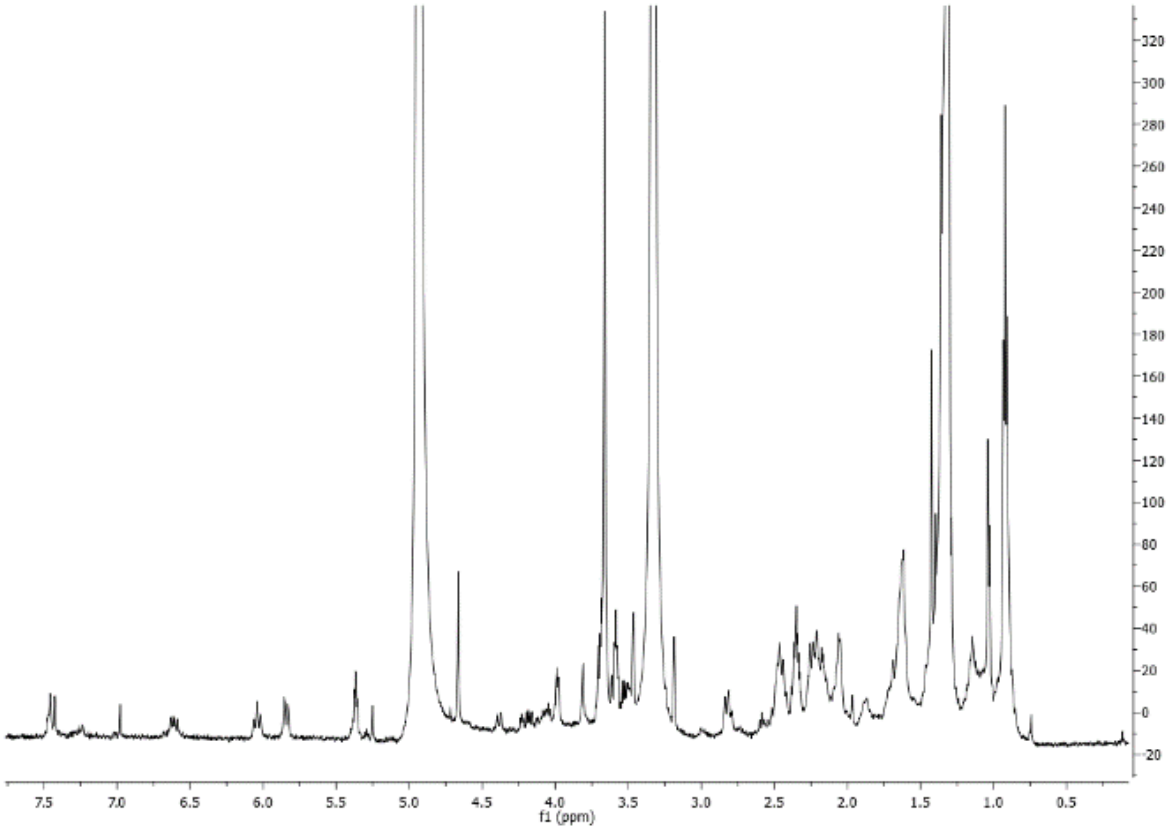

Figure 15 ^{1}H NMR spectrum of F3 recorded in CD_3OD at 400 Mhz.

7.2.5 Statistical analysis

The results are shown as average ± standard error, the number of replicates was five, unless differently specified. Statistical differences were assigned according to one-way analysis of variance (ANOVA) and post hoc Tukey`s test, assuming $p < 0.05$ as level of significance. The normality of the data distribution was assessed using the Shapiro-Wilk test, and the homogeneity of variances among groups was evaluated using Levene's test. Statistical analysis has been conducted with the statistical software PAST.

7.3 Results

7.3.1 Plant protection activity and rain-fastness of L. capsici *AZ78*

In absence of the simulated rain (Figure 16), AZ78 guaranteed a plant protection efficacy of 90.5%, comparable to copper hydroxide at 2 g L^{-1}, 1 g L^{-1} and 0.5 g L^{-1} (93.5%, 87.8% and 91.9%, respectively) and AZ78 in combination with 0.5 g L^{-1} of copper hydroxide (95.9%). However, the plant protection efficacy reached by AZ78 in combination with 1g L^{-1} of copper hydroxide was significantly higher than copper hydroxide 1 g L^{-1} and AZ78 both as stand alone, reducing *P. viticola* sporulation by 97.3%.

Noteworthy, no statistically significant differences were observed after the exposure to the simulated rain with plant protection efficacy values ranging from 84.4% in 0.5 g L^{-1} copper hydroxide treated plants to 94.2% in plants treated with AZ78 in combination with 0.5 g L^{-1} of copper hydroxide.

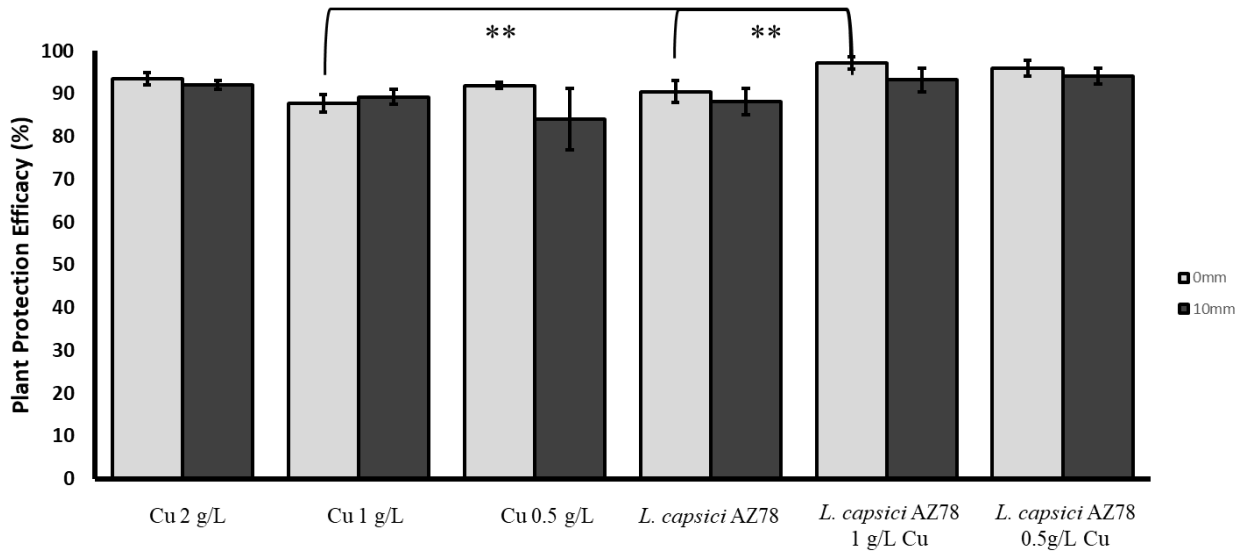

Figure 16 Plant protection activity against *P. viticola* on plants treated with *L. capsici* AZ78, as stand-alone or in combination with copper hydroxide, without (light grey bars) and with an equivalent of 10mm simulated rain (dark grey bars). Columns represent mean ± standard error of five replicates (plants). Statistical differences were determined using t-test (**: $p<0.01$, n=5)

7.3.2 Direct toxicity against P. viticola *sporangia*

Fluorescent microscope observation highlighted an extreme reduction in viability of *Plasmopara viticola* sporangia exposed to AZ78 (Figure 17). The application of AZ78 cell suspension caused a reduction by 91% of viable *P. viticola* sporangia (7% viable sporangia) compared to the untreated control (77% of viable sporangia), significantly higher than copper hydroxide at 2 g L^{-1}, responsible for a reduction by 45% of viable sporangia compared to the untreated control (42% viable sporangia).

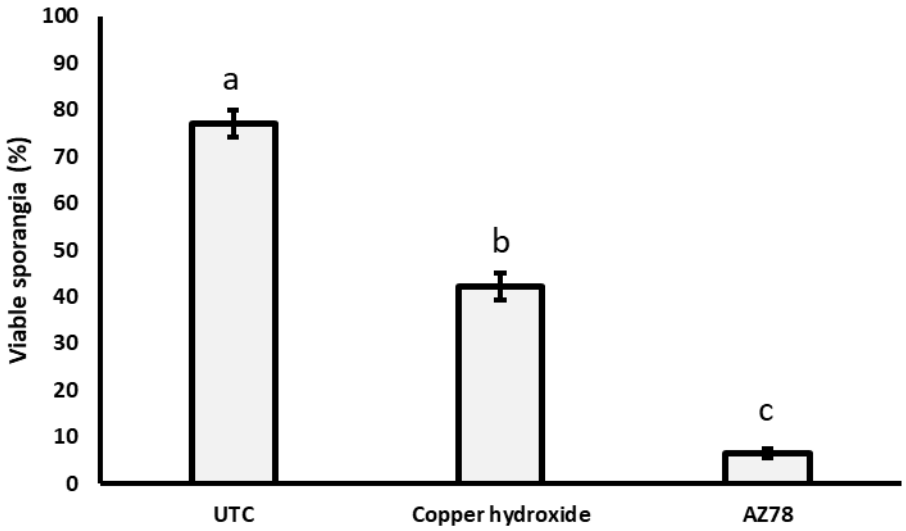

Figure 17 Direct toxicity of *L. capsici* AZ78 on *Plasmopara viticola* sporangia, compared to untreated control (UTC) and copper hydroxide (2 g L^{-1}). Columns represent mean ± standard error of five replicates (leaf discs) of at least 100 sporangia each. Columns with different letters are significantly different according to Tukey's HSD test **(***p*< 0.05).

7.3.3 Induction of callose deposition on AZ78-treated leaf discs

The aniline blue staining on grapevine leaf discs under fluorescence microscope revealed that preventive application of AZ78 cells induces systemic plant defense response by stimulating callose formation around stomatal guard cells, resulting in stomata closure and preventing pathogen entry. This supports earlier findings showing the high efficacy of AZ78 treatments against *P. viticola*.

Untreated and uninoculated leaf discs showed no callose or pathogen presence (Figure 18). On the other hand, untreated but inoculated discs highlighted the presence of zoospore without callose formation after 24h. The positive control BION 50 WG (acibenzolar-S-methyl 50%, Syngenta; 50 mg/L), induced callose deposition, consistent with its bio stimulant activity. Similarly, AZ78-treated leaf tissues were devoid of pathogen mycelia, with pronounced callose deposition around stomata when applied before pathogen inoculation.

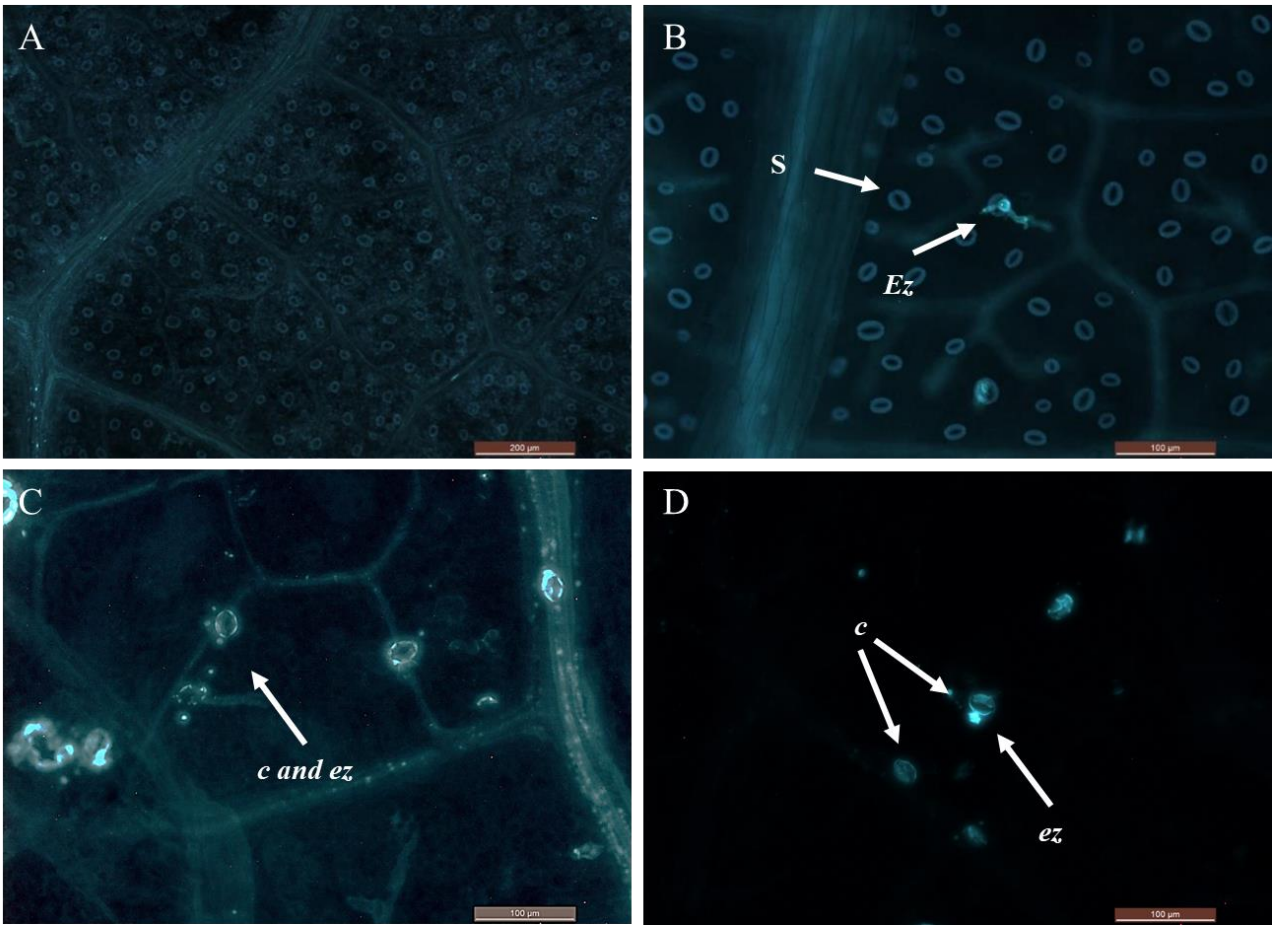

Figure 18 Aniline blue staining on grapevine leaf discs before *P. viticola* infection (**A**), 24 hours after infection on an untreated leaf disc (**B**), with visible stomata (*s*) and encysted, germinated zoospore (*ez*). Callose deposition (*c*) on leaf discs treated with BION *WG* (acibenzolar-S-methyl 50%, Syngenta; 50 mg/L, **C**) and *L. capsici* AZ78 bacterial suspension (**D**) 24 hours after *P. viticola* infection.

7.3.4 Induction of Reactive Oxygen Species production

DAB staining allows to visualize ROS production by the formation of a dark brown precipitate, as result of the oxidation of DAB. By bright field microscopy, the untreated and uninoculated leaf discs exhibited no ROS accumulation 24 hours post inoculation (Figure 19). In contrast, the inoculated leaf discs displayed a minimal level of ROS accumulation. Laminarin-treated leaf discs demonstrated a moderate increase in ROS accumulation while leaf discs treated with AZ78 cells exhibited a significantly higher level of ROS accumulation compared to the untreated control (), when applied 24 hours before inoculation, in particular in the proximity of the stomata guard cells.

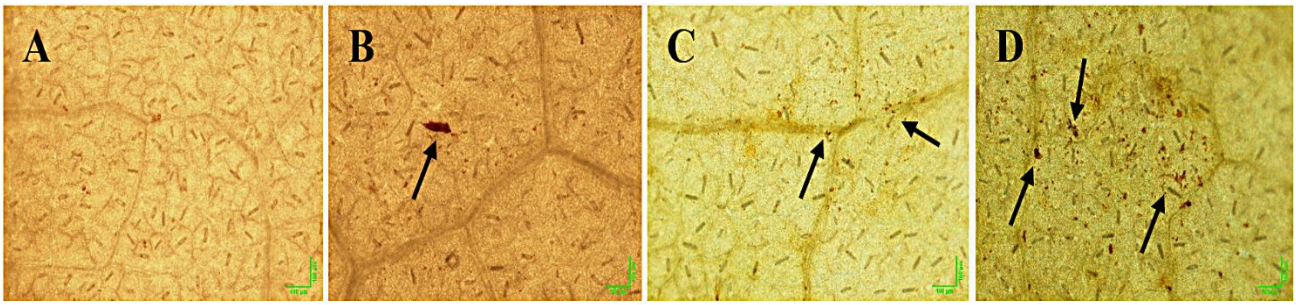

Figure 19 ROS accumulation in grapevine leaf discs inoculated with *Plasmopara viticola*. Untreated, mock inoculated (**A**), untreated, inoculated with *P. viticola* (**B**), leaf discs treated with laminarin (**C**), leaf discs treated with *L. capsici* AZ78 cell suspension 24 hours before *P. viticola* inoculation (**D**). Black arrows indicate ROS accumulation.

7.3.5 Isolation and characterization of secondary metabolites with anti-oomycete activity

Two pure compounds were eluted at retention time (rt) of 5.73 and 7.79 min by HPLC, pooled, concentrated under reduced pressure, lyophilized, and identified as dihydromaltophylin (DMP) (0.53 mg, rt 5.73) and maltophylin (MP) (0.83 mg, rt 7.79 min), respectively (Figure 20).

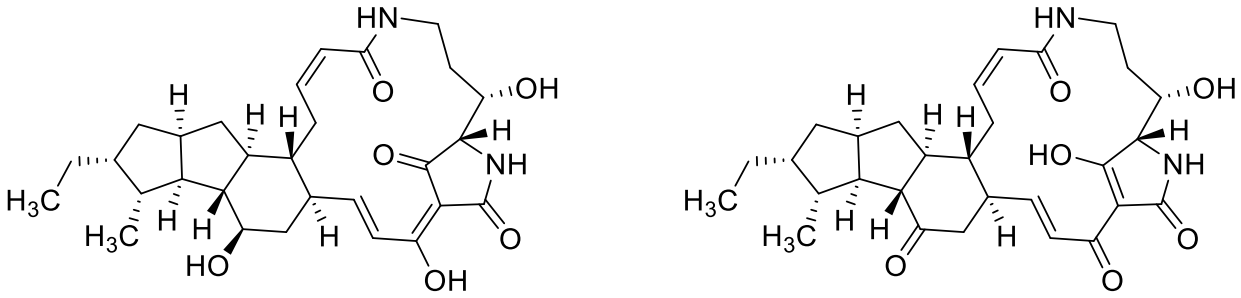

Figure 20 Chemical structures of the compounds isolated from *L. capsici* AZ78 with acute anti-oomycete activity.

Their ESI-MS spectra showed the dimer protonated adduct $[2M+H]^+$ and the protonated adduct $[M+H]^+$ ions at *m*/*z* 1025 and 513 for dihydromaltophylin (Figure 21) and at *m*/*z* 1021 and 511 for maltophylin (Figure 22). The isolated compounds correspond to polycyclic tetramate macrolactams, a class of metabolites commonly produced by bacteria (Blodgett *et al.*, 2010) characterized by a tetramic acid moiety linked to a macrocyclic lactam.

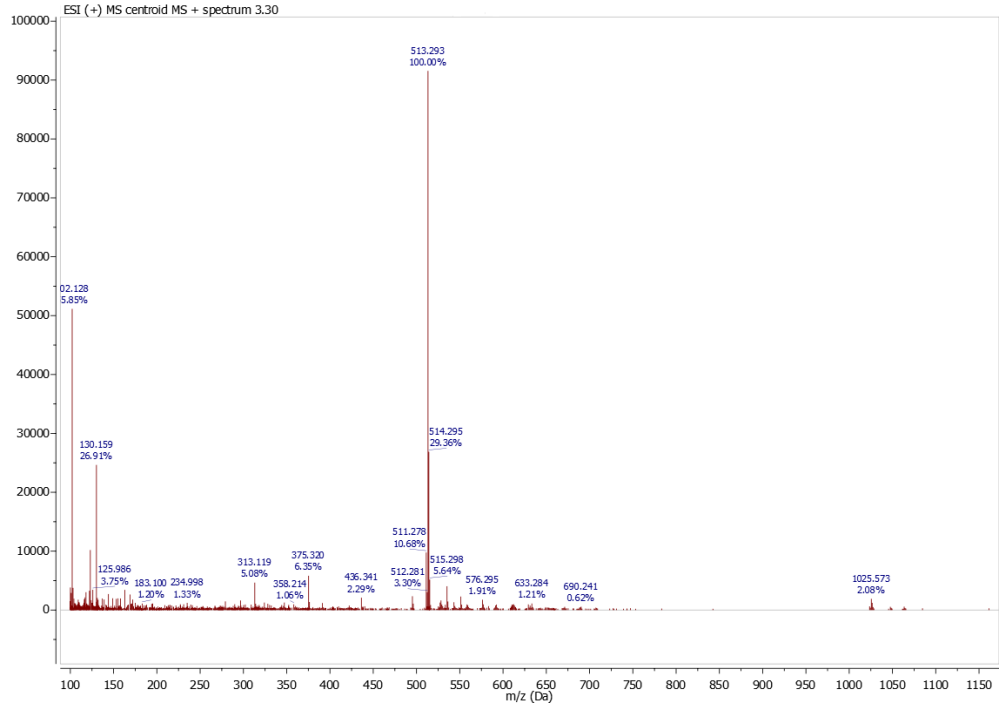

Figure 21 ESI MS spectrum of dihydromaltophylin (DMP) recorded in positive mode.

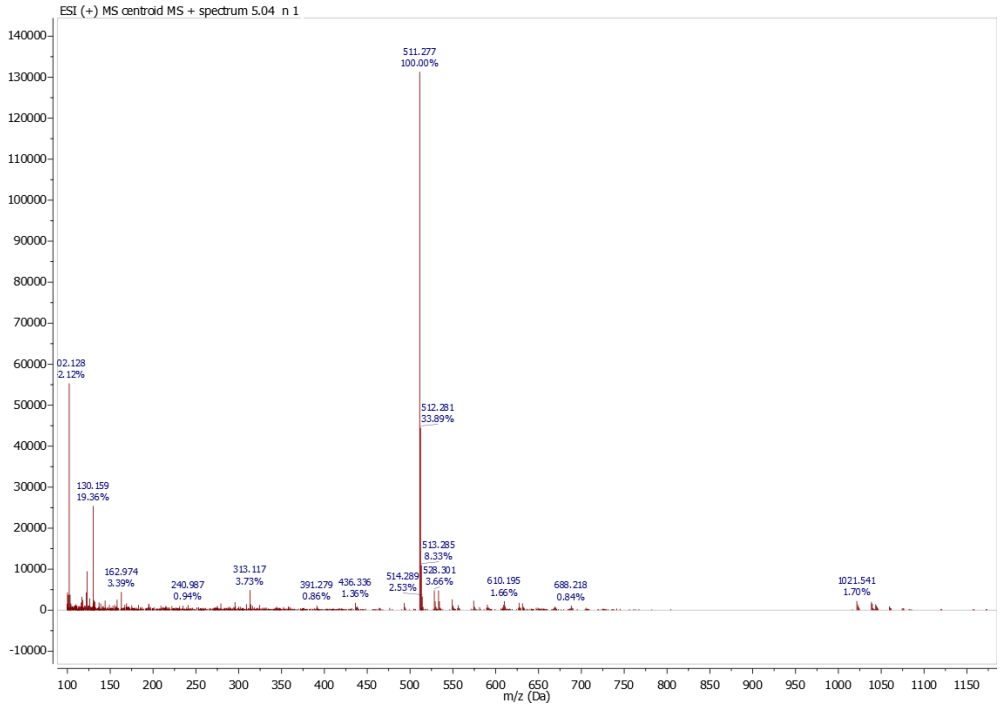

Figure 22 ESI MS spectrum of maltophylin (MP) recorded in positive mode.

Mixtures of DMP and MP at concentrations of 500 mg L^{-1}, 50 mg L^{-1}, 5 mg L^{-1}, 2.5 mg L^{-1}, and 0.5 mg L^{-1} were tested for the control of *P. viticola* on grapevine leaf discs according to the procedure reported above. DMSO at concentrations of 0.1% and 0.05% as well as distilled water were used as the untreated controls, whereas Coprantol Hi Bio (Copper hydroxide 20%, Syngenta; 2 g L^{-1}) and Zoxamide (Sigma, 81.5 mg L^{-1}) were used as positive controls.

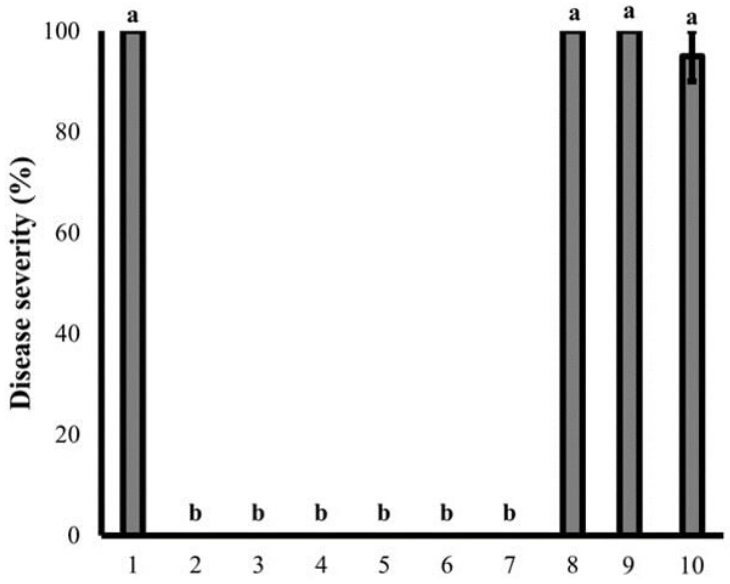

Figure 23 Disease severity on grapevine leaf discs treated with dihydromaltophylin and maltophylin mixture against Plasmopara viticola. (**1**) untreated; (**2**) Coprantol Hi Bio (Copper hydroxide 20%, Syngenta; 2 g L^{-1}); and (**3**) Zoxamide (Sigma, 81.5 mg L^{-1}). The dihydromaltophylin and maltophylin mixture were used at the following concentrations (**4**) 500 mg L^{-1}; (**5**) 50 mg L^{-1}; (**6**) 5 mg L^{-1}; (**7**) 2.5 mg L^{-1}; (**8**) 0.5 mg L^{-1}; (**9**) DMSO 0.1%; and (**10**) DMSO 0.05%. Data are shown as the mean ± SE (n = 5), and different letters indicate significant differences according to Tukey's HSD test ($p < 0.05$).

The mixture of DMP and MP, at all concentrations except 0.5 mg L^{-1}, exhibited 0% disease severity, hence complete growth inhibition, with the DMP and MP mixture as effective as the copper hydroxide and zoxamide in controlling *P. viticola* (Figure 23). At low concentrations (0.1% and 0.05%), DMSO did not cause any phytotoxic symptoms on leaf tissues and disease severity remained comparable to the untreated control treated with only the plant pathogen, which exhibited 100% disease severity, thus highlighting that these lower concentrations of DMSO were ineffective in disease control.

Proven the anti-oomycete activity of DMP and MP, the mode of action of these secondary metabolites in controlling *P. viticola* has been further investigated, similarly as shown above.

Fluorescent microscopy revealed the toxicity of DMP and MP towards *P. viticola* sporangia (Figure 24). In fact, the percentage of devitalized sporangia was comparable to copper hydroxide in

DMP/MP mixture at 5 and 50 mg L^{-1}, while it was significantly higher at 500 mg L^{-1}. The toxic activity was correlated with the DMP/MP mixture concentration, until reaching a comparable toxicity to DMSO 0.1% and 0.05% at 0.5 mg L^{-1}. Interestingly, DMSO itself had a significant reduction in sporangia viability compared to the untreated control. However, this moderate toxicity was not sufficient in order to play a role in controlling *P. viticola*, as shown in the previous leaf disc assay.

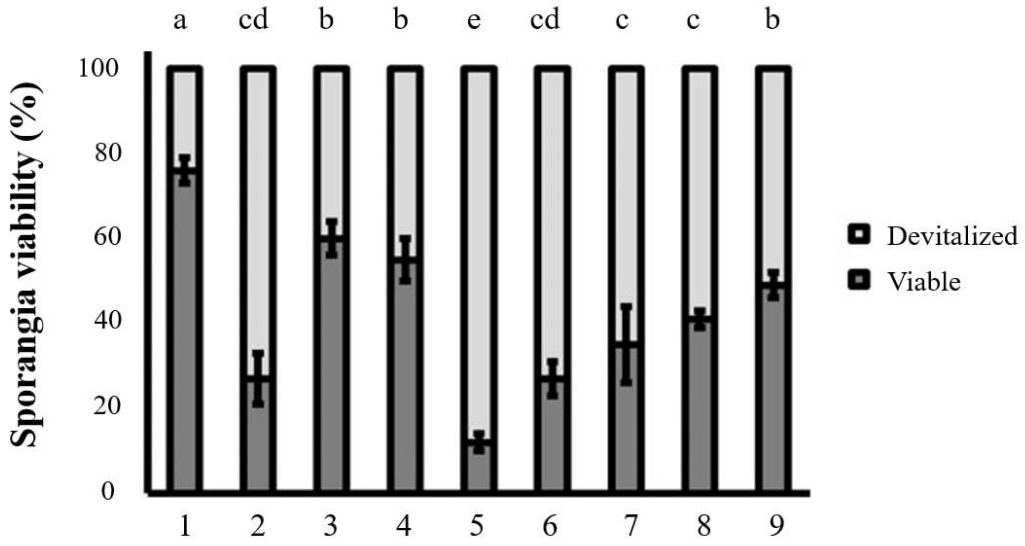

Figure 24 Percentage of viable and devitalized *P. viticola* sporangia following the application of DMP and MP. (**1**) untreated; (**2**) Coprantol Hi Bio (Copper hydroxide 20%, Syngenta; solution 2 g L^{-1}); (**3**) DMSO 0.1%; and (**4**) DMSO 0.05%. The dihydromaltophylin and maltophylin mixture were used at the following concentrations (**5**) 500 mg L^{-1}; (**6**) 50 mg L^{-1}; (**7**) 5 mg L^{-1}; (**8**) 2.5 mg L^{-1}; (**9**) 0.5 mg L^{-1}. Data are presented as mean ± SE (n = 5), different letters indicate significant differences according to Tukey's HSD test ($p < 0.05$).

Moreover, following the same protocol shown before, DMP/MP mixture have been tested for its capacity of inducing plant defenses. By aniline blue staining under fluorescent microscope, callose deposition was evident on DMP/MP treated leaf discs at a concentration of 500, 25 and 2.5 mg L^{-1} (Figure 25B-C-D) 24 hours after *P. viticola* inoculation, while in leaf discs treated with DMSO 10% and DMP/MP mixture at 0.5 mg L^{-1} the production of callose was absent.

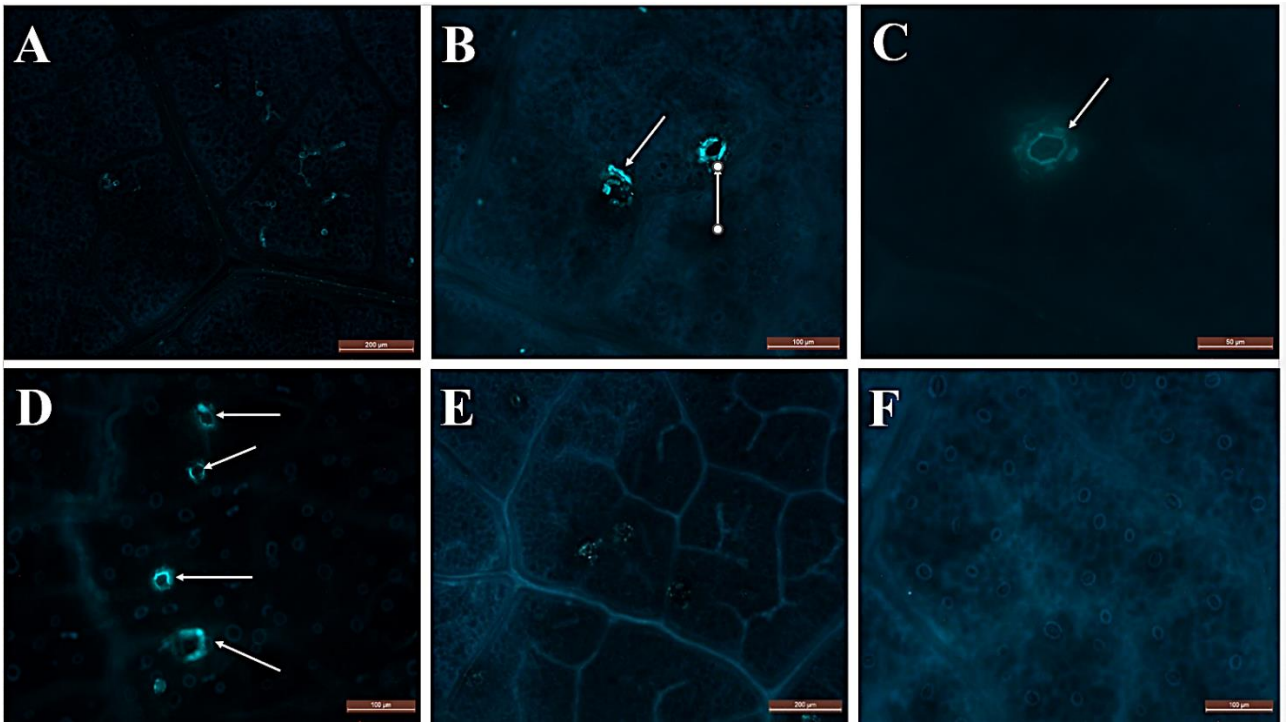

Figure 25 Callose deposition in grapevine leaf discs treated with DMP/MP mixture. **(A)** untreated control; leaf discs treated with DMP/MP at 500 mg L^{-1} **(B),** 25 mg L^{-1} **(C),** 2.5 mg mL^{-1} **(D),** 0.5 mg L^{-1} **(E)** and DMSO 10% **(F)**. White arrows indicate the deposition of callose.

7.4 Discussion

In the past decade, *Lysobacter capsici* AZ78 has been standing out as one of the most promising mBCA for the control of *P. viticola* (see Chapter 4.6 for details). In fact, its excellent disease suppression in laboratory but also open field conditions and the tolerance to copper-based fungicides put AZ78 in the spotlight as partner in integrated pest management strategies.

In this study, the plant protection activity, rain fastness and the mode of action of AZ78 have been investigated, with more than promising outcomes.

A formulated AZ78 cell suspension has been applied on greenhouse cultivated grapevine plants in the presence of a simulated rain, in order to test the rain fastness of the mBCA. While the rain-fastness capacity of copper-based fungicide is well known, especially with limited precipitations,

it is notable the rain fastness offered by AZ78, even with a still elementary liquid formulation. In fact, no statistically significant difference was reported among the treatments after the simulated rain, with plant protection efficacy beyond 80%.

However, during a field trial, the AZ78 population on the phyllosphere decreased of around 100 times after one week from the applications, possibly due to high temperatures, while the use of formulation additives protected AZ78 against environmental factors and improved its persistence on the leave by more than 10 times compared to the pure cell suspension (Segarra *et al.,* 2016), highlighting the crucial role of formulation in a plant protection product.

On the other hand, though, the combination of copper hydroxide and AZ78 provided a significant increase in the control activity against *P. viticola* in absence of the simulated rain, suggesting a synergic effect. Likely, exposing the plants to prolonged simulated rain will further challenge the rain fastness capacity of the different formulations, thus highlighting the synergic activity provided by the combination of AZ78 and copper hydroxide. If confirmed, this will allow the implementation of low copper-input disease management strategies, without compromising the plant protection activity, thanks to genes encoding for copper oxidase (*copA*) and copper exporting PIB-type ATPases (*ctpA*) (Puopolo *et al.,* 2014a; Segarra *et al.,* 2016).

On the other hand, given the excellent plant protection activity of AZ78 at 1×10^{8} CFU mL^{-1}, a reduction in AZ78 cells´ concentration may be evaluated, especially when in combination with copper, in order to facilitate the large-scale production of the mBCA.

The characterization of the AZ78 mode of action offered very promising insights, since the control of *P. viticola* occurs acting directly against the pathogen, but also priming plant defenses.

The results in this study suggest an extremely high toxic activity by AZ78 cells towards *P. viticola* sporangia, already after an incubation of 5 minutes. However, AZ78, such as other *Lysobacter* strains, is well known to possess anti-fungal and anti-oomycete activity. Although direct predation by the bacterial cells has not been reported yet, the release of secondary metabolites with strong inhibitory activity has been repeatedly found in several previous studies (Puopolo *et al.,* 2014b; Puopolo *et al.,* 2016, Brescia *et al.,* 2021).

In support of these hypothesis are the findings regarding the anti-oomycete activity of the secondary metabolites isolated from AZ78 cell suspensions, among which dihydromaltophylin and maltophylin were selected as the most promising ones. These two compounds not only exhibited excellent plant protection activity in leaf disc bioassay at extremely low concentrations (100% growth inhibition at 2.5 mg L^{-1}), but also induced plant defense response such as callose deposition.

The role of DMP (also reported in literature as HSAF) for the antifungal and antimicrobial activity has been already reported multiple times. HSAF was first isolated from *Lysobacter enzymogenes* C3 and characterized by a unique macrocyclic lactam system containing a tetramic acid moiety and a 5,5,6-tricyclic skeleton. The mode of action has been identified as the disruption of sphingolipids biosynthesis in fungi (Yu *et al.,* 2007) and HSAF has been reported in other *Lysobacter* spp. such *as L. enzymogenes* OH11 (Lou *et al.,* 2010), *Lyosbacter* sp. Strain SB-K88 (Islam *et al.,* 2005, Islam 2010). Moreover, HSAF has been linked to the induction of apoptosis in *Candida albicans* through the production of ROS (Ding *et al.,* 2016) and of aberrant deposition of thick cell wall patches to inhibit the growth of *Aspergillus nidulans*, mediated by disrupting the biosynthesis of ceramide (Li *et al.,* 2006).

In the present study we highlighted the strong production of ROS after the application of *L. capsici* AZ78 cell suspension, as a response to the inoculation of *P. viticola*, which may likely be caused by the release of DMP/MP by the bacterial cells.

Moreover, callose deposition plays an important role in the defense against various pathogens, including fungi and oomycete, in particular strengthening cell walls, creating a physical barrier to impede pathogen penetration. (Kortekamp *et al.*, 1997). This mechanism in grapevine allows the stomatal guard cells to thicken until complete stomatal closure (Allègre *et al.*, 2009).

AZ78 was already reported as an inducer of callose deposition as defense mechanism against *P. viticola* (Brescia *et al.*, 2018), as confirmed by this study, while, by the time writing this is the first report of the role of HSAF/DMP and MP in the induction of callose. Hence, we can propose these secondary metabolites as main factors in this kind of defense response as a consequence of *P. viticola* infection.

7.5 Conclusions

The search for effective biocontrol agents in viticulture is currently one of the main topics in research community, given the countless challenges that farmers have to face in the disease management against destructive pathogens such as *Plasmopara viticola*, in order to develop efficient and environmentally friendly IPM strategies, where biocontrol agents or botanicals can line-up besides conventional active ingredients.

In this study we further characterized one of the most promising mBCA for the control of downy mildew of grapevine, *Lysobacter capsici* AZ78, giving deeper insights into its mode of action and effects towards the plant. In fact, we demonstrated that not only this Gram-negative bacterium and its secondary metabolites (dihydromaltophylin and maltophylin), possess an extremely toxic activity towards *P. viticola* sporangia but they play a crucial role in inducing plat defenses, such as the deposition of callose and the production of reactive oxygen species, suggesting a multi-level mode of action.

Its plant protection activity, besides the capacity of AZ78 to tolerate copper, its persistence on the phyllosphere in combination with a protectant formulation, makes *L. capsici* AZ78 an ideal candidate for the development of a mBCA-based biofungicide, to be included in the portfolio of plant protection products, with the aim of implementing sustainable, eco-friendly IPM strategies.

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CHAPTER 8. DISCUSSION

The management of *Plasmopara viticola*, the causative agent of grapevine downy mildew, presents a significant challenge to viticulture worldwide. This study provides a detailed analysis of the genetic structure, reproductive strategies, and fungicide resistance of *P. viticola* populations in the Trentino South Tyrol region, alongside evaluations of biological control agents (BCAs) and natural compounds as alternatives to conventional fungicides. By integrating insights from genetic studies, fungicide resistance assays, and biocontrol trials, this discussion highlights the critical need for sustainable, integrated and region-specific disease management strategies to control *P. viticola*.

The genetic population structure and epidemic dynamics of *Plasmopara viticola* have been explored at multiple spatial scales, including continental (Gobbin *et al*., 2006; Fontaine *et al*., 2013; Li *et al*., 2016), regional (Gobbin *et al*., 2003; Delmas *et al*., 2017; Maddalena *et al*., 2020), and localized field or single-plant levels (Gobbin *et al*., 2003; Matasci *et al*., 2010). These investigations consistently highlight the panmictic nature of *P. viticola* populations and emphasize the significant roles of both sexual and asexual reproduction in shaping their genetic diversity. Despite these advancements, research focusing specifically on the population structure of *P. viticola* within Italy, a prominent wine-producing nation, remains scarce. Previous studies have either examined a limited geographical scope, as in Gobbin *et al*. (2003), or included Italian populations as part of broader European analyses, such as the study by Fontaine *et al*. (2013), which used a small number of isolates from Italy. Additionally, most of these studies employed a limited number of genetic markers, typically analyzing only 5 to 8 SSR *loci*.

An exception is the work by Maddalena *et al*. (2020), which provided a more extensive examination of P. viticola populations in Italy. This study utilized 31 SSR *loci* and sampled

populations across 12 regions, achieving a more representative geographic coverage. However, several regions, including Trentino-South Tyrol, a key area for Italian viticulture, were not included. Given the importance of Trentino-South Tyrol in wine production, its exclusion represents a critical gap in our understanding of the genetic diversity and population dynamics of *P. viticola*.

The genetic analysis revealed a strikingly low genetic diversity within *P. viticola* populations in Trentino South Tyrol, with an observed heterozygosity (HE) of 0.23. This is substantially lower than values reported in other European regions, likely reflecting a bottleneck effect following the pathogen's introduction from North America European (Gobbin *et al*., 2006; Rouxel *et al*., 2012, 2013; Fontaine *et al*., 2013; Maddalena *et al*., 2020; Fontaine *et al*.,2021). Further constraints on gene flow within the region may exacerbate this reduced genetic variability. The limited genetic differentiation observed between geographically close populations aligns with earlier studies indicating that spore dispersal mechanisms, such as rain-driven dissemination, contribute to the spread of *P. viticola*. Identical genotypic profiles detected across distant locations further underscore the pathogen's ability to overcome physical barriers through environmental factors like wind and rainfall (Gobbin *et al*., 2006).

The predominantly asexual reproductive strategy of *P. viticola* populations was evident, with negative F_{IS} values at most *loci* suggesting an excess of heterozygotes. Although the lack of significant deviation from Hardy-Weinberg equilibrium at several *loci* supports the hypothesis of panmixia in these populations, it is important to note that asexual reproduction can still maintain high levels of genetic diversity through the accumulation of mutations over time (Maddalena *et al*., 2020). This accumulation of mutations may pose a significant threat, as it can contribute to the development of fungicide resistance. As such, the combination of asexual reproduction and the potential for rapid

accumulation of mutations makes *P. viticola* populations particularly susceptible to the emergence of fungicide resistance, a concern highlighted by the results of our bioassay.

The bioassays conducted to assess fungicide resistance revealed significant heterogeneity in the sensitivity of *P. viticola* isolates to various fungicide classes. Notably, resistance was observed in isolates collected in both 2021 and 2022, with a considerable proportion of isolates exhibiting adaptation to multiple fungicides, including mandipropamid, ametoctradin, and amisulbrom. These findings are concerning, as resistance to multiple fungicides suggests that *P. viticola* populations in the region are already undergoing adaptation to commonly used fungicides, posing a risk for future fungicide failure in vineyard management. The recorded EC_{50} values for mandipropamid, which exceeded the resistance threshold in 50% of the isolates, further highlights the widespread adaptation to this active ingredient, as reported by Delmas *et al*. (2017).

Interestingly, the results of the fungicide resistance bioassays revealed that the patterns of resistance were not consistently associated with any particular disease management strategy. For example, isolates from vineyards under organic farming (O and O+ management) as well as those under integrated pest management exhibited similar levels of adaptation to fungicides. This suggests that fungicide resistance in this region is not exclusively driven by the frequency or intensity of fungicide applications within a given management strategy, but rather by broader regional factors such as the proximity of vineyards, cross-contamination from neighboring sites, and the inherent capacity of the pathogen to disperse over distances. These findings underscore the importance of considering vineyard management strategies on a regional scale, as the spread of resistant genotypes is influenced by the spatial arrangement and highlight the need for coordinated resistance management strategies at a regional scale.

The highlighted critical scenario posed by wide-spread fungicide resistances to single-site fungicides, further stresses the urgent need of a wider portfolio of plan protection products. In this context biofungicides based on plant extracts and biocontrol agents have huge room for emerging and establishing on the market if demonstrating satisfying plant protection performances.

However, literature review displayed only few candidates with satisfying results at field scale, despite the high number of candidates showing anti-oomycete activity in controlled conditions. Indeed, it is not seldom that plant protection efficacy above 90% in greenhouse cultivated plants (Thuerig *et al*., 2015; Mulholland *et al*., 2017; Rienth *et al*., 2019; Heng *et al*., 2021), which is hard to replicate in field conditions due to rain fastness and persistence.

So far, plant extracts from *Salvia officinalis, Magnolia officinalis, Larix decidua* (Dagostin *et al*., 2010; Thuerig *et al*., 2018a, 2018b) offered the best performances in the field, with efficacies beyond 70% (See Chapter 4.5.1 for details), while among mBCA noteworthy results were achieved by *Streptomyces atratus* PY-1 (Liang et al., 2016), *Lysobacter capsici* AZ78 (Markellou et al., 2022) and *Penicillium chrysogenum* (Tamm et al., 2011).

In this thesis we proposed four novel candidates, with extremely promising anti-oomycete activity, although their research and development.

Extracts from *Glechoma hederacea* were able to significantly reduce *P. viticola* disease severity, suggesting a crucial role to caffeic acid and methyl caffeate as already reported in previous studies (Mattivi et al., 2011; Balachandran et al., 2012; Sanchez-Maldonado et al., 2016; Gabaston et al., 2017), suggesting a synergic effect of the isolated metabolites in the control of *P. viticola*.

Interestingly, 12110, 12035 and *L. capsici* AZ78 displayed a multi-level mode of action, with plant defence induction alongside an acute direct toxicity towards *P. viticola*. In particular 12110 was

able to induce callose deposition and ROS production, both renown defense plant responses as a consequence of a biotic stress ((Kortekamp et al., 1997; Allègre et al., 2009; Palmieri et al., 2012), while 12035 only significant ROS production.

Finally, *L. capsici* AZ78, proved itself as one of the most promising candidates, given its remarkable plant protection activity, persistence on the phyllosphere if in combination with a protectant formulation (Segarra et al., 2016), its tolerance towards copper (Puopolo et al., 2014), but also a variegated mode of action by the release of lytic enzymes and as plant defense inducer. In this study, we highlighted the crucial role of dihydromaltophylin (HASF) and maltophylin under both aspects.

CHAPTER 9. EXTENDED CONCLUSIONS

In the coming years, managing grapevine downy mildew will present increasingly critical challenges for viticulture. Key drivers of this complexity include the progressive reduction of copper inputs - mandated due to their detrimental effects on human health and the environment - and the rise in fungicide resistant *Plasmopara viticola* populations. Moreover, regulatory restrictions are leading to the withdrawal of several synthetic fungicide active ingredients that have historically been integral to disease management.

The outburst of *Plasmopara viticola* populations resistant to chemical fungicides is one of the major challenges in conventional disease management, as the annual monitoring bulletins are reporting. Not only active ingredients widely used in the past decades, but also newly introduced molecules are facing a dramatic decrease in their efficacy in the vineyards to the presence of resistant *P. viticola* isolates. Moreover, it has been widely demonstrated that the genetic diversity of *P. viticola* is extremely limited in Europe and the combination of sexual and asexual reproduction is a key factor in the outburst and diffusion of fungicide resistant populations.

For this reason, implementing IPM strategies is crucial in order to mitigate this phenomenon and preserve as long as possible the efficacy of the already existing active ingredients, combining regulatory limitations with anti-fungicide resistance disease management.

Not only smarter and more efficient fungicide applications are extremely crucial, but all these factors collectively underscore the urgency for the research community to accelerate the development of sustainable plant protection products.

While substantial progress has been made in identifying potential alternatives, such as plant extracts and microbial agents with activity against *P. viticola*, their transition from controlled experimental conditions to practical vineyard applications remains limited. Only a small subset of these candidates has demonstrated satisfactory efficacy under field conditions, and fewer still have progressed to commercial biopesticides. At present, there are no microbial biopesticides specifically targeting *P. viticola* available on the market, representing a significant gap in sustainable viticultural practices.

The foremost challenge lies in overcoming the inherent limitations of biological solutions, such as their lower persistence, susceptibility to environmental degradation, and reduced efficacy under adverse weather conditions. To address these constraints, researchers and companies must focus on the development of advanced formulations that enhance the stability, rain fastness, and shelf life of these biocontrol products. Additionally, ensuring that such innovations are economically viable for farmers is essential to drive widespread adoption.

Achieving these goals will require a multidisciplinary approach, integrating advancements in microbiology, chemistry, and agricultural engineering. Innovations in formulation technologies, such as encapsulation methods and stabilizing additives, may hold the key to unlocking the full potential of biopesticides. Furthermore, collaboration between academia, industry, and regulatory bodies will be critical to streamline the path from laboratory discovery to market availability.

Ultimately, the development of robust, cost-effective, and environmentally friendly biocontrol solutions can lead to a paradigm shift in the management of grapevine downy mildew. Such advancements will not only reduce reliance on traditional chemical fungicides but also contribute to a broader vision of sustainable agriculture, balancing productivity with ecological stewardship. By

addressing these pressing challenges, the viticulture sector can ensure its resilience in the face of emerging threats, while aligning with the global imperative for more sustainable farming practices.

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APPENDIX A. LIST OF PUBLICATIONS

Chapter 4 and Chapter 5 are published articles, Nadalini and Puopolo (2024) and Zorrilla *et al.*, (2024) respectively. Moreover, Chapter 3 refers to the unpublished manuscript in preparation reported below as Nadalini *et al.*, (2025).

Chapter 6 was published as a Conference proceeding for the XVI IOBC-WPRS meeting in Wageningen (NL) as Nadalini and Puopolo (2023).

Papers in peer-review journals

- Zorrilla, J.G., Giovannini, O., **Nadalini, S.**, *et al.*, 2024. Suppressive activity of *Glechoma hederacea* extracts against the phytopathogenic oomycete *Plasmopara viticola*, and first screening of the active metabolites. *Agriculture*, 14(1), 58; https://doi.org/10.2290/agriculture1410058
- **Nadalini, S.**, Puopolo, G., 2024. Biological control of *Plasmopara viticola*: where are we now? Book chapter in: *Biocontrol agents for improved agriculture* (A volume in *Plant and soil microbiome*), 67-100. https://doi.org/10.1016/b978-0-443-15199-6.00013-0
- **Nadalini, S.**, Tomada, S., Moffa, A., *et al.*, 2025. Tentative title: Low genetic diversity and adaptation to single-site fungicides of *Plasmopara viticola* populations in Trentino South Tyrol (unpublished). In preparation.

Conference proceedings

- **Nadalini, S**., Puopolo, G., 2023. 12110 and 12035: two novel candidates as potential biofungicides to control *Plasmopara viticola*. *IOBC-WPRS Bulletin Vol. 165. Proceedings of the XVI meeting*, Wageningen, the Netherlands, 6-9 June 2023.

Oral presentations at conferences

- **Nadalini, S**., Bejarano Ramos, A., Puopolo, G., *et al.*, 2022. The perception of indole negatively modulates biocontrol activities in the plant beneficial rhizobacterium *Lysobacter capsici* AZ78. *Biocontrol & Natural Products Conference*, Perpignan, France 20-23 September 2022.
- **Nadalini S**., Puopolo G., 2023. 12110 and 12035: two novel candidates as potential biofungicides to control *Plasmopara viticola*. *IOBC-WPRS Conference*, Wageningen, the Netherlands, 6-9 June 2023.

Poster presentations at conferences

- **Nadalini S**., Tomada S. *et al.*, 2023. Monitoring *Plasmopara viticola* populations resistant to single-site chemical fungicides in Trentino Alto Adige. *XXVII SIPaV Congress 2023*, Napoli, Italy 18-20 September 2023.

APPENDIX B. SUPPLEMENTARY FIGURES AND TABLES

Supplementary table 1 Information about *Plasmopara viticola* samples. Identification number (ID), winegrowing areas, disease management strategy, grapevine variety and sampling vegetative season (year).

Sample ID	Sample code	Winegrowing areas	Management system	variety	Vegetative season
1	Ter_B_2021	Terlan	Organic+	Pinot blanc	2021
2	Ter_B_2021	Terlan	Organic+	Pinot blanc	2021
3	Ter_B_2021	Terlan	Organic+	Pinot blanc	2021
4	Ter_B_2021	Terlan	Organic+	Pinot blanc	2021
5	Ter_B_2021	Terlan	Organic+	Pinot blanc	2021
6	Ter_B_2021	Terlan	Organic+	Pinot blanc	2021
7	Ter_B_2021	Terlan	Organic+	Pinot blanc	2021
8	Ter_B_2021	Terlan	Organic+	Pinot blanc	2021
9	Ter_B_2021	Terlan	Organic+	Pinot blanc	2021
10	Ter_B_2021	Terlan	Organic+	Pinot blanc	2021
11	Ter_B_2021	Terlan	Organic+	Pinot blanc	2021
12	Ter_B_2021	Terlan	Organic+	Pinot blanc	2021
13	Ter_B_2021	Terlan	Organic+	Pinot blanc	2021
14	Ter_B_2021	Terlan	Organic+	Pinot blanc	2021
15	Ter_B_2021	Terlan	Organic+	Pinot blanc	2021
16	Ter_B_2022	Terlan	Organic+	Pinot blanc	2022
17	Ter_B_2022	Terlan	Organic+	Pinot blanc	2022
18	Ter_B_2022	Terlan	Organic+	Pinot blanc	2022
19	Ter_B_2022	Terlan	Organic+	Pinot blanc	2022
20	Ter_B_2022	Terlan	Organic+	Pinot blanc	2022
21	Ter_B_2022	Terlan	Organic+	Pinot blanc	2022
22	Ter_B_2022	Terlan	Organic+	Pinot blanc	2022
23	Ter_B_2022	Terlan	Organic+	Pinot blanc	2022
24	Ter_B_2022	Terlan	Organic+	Pinot blanc	2022
25	Ter_B_2022	Terlan	Organic+	Pinot blanc	2022
26	Ter_I_2021	Terlan	Integrated	Pinot blanc	2021
27	Ter_I_2021	Terlan	Integrated	Pinot blanc	2021
28	Ter_I_2021	Terlan	Integrated	Pinot blanc	2021
29	Ter_I_2021	Terlan	Integrated	Pinot blanc	2021
30	Ter_I_2021	Terlan	Integrated	Pinot blanc	2021
31	Ter_I_2021	Terlan	Integrated	Pinot blanc	2021
32	Ter_I_2021	Terlan	Integrated	Pinot blanc	2021

33	Ter_I_2021	Terlan	Integrated	Pinot blanc	2021
34	Ter_I_2021	Terlan	Integrated	Pinot blanc	2021
35	Ter_I_2021	Terlan	Integrated	Pinot blanc	2021
36	Ter_I_2021	Terlan	Integrated	Pinot blanc	2021
37	Ter_I_2021	Terlan	Integrated	Pinot blanc	2021
38	Ter_I_2021	Terlan	Integrated	Pinot blanc	2021
39	Ter_I_2021	Terlan	Integrated	Pinot blanc	2021
40	Ter_I_2021	Terlan	Integrated	Pinot blanc	2021
41	Ter_I_2022	Terlan	Integrated	Pinot blanc	2022
42	Ter_I_2022	Terlan	Integrated	Pinot blanc	2022
43	Ter_I_2022	Terlan	Integrated	Pinot blanc	2022
44	Ter_I_2022	Terlan	Integrated	Pinot blanc	2022
45	Ter_I_2022	Terlan	Integrated	Pinot blanc	2022
46	Ter_I_2022	Terlan	Integrated	Pinot blanc	2022
47	Ter_I_2022	Terlan	Integrated	Pinot blanc	2022
48	Ter_I_2022	Terlan	Integrated	Pinot blanc	2022
49	Ter_I_2022	Terlan	Integrated	Pinot blanc	2022
50	Ter_I_2022	Terlan	Integrated	Pinot blanc	2022
51	Ter_O_2021	Terlan	Organic	Pinot blanc	2021
52	Ter_O_2021	Terlan	Organic	Pinot blanc	2021
53	Ter_O_2021	Terlan	Organic	Pinot blanc	2021
54	Ter_O_2021	Terlan	Organic	Pinot blanc	2021
55	Ter_O_2021	Terlan	Organic	Pinot blanc	2021
56	Ter_O_2021	Terlan	Organic	Pinot blanc	2021
57	Ter_O_2021	Terlan	Organic	Pinot blanc	2021
58	Ter_O_2021	Terlan	Organic	Pinot blanc	2021
59	Ter_O_2021	Terlan	Organic	Pinot blanc	2021
60	Ter_O_2021	Terlan	Organic	Pinot blanc	2021
61	Ter_O_2021	Terlan	Organic	Pinot blanc	2021
62	Ter_O_2021	Terlan	Organic	Pinot blanc	2021
63	Ter_O_2021	Terlan	Organic	Pinot blanc	2021
64	Ter_O_2021	Terlan	Organic	Pinot blanc	2021
65	Ter_O_2021	Terlan	Organic	Pinot blanc	2021
66	Ter_O_2022	Terlan	Organic	Pinot blanc	2022
67	Ter_O_2022	Terlan	Organic	Pinot blanc	2022
68	Ter_O_2022	Terlan	Organic	Pinot blanc	2022
69	Ter_O_2022	Terlan	Organic	Pinot blanc	2022
70	Ter_O_2022	Terlan	Organic	Pinot blanc	2022
71	Ter_O_2022	Terlan	Organic	Pinot blanc	2022
72	Ter_O_2022	Terlan	Organic	Pinot blanc	2022

73	Ter_O_2022	Terlan	Organic	Pinot blanc	2022
74	Ter_O_2022	Terlan	Organic	Pinot blanc	2022
75	Ter_O_2022	Terlan	Organic	Pinot blanc	2022
76	Tr_B_2021	Tramin	Organic+	Pinot blanc	2021
77	Tr_B_2021	Tramin	Organic+	Pinot blanc	2021
78	Tr_B_2021	Tramin	Organic+	Pinot blanc	2021
79	Tr_B_2021	Tramin	Organic+	Pinot blanc	2021
80	Tr_B_2021	Tramin	Organic+	Pinot blanc	2021
81	Tr_B_2021	Tramin	Organic+	Pinot blanc	2021
82	Tr_B_2021	Tramin	Organic+	Pinot blanc	2021
83	Tr_B_2021	Tramin	Organic+	Pinot blanc	2021
84	Tr_B_2021	Tramin	Organic+	Pinot blanc	2021
85	Tr_B_2021	Tramin	Organic+	Pinot blanc	2021
86	Tr_B_2021	Tramin	Organic+	Pinot blanc	2021
87	Tr_B_2021	Tramin	Organic+	Pinot blanc	2021
88	Tr_B_2021	Tramin	Organic+	Pinot blanc	2021
89	Tr_B_2021	Tramin	Organic+	Pinot blanc	2021
90	Tr_B_2021	Tramin	Organic+	Pinot blanc	2021
91	Tr_B_2022	Tramin	Organic+	Pinot blanc	2022
92	Tr_B_2022	Tramin	Organic+	Pinot blanc	2022
93	Tr_B_2022	Tramin	Organic+	Pinot blanc	2022
94	Tr_B_2022	Tramin	Organic+	Pinot blanc	2022
95	Tr_B_2022	Tramin	Organic+	Pinot blanc	2022
96	Tr_B_2022	Tramin	Organic+	Pinot blanc	2022
97	Tr_B_2022	Tramin	Organic+	Pinot blanc	2022
98	Tr_B_2022	Tramin	Organic+	Pinot blanc	2022
99	Tr_B_2022	Tramin	Organic+	Pinot blanc	2022
100	Tr_B_2022	Tramin	Organic+	Pinot blanc	2022
101	Tr_I_2021	Tramin	Integrated	Pinot blanc	2021
102	Tr_I_2021	Tramin	Integrated	Pinot blanc	2021
103	Tr_I_2021	Tramin	Integrated	Pinot blanc	2021
104	Tr_I_2021	Tramin	Integrated	Pinot blanc	2021
105	Tr_I_2021	Tramin	Integrated	Pinot blanc	2021
106	Tr_I_2021	Tramin	Integrated	Pinot blanc	2021
107	Tr_I_2021	Tramin	Integrated	Pinot blanc	2021
108	Tr_I_2021	Tramin	Integrated	Pinot blanc	2021
109	Tr_I_2021	Tramin	Integrated	Pinot blanc	2021
110	Tr_I_2021	Tramin	Integrated	Pinot blanc	2021
111	Tr_I_2021	Tramin	Integrated	Pinot blanc	2021
112	Tr_I_2021	Tramin	Integrated	Pinot blanc	2021

113	Tr_I_2021	Tramin	Integrated	Pinot blanc	2021
114	Tr_I_2021	Tramin	Integrated	Pinot blanc	2021
115	Tr_I_2021	Tramin	Integrated	Pinot blanc	2021
116	Tr_I_2022	Tramin	Integrated	Pinot blanc	2022
117	Tr_I_2022	Tramin	Integrated	Pinot blanc	2022
118	Tr_I_2022	Tramin	Integrated	Pinot blanc	2022
119	Tr_I_2022	Tramin	Integrated	Pinot blanc	2022
120	Tr_I_2022	Tramin	Integrated	Pinot blanc	2022
121	Tr_I_2022	Tramin	Integrated	Pinot blanc	2022
122	Tr_I_2022	Tramin	Integrated	Pinot blanc	2022
123	Tr_I_2022	Tramin	Integrated	Pinot blanc	2022
124	Tr_I_2022	Tramin	Integrated	Pinot blanc	2022
125	Tr_I_2022	Tramin	Integrated	Pinot blanc	2022
126	Tr_O_2021	Tramin	Organic	Pinot blanc	2021
127	Tr_O_2021	Tramin	Organic	Pinot blanc	2021
128	Tr_O_2021	Tramin	Organic	Pinot blanc	2021
129	Tr_O_2021	Tramin	Organic	Pinot blanc	2021
130	Tr_O_2021	Tramin	Organic	Pinot blanc	2021
131	Tr_O_2021	Tramin	Organic	Pinot blanc	2021
132	Tr_O_2021	Tramin	Organic	Pinot blanc	2021
133	Tr_O_2021	Tramin	Organic	Pinot blanc	2021
134	Tr_O_2021	Tramin	Organic	Pinot blanc	2021
135	Tr_O_2021	Tramin	Organic	Pinot blanc	2021
136	Tr_O_2021	Tramin	Organic	Pinot blanc	2021
137	Tr_O_2021	Tramin	Organic	Pinot blanc	2021
138	Tr_O_2021	Tramin	Organic	Pinot blanc	2021
139	Tr_O_2021	Tramin	Organic	Pinot blanc	2021
140	Tr_O_2021	Tramin	Organic	Pinot blanc	2021
141	Tr_O_2022	Tramin	Organic	Pinot blanc	2022
142	Tr_O_2022	Tramin	Organic	Pinot blanc	2022
143	Tr_O_2022	Tramin	Organic	Pinot blanc	2022
144	Tr_O_2022	Tramin	Organic	Pinot blanc	2022
145	Tr_O_2022	Tramin	Organic	Pinot blanc	2022
146	Tr_O_2022	Tramin	Organic	Pinot blanc	2022
147	Tr_O_2022	Tramin	Organic	Pinot blanc	2022
148	Tr_O_2022	Tramin	Organic	Pinot blanc	2022
149	Tr_O_2022	Tramin	Organic	Pinot blanc	2022
150	Tr_O_2022	Tramin	Organic	Pinot blanc	2022
151	Mag_B_2021	Magreid	Organic+	Pinot blanc	2021
152	Mag_B_2021	Magreid	Organic+	Pinot blanc	2021

153	Mag_B_2021	Magreid	Organic+	Pinot blanc	2021
154	Mag_B_2021	Magreid	Organic+	Pinot blanc	2021
155	Mag_B_2021	Magreid	Organic+	Pinot blanc	2021
156	Mag_B_2021	Magreid	Organic+	Pinot blanc	2021
157	Mag_B_2021	Magreid	Organic+	Pinot blanc	2021
158	Mag_B_2021	Magreid	Organic+	Pinot blanc	2021
159	Mag_B_2021	Magreid	Organic+	Pinot blanc	2021
160	Mag_B_2021	Magreid	Organic+	Pinot blanc	2021
161	Mag_B_2021	Magreid	Organic+	Pinot blanc	2021
162	Mag_B_2021	Magreid	Organic+	Pinot blanc	2021
163	Mag_B_2021	Magreid	Organic+	Pinot blanc	2021
164	Mag_B_2021	Magreid	Organic+	Pinot blanc	2021
165	Mag_B_2021	Magreid	Organic+	Pinot blanc	2021
166	Mag_B_2022	Magreid	Organic+	Pinot blanc	2022
167	Mag_B_2022	Magreid	Organic+	Pinot blanc	2022
168	Mag_B_2022	Magreid	Organic+	Pinot blanc	2022
169	Mag_B_2022	Magreid	Organic+	Pinot blanc	2022
170	Mag_B_2022	Magreid	Organic+	Pinot blanc	2022
171	Mag_B_2022	Magreid	Organic+	Pinot blanc	2022
172	Mag_B_2022	Magreid	Organic+	Pinot blanc	2022
173	Mag_B_2022	Magreid	Organic+	Pinot blanc	2022
174	Mag_B_2022	Magreid	Organic+	Pinot blanc	2022
175	Mag_B_2022	Magreid	Organic+	Pinot blanc	2022
176	Mag_I_2021	Magreid	Integrated	Pinot blanc	2021
177	Mag_I_2021	Magreid	Integrated	Pinot blanc	2021
178	Mag_I_2021	Magreid	Integrated	Pinot blanc	2021
179	Mag_I_2021	Magreid	Integrated	Pinot blanc	2021
180	Mag_I_2021	Magreid	Integrated	Pinot blanc	2021
181	Mag_I_2021	Magreid	Integrated	Pinot blanc	2021
182	Mag_I_2021	Magreid	Integrated	Pinot blanc	2021
183	Mag_I_2021	Magreid	Integrated	Pinot blanc	2021
184	Mag_I_2021	Magreid	Integrated	Pinot blanc	2021
185	Mag_I_2021	Magreid	Integrated	Pinot blanc	2021
186	Mag_I_2021	Magreid	Integrated	Pinot blanc	2021
187	Mag_I_2021	Magreid	Integrated	Pinot blanc	2021
188	Mag_I_2021	Magreid	Integrated	Pinot blanc	2021
189	Mag_I_2021	Magreid	Integrated	Pinot blanc	2021
190	Mag_I_2021	Magreid	Integrated	Pinot blanc	2021
191	Mag_I_2022	Magreid	Integrated	Pinot blanc	2022
192	Mag_I_2022	Magreid	Integrated	Pinot blanc	2022

193	Mag_I_2022	Magreid	Integrated	Pinot blanc	2022
194	Mag_I_2022	Magreid	Integrated	Pinot blanc	2022
195	Mag_I_2022	Magreid	Integrated	Pinot blanc	2022
196	Mag_I_2022	Magreid	Integrated	Pinot blanc	2022
197	Mag_I_2022	Magreid	Integrated	Pinot blanc	2022
198	Mag_I_2022	Magreid	Integrated	Pinot blanc	2022
199	Mag_O_2021	Magreid	Organic	Pinot blanc	2021
200	Mag_O_2021	Magreid	Organic	Pinot blanc	2021
201	Mag_O_2021	Magreid	Organic	Pinot blanc	2021
202	Mag_O_2021	Magreid	Organic	Pinot blanc	2021
203	Mag_O_2021	Magreid	Organic	Pinot blanc	2021
204	Mag_O_2021	Magreid	Organic	Pinot blanc	2021
205	Mag_O_2021	Magreid	Organic	Pinot blanc	2021
206	Mag_O_2021	Magreid	Organic	Pinot blanc	2021
207	Mag_O_2021	Magreid	Organic	Pinot blanc	2021
208	Mag_O_2021	Magreid	Organic	Pinot blanc	2021
209	Mag_O_2021	Magreid	Organic	Pinot blanc	2021
210	Mag_O_2021	Magreid	Organic	Pinot blanc	2021
211	Mag_O_2021	Magreid	Organic	Pinot blanc	2021
212	Mag_O_2021	Magreid	Organic	Pinot blanc	2021
213	Mag_O_2021	Magreid	Organic	Pinot blanc	2021
214	Mag_O_2022	Magreid	Organic	Pinot blanc	2022
215	Mag_O_2022	Magreid	Organic	Pinot blanc	2022
216	Mag_O_2022	Magreid	Organic	Pinot blanc	2022
217	Mag_O_2022	Magreid	Organic	Pinot blanc	2022
218	Mag_O_2022	Magreid	Organic	Pinot blanc	2022
219	Mag_O_2022	Magreid	Organic	Pinot blanc	2022
220	Mag_O_2022	Magreid	Organic	Pinot blanc	2022
221	Mag_O_2022	Magreid	Organic	Pinot blanc	2022
222	Mag_O_2022	Magreid	Organic	Pinot blanc	2022
223	Mag_O_2022	Magreid	Organic	Pinot blanc	2022
224	SaM_B_2021	San Michele A/A	Organic+	Chardonnay	2021
225	SaM_B_2021	San Michele A/A	Organic+	Chardonnay	2021
226	SaM_B_2021	San Michele A/A	Organic+	Chardonnay	2021
227	SaM_B_2021	San Michele A/A	Organic+	Chardonnay	2021
228	SaM_B_2021	San Michele A/A	Organic+	Chardonnay	2021
229	SaM_B_2022	San Michele A/A	Organic+	Chardonnay	2022
230	SaM_B_2022	San Michele A/A	Organic+	Chardonnay	2022
231	SaM_B_2022	San Michele A/A	Organic+	Chardonnay	2022
232	SaM_B_2022	San Michele A/A	Organic+	Chardonnay	2022

233	SaM_B_2022	San Michele A/A	Organic+	Chardonnay	2022
234	SaM_I_2021	San Michele A/A	Integrated	Chardonnay	2021
235	SaM_I_2021	San Michele A/A	Integrated	Chardonnay	2021
236	SaM_I_2021	San Michele A/A	Integrated	Chardonnay	2021
237	SaM_I_2021	San Michele A/A	Integrated	Chardonnay	2021
238	SaM_I_2021	San Michele A/A	Integrated	Chardonnay	2021
239	SaM_I_2022	San Michele A/A	Integrated	Chardonnay	2022
240	SaM_I_2022	San Michele A/A	Integrated	Chardonnay	2022
241	SaM_I_2022	San Michele A/A	Integrated	Chardonnay	2022
242	SaM_I_2022	San Michele A/A	Integrated	Chardonnay	2022
243	SaM_I_2022	San Michele A/A	Integrated	Chardonnay	2022
244	SaM_O_2021	San Michele A/A	Organic	Chardonnay	2021
245	SaM_O_2021	San Michele A/A	Organic	Chardonnay	2021
246	SaM_O_2021	San Michele A/A	Organic	Chardonnay	2021
247	SaM_O_2021	San Michele A/A	Organic	Chardonnay	2021
248	SaM_O_2021	San Michele A/A	Organic	Chardonnay	2021
249	SaM_O_2022	San Michele A/A	Organic	Chardonnay	2022
250	SaM_O_2022	San Michele A/A	Organic	Chardonnay	2022
251	SaM_O_2022	San Michele A/A	Organic	Chardonnay	2022
252	SaM_O_2022	San Michele A/A	Organic	Chardonnay	2022
253	SaM_O_2022	San Michele A/A	Organic	Chardonnay	2022
254	Vdl_B_2021	Valle dei laghi	Organic+	Chardonnay	2021
255	Vdl_B_2021	Valle dei laghi	Organic+	Chardonnay	2021
256	Vdl_B_2021	Valle dei laghi	Organic+	Chardonnay	2021
257	Vdl_B_2021	Valle dei laghi	Organic+	Chardonnay	2021
258	Vdl_B_2022	Valle dei laghi	Organic+	Chardonnay	2022
259	Vdl_B_2022	Valle dei laghi	Organic+	Chardonnay	2022
260	Vdl_B_2022	Valle dei laghi	Organic+	Chardonnay	2022
261	Vdl_B_2022	Valle dei laghi	Organic+	Chardonnay	2022
262	Vdl_I_2021	Valle dei laghi	Integrated	Chardonnay	2021
263	Vdl_I_2021	Valle dei laghi	Integrated	Chardonnay	2021
264	Vdl_I_2021	Valle dei laghi	Integrated	Chardonnay	2021
265	Vdl_I_2021	Valle dei laghi	Integrated	Chardonnay	2021
266	Vdl_I_2022	Valle dei laghi	Integrated	Chardonnay	2022
267	Vdl_I_2022	Valle dei laghi	Integrated	Chardonnay	2022
268	Vdl_I_2022	Valle dei laghi	Integrated	Chardonnay	2022
269	Vdl_I_2022	Valle dei laghi	Integrated	Chardonnay	2022
270	Vdl_I_2022	Valle dei laghi	Integrated	Chardonnay	2022
271	Vdl_O_2021	Valle dei laghi	Organic	Chardonnay	2021
272	Vdl_O_2021	Valle dei laghi	Organic	Chardonnay	2021

273	Vdl_O_2021	Valle dei laghi	Organic	Chardonnay	2021
274	Vdl_O_2021	Valle dei laghi	Organic	Chardonnay	2021
275	Vdl_O_2021	Valle dei laghi	Organic	Chardonnay	2021
276	Vdl_O_2022	Valle dei laghi	Organic	Chardonnay	2022
277	Vdl_O_2022	Valle dei laghi	Organic	Chardonnay	2022
278	Vdl_O_2022	Valle dei laghi	Organic	Chardonnay	2022
279	Rov_B_2021	Rovereto	Organic+	Chardonnay	2021
280	Rov_B_2021	Rovereto	Organic+	Chardonnay	2021
281	Rov_B_2021	Rovereto	Organic+	Chardonnay	2021
282	Rov_B_2021	Rovereto	Organic+	Chardonnay	2021
283	Rov_B_2021	Rovereto	Organic+	Chardonnay	2021
284	Rov_B_2022	Rovereto	Organic+	Chardonnay	2022
285	Rov_B_2022	Rovereto	Organic+	Chardonnay	2022
286	Rov_B_2022	Rovereto	Organic+	Chardonnay	2022
287	Rov_B_2022	Rovereto	Organic+	Chardonnay	2022
288	Rov_B_2022	Rovereto	Organic+	Chardonnay	2022
289	Rov_I_2021	Rovereto	Integrated	Chardonnay	2021
290	Rov_I_2021	Rovereto	Integrated	Chardonnay	2021
291	Rov_I_2021	Rovereto	Integrated	Chardonnay	2021
292	Rov_I_2021	Rovereto	Integrated	Chardonnay	2021
293	Rov_I_2021	Rovereto	Integrated	Chardonnay	2021
294	Rov_I_2022	Rovereto	Integrated	Chardonnay	2022
295	Rov_I_2022	Rovereto	Integrated	Chardonnay	2022
296	Rov_I_2022	Rovereto	Integrated	Chardonnay	2022
297	Rov_I_2022	Rovereto	Integrated	Chardonnay	2022
298	Rov_O_2021	Rovereto	Organic	Chardonnay	2021
299	Rov_O_2021	Rovereto	Organic	Chardonnay	2021
300	Rov_O_2021	Rovereto	Organic	Chardonnay	2021
301	Rov_O_2021	Rovereto	Organic	Chardonnay	2021
302	Rov_O_2021	Rovereto	Organic	Chardonnay	2021
303	Rov_O_2022	Rovereto	Organic	Chardonnay	2022
304	Rov_O_2022	Rovereto	Organic	Chardonnay	2022
305	Rov_O_2022	Rovereto	Organic	Chardonnay	2022
306	Rov_O_2022	Rovereto	Organic	Chardonnay	2022
307	Rov_O_2022	Rovereto	Organic	Chardonnay	2022

Supplementary table 2 Allelic profile of the 13 microsatellite *loci* of 307 *P. viticola* strains. Missing data "0".

Sample ID	ISA		PV13		PV17		PV31		PV139		PV141		PV91		PV127		PV104		PV88		PV103		PV83		PV101	
1	135	135	217	217	146	146	243	243	133	135	191	191	140	142	220	220	324	324	205	207	287	287	243	243	263	263
2	127	131	217	217	148	148	243	243	133	133	191	193	142	142	220	220	324	324	205	207	287	287	241	243	266	266
3	131	135	217	217	148	148	243	243	133	133	191	193	140	142	220	220	324	324	207	207	297	297	243	243	263	263
4	131	135	217	217	146	148	243	243	133	133	191	191	140	140	220	220	324	324	207	207	287	287	243	243	263	263
5	135	135	217	217	146	148	243	243	133	133	191	191	140	142	220	220	324	324	207	207	287	287	243	243	263	263
6	135	135	217	217	146	146	243	243	135	135	191	191	140	142	220	220	324	324	207	207	287	297	243	243	263	263
7	131	135	217	217	146	148	243	243	133	133	191	191	140	140	220	220	324	324	207	207	287	287	243	243	263	263
8	135	135	217	219	146	148	243	243	133	133	191	193	140	142	220	220	324	324	207	207	287	287	243	243	263	263
9	135	135	217	219	146	148	243	243	133	133	191	191	140	140	220	220	324	324	207	207	287	297	243	243	263	266
10	135	135	217	217	148	148	243	243	133	133	191	191	140	140	220	220	324	324	207	207	287	297	243	243	263	263
11	131	131	217	217	146	146	243	243	133	133	191	191	140	140	220	220	324	324	205	207	287	297	243	243	263	266
12	131	135	217	219	146	148	243	243	133	133	191	193	140	142	220	220	324	324	207	207	287	287	243	243	263	266
13	131	135	217	217	148	148	241	243	133	133	191	193	140	140	220	220	324	324	207	207	287	287	243	243	263	263
14	131	135	217	217	146	146	243	243	133	133	191	193	140	142	220	220	324	324	205	207	287	297	243	243	263	266
15	135	135	217	217	146	148	243	243	133	133	191	193	140	142	220	220	324	324	207	207	287	287	243	243	263	266
16	135	135	217	217	146	146	243	243	133	133	191	191	140	140	220	220	324	324	207	207	287	287	243	243	263	266
17	131	135	217	217	146	146	243	243	133	133	191	193	140	140	220	220	324	324	207	207	287	287	243	243	263	263
18	135	135	217	217	146	146	243	243	133	133	191	191	140	142	220	220	324	324	207	207	287	287	243	243	263	266
19	127	135	217	217	146	146	243	243	133	133	191	191	140	142	220	220	324	324	207	207	297	297	243	243	263	263
20	127	135	217	217	146	146	243	243	133	133	191	191	140	142	220	220	324	324	207	207	297	297	243	243	263	263

Sample ID	ISA		PV13		PV17		PV31		PV139		PV141		PV91		PV127		PV104		PV88		PV103		PV83		PV101	
21	135	135	217	219	146	148	243	243	133	133	191	191	140	140	220	220	324	324	207	207	287	297	243	243	263	263
22	135	135	217	217	146	148	243	243	133	133	191	193	140	140	220	220	324	324	207	207	287	297	243	243	263	266
23	135	135	217	217	146	148	243	243	135	135	191	193	140	142	220	224	324	324	205	207	287	287	243	243	263	266
24	135	135	217	217	146	146	243	243	133	133	191	193	140	140	220	220	324	324	207	207	287	297	243	243	263	263
25	135	135	217	217	146	148	243	243	133	133	191	191	140	140	220	220	324	324	207	207	287	297	243	243	263	266
26	135	135	217	217	146	146	243	243	133	133	191	191	140	140	220	220	324	324	207	207	287	287	243	243	263	266
27	135	135	217	217	146	148	243	243	133	133	191	191	140	140	220	220	324	324	207	207	287	297	243	243	263	266
28	131	135	217	217	146	146	243	243	133	133	191	191	140	142	218	220	324	324	207	207	287	287	243	243	263	263
29	135	135	217	217	146	148	243	243	133	133	193	193	140	140	220	220	324	324	207	207	287	287	243	243	266	266
30	135	135	217	219	146	146	241	243	133	133	191	191	140	140	220	220	324	324	207	207	287	287	243	243	263	263
31	127	135	217	217	146	148	243	243	133	133	191	191	142	142	220	220	324	324	207	207	287	287	243	243	263	263
32	135	135	217	217	146	146	243	243	133	133	191	191	140	140	218	220	324	324	205	207	287	287	243	243	263	263
33	131	135	217	217	146	146	243	243	133	133	191	193	140	140	220	220	324	324	205	207	287	287	243	243	263	266
34	135	135	217	217	146	148	243	243	133	133	191	193	142	142	218	220	324	324	205	207	287	287	243	243	263	263
35	135	135	217	217	146	146	243	243	133	133	191	191	140	140	220	220	324	324	207	207	297	297	243	243	263	266
36	135	135	217	219	146	146	241	243	133	133	191	193	140	142	218	220	324	324	207	207	287	297	243	243	263	263
37	135	135	217	219	146	148	243	243	133	133	191	191	140	142	220	220	324	324	207	207	287	287	243	243	263	263
38	135	135	217	217	146	148	243	243	133	133	191	193	140	140	220	220	324	324	207	207	287	297	243	243	263	263
39	135	135	217	219	146	146	241	243	133	133	191	193	140	142	218	220	324	324	207	207	287	297	243	243	263	263
40	135	135	217	219	146	148	243	243	133	133	191	191	140	142	220	220	324	324	205	207	287	297	243	243	263	266
41	131	135	217	217	146	146	241	243	133	133	191	191	140	140	220	220	324	324	205	207	287	297	243	243	266	266
42	131	135	217	217	146	146	243	243	133	133	191	191	140	140	220	220	324	324	205	207	287	287	243	243	263	263

Sample ID	ISA		PV13		PV17		PV31		PV139		PV141		PV91		PV127		PV104		PV88		PV103		PV83		PV101	
43	131	135	217	217	146	146	241	243	133	133	191	191	140	140	220	220	324	324	205	207	287	297	243	243	266	266
44	135	135	217	219	146	148	243	243	133	133	191	191	140	142	220	220	324	324	207	207	287	297	243	243	263	266
45	127	135	217	217	146	146	241	243	133	133	191	191	142	142	220	220	324	324	205	207	287	287	243	243	263	263
46	135	135	217	217	146	146	243	243	133	135	191	193	140	142	220	220	324	324	207	207	287	297	243	243	263	266
47	127	135	217	219	146	148	243	243	133	133	191	191	140	142	220	220	324	324	205	207	287	297	240	243	263	263
48	135	135	217	217	146	146	243	243	133	133	191	191	142	142	220	220	324	324	205	207	287	297	243	243	263	263
49	135	135	217	217	146	146	243	243	133	133	193	193	140	140	220	220	324	324	207	207	287	287	243	243	263	263
50	135	135	217	217	0	0	0	0	133	133	191	191	140	142	220	220	324	324	207	207	287	297	243	243	263	263
51	127	127	217	219	148	148	243	243	133	133	191	193	140	140	220	220	324	324	207	207	287	297	243	243	263	266
52	131	135	217	219	146	146	243	243	133	133	191	191	142	142	220	220	324	324	207	207	287	287	243	243	263	266
53	135	135	217	217	146	146	243	243	133	133	191	193	140	140	218	220	324	324	207	207	287	287	243	243	263	263
54	135	135	217	217	146	146	243	243	133	133	191	191	140	142	220	220	324	324	207	207	287	287	243	243	263	263
55	135	135	217	217	146	148	243	243	133	133	191	191	142	142	220	220	324	324	207	207	287	297	243	243	263	263
56	135	135	217	217	146	148	243	243	133	133	191	191	140	142	220	220	324	324	205	207	287	287	243	243	263	266
57	131	135	217	219	146	148	243	243	133	133	191	191	140	142	220	220	324	324	207	207	287	297	243	243	263	263
58	127	135	217	217	146	148	243	243	133	133	191	193	140	140	220	220	324	324	207	207	287	297	243	243	263	266
59	131	135	217	217	148	148	243	243	133	133	191	191	140	140	220	220	324	324	207	207	287	287	243	243	263	263
60	135	135	217	217	146	146	243	243	133	133	191	191	140	140	220	220	324	324	207	207	287	297	243	243	263	266
61	131	135	217	217	146	146	243	243	133	133	191	191	140	140	218	220	324	324	205	207	287	297	243	243	263	263
62	131	135	217	217	146	146	243	243	133	133	191	191	140	140	220	220	324	324	207	207	287	287	243	243	263	266
63	127	135	217	217	146	148	243	243	133	133	191	193	140	142	218	220	324	324	207	207	287	287	243	243	263	266
64	131	131	217	217	146	146	243	243	133	133	191	191	140	140	220	220	324	324	207	207	287	287	243	243	263	263

Sample ID	ISA		PV13		PV17		PV31		PV139		PV141		PV91		PV127		PV104		PV88		PV103		PV83		PV101	
65	135	135	217	217	148	148	243	243	133	133	191	191	140	142	218	220	324	324	205	207	287	287	243	243	263	263
66	135	135	217	217	146	146	243	243	133	133	191	191	140	140	220	220	324	324	207	207	287	287	243	243	263	266
67	135	135	217	219	146	146	243	243	133	133	191	191	142	142	220	220	324	324	207	207	287	287	243	243	263	263
68	135	135	217	219	146	146	243	243	133	133	191	191	142	142	220	220	324	324	207	207	287	287	243	243	263	263
69	135	135	217	217	146	146	243	243	133	133	191	191	140	140	220	220	324	324	207	207	287	287	243	243	263	266
70	135	135	217	219	146	146	243	243	133	133	191	191	142	142	220	220	324	324	207	207	287	287	243	243	263	263
71	135	135	217	217	146	148	243	243	133	135	191	191	140	142	220	220	324	324	205	207	287	287	243	243	263	263
72	135	135	217	217	148	148	243	243	133	133	191	191	142	142	220	220	324	324	207	207	287	287	243	243	263	263
73	135	135	217	217	148	148	243	243	133	135	191	191	140	142	220	220	324	324	207	207	287	287	243	243	266	266
74	135	135	217	217	146	146	243	243	133	133	191	191	142	142	218	220	324	324	207	207	287	287	243	243	263	263
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76	135	135	217	219	146	146	243	243	133	133	191	193	140	140	220	220	324	324	205	207	287	297	243	243	263	266
77	127	135	217	217	146	148	243	243	133	133	191	191	140	142	220	220	324	324	207	207	287	297	243	243	263	266
78	135	135	217	217	146	146	243	245	133	133	191	191	140	142	220	220	324	324	207	207	287	287	243	243	263	263
79	135	135	217	217	146	148	243	243	133	133	191	191	140	142	220	220	324	324	205	207	287	297	243	243	263	266
80	135	135	217	217	146	146	243	245	133	133	191	191	140	142	220	220	324	324	207	207	287	297	243	243	263	263
81	135	135	217	219	146	146	243	243	133	133	191	191	140	142	220	220	324	324	205	207	287	287	243	243	263	263
82	135	135	217	217	146	148	243	243	133	133	191	191	140	142	220	220	324	324	207	207	287	297	243	243	263	263
83	127	135	217	217	146	150	243	243	133	133	191	191	140	142	220	220	324	324	207	207	287	287	243	243	263	266
84	127	135	217	217	146	146	243	243	133	133	191	191	140	140	220	220	324	324	207	207	287	287	243	243	263	263
85	135	135	217	217	146	146	243	243	133	133	191	191	140	142	220	220	324	324	207	207	287	287	243	243	263	266
86	135	135	217	219	146	146	243	243	133	133	191	191	142	142	220	220	324	324	207	207	287	297	243	243	263	266

Sample ID	ISA		PV13		PV17		PV31		PV139		PV141		PV91		PV127		PV104		PV88		PV103		PV83		PV101	
87	131	135	217	217	146	146	243	243	133	133	191	193	140	140	220	220	324	324	207	207	287	287	243	243	263	263
88	135	135	217	217	146	146	243	243	133	133	191	191	140	142	220	220	324	324	207	207	297	297	243	243	263	266
89	135	135	217	219	146	148	243	243	133	133	191	193	140	142	220	220	324	324	207	207	287	297	243	243	263	266
90	135	135	217	219	146	148	243	243	133	133	191	193	140	142	220	220	324	324	205	207	287	297	243	243	263	266
91	135	135	217	217	146	148	243	243	133	133	191	193	140	142	220	220	324	324	205	207	287	287	243	243	263	263
92	127	135	217	217	146	148	243	243	133	133	191	191	140	140	220	220	324	324	205	207	297	297	243	243	263	266
93	131	135	217	217	146	148	243	243	133	133	191	193	140	142	220	220	324	324	207	207	287	287	243	243	263	266
94	131	135	217	217	146	146	243	243	133	133	191	191	140	140	218	220	324	324	207	207	287	297	243	243	263	266
95	131	135	217	219	146	148	243	243	133	133	193	193	140	140	220	220	324	324	205	207	287	297	243	243	263	263
96	135	135	217	217	146	148	243	243	133	133	191	191	140	142	220	220	324	324	207	207	287	297	243	243	263	266
97	135	135	217	217	146	146	243	243	133	133	191	191	140	142	220	220	324	324	207	207	287	297	243	243	263	266
98	127	135	217	217	146	146	243	243	133	133	191	191	140	142	220	220	324	324	207	207	287	297	243	243	266	266
99	135	135	217	217	146	148	243	243	133	133	191	193	140	142	220	220	324	324	207	207	287	297	240	243	263	263
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102	131	135	217	217	146	148	243	243	133	133	191	191	140	140	220	220	324	324	207	207	287	297	243	243	263	263
103	135	135	217	219	146	146	243	243	133	133	191	193	140	140	218	220	324	324	207	207	287	287	243	243	263	263
104	135	135	217	217	146	148	243	243	133	133	191	191	140	140	220	220	324	324	207	207	287	287	243	243	263	263
105	135	135	217	217	146	146	243	243	133	133	191	191	140	142	220	220	324	324	207	207	287	287	243	243	263	266
106	135	135	217	217	146	148	243	243	133	133	191	193	140	140	220	220	324	324	207	207	287	287	243	243	263	263
107	135	135	217	217	146	146	241	243	133	133	191	191	140	142	220	220	324	324	205	207	287	287	241	243	263	266
108	135	135	217	217	146	146	243	243	133	133	191	191	140	142	220	220	324	324	207	207	287	297	243	243	266	266

Sample ID	ISA		PV13		PV17		PV31		PV139		PV141		PV91		PV127		PV104		PV88		PV103		PV83		PV101	
109	131	135	217	217	146	148	243	243	133	133	191	193	140	142	220	220	324	324	207	207	287	287	243	243	263	266
110	135	135	217	217	148	148	243	243	133	133	191	191	140	142	218	220	324	324	207	207	287	287	243	243	263	263
111	131	135	217	217	148	148	243	245	133	133	191	193	140	142	220	220	324	324	207	207	287	297	243	243	263	266
112	131	135	217	217	146	146	243	243	131	133	191	193	142	142	220	220	324	324	207	207	287	287	243	243	263	263
113	135	135	217	217	146	146	243	243	133	133	191	191	140	142	220	220	324	324	207	207	287	297	243	243	263	263
114	131	135	217	219	148	148	243	243	133	133	191	191	140	142	218	220	324	324	207	207	287	287	243	243	263	266
115	131	135	217	219	146	146	243	243	133	133	191	191	140	142	220	220	324	324	207	207	287	297	243	243	263	263
116	135	135	217	217	146	146	243	243	133	133	191	191	140	140	220	220	324	324	207	207	297	297	243	243	263	266
117	135	135	217	219	146	148	243	243	133	133	191	191	140	142	218	220	324	324	207	207	287	287	243	243	263	263
118	135	135	217	217	146	148	243	243	133	133	191	191	140	140	220	220	324	324	207	207	287	287	243	243	263	266
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120	127	135	217	217	146	148	243	243	133	133	191	193	140	140	220	220	324	324	207	207	287	287	243	243	263	266
121	135	135	217	219	146	146	243	243	133	133	191	191	140	140	220	220	324	324	207	207	287	297	243	243	0	0
122	135	135	217	217	144	146	243	243	133	133	191	191	142	142	220	220	324	324	207	207	287	297	243	243	263	263
123	135	135	217	217	146	148	243	243	133	133	191	191	140	142	220	220	324	324	207	207	287	287	243	243	263	266
124	131	135	217	217	146	146	243	243	133	133	191	193	140	142	220	220	324	324	205	207	287	297	243	243	263	266
125	0	0	0	0	0	0	0	0	133	133	191	193	140	142	220	220	324	324	207	207	287	297	243	243	263	266
126	135	135	217	217	146	148	243	243	133	133	191	191	142	142	220	220	324	324	207	207	287	287	241	243	263	263
127	127	131	217	217	146	148	243	243	133	133	191	191	140	142	220	220	324	324	207	207	287	287	243	243	263	263
128	135	135	217	217	146	146	243	243	133	133	191	191	140	140	220	220	324	324	207	207	287	287	243	243	263	263
129	135	135	217	217	148	148	243	243	133	133	191	193	142	142	220	220	324	324	205	207	287	287	243	243	263	263
130	135	135	217	217	146	146	243	245	133	133	191	191	140	140	220	220	324	324	207	207	287	287	243	243	263	263

Sample ID	ISA		PV13		PV17		PV31		PV139		PV141		PV91		PV127		PV104		PV88		PV103		PV83		PV101	
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133	127	135	217	219	146	148	243	243	133	133	193	193	142	142	220	220	324	324	207	207	287	287	243	243	263	263
134	135	135	217	217	146	148	243	243	133	133	191	191	140	140	220	220	324	324	207	207	287	287	243	243	266	266
135	131	135	217	217	146	148	243	243	133	133	191	193	140	142	220	220	324	324	207	207	287	287	243	243	263	263
136	131	135	217	217	146	148	243	243	133	133	191	191	140	140	218	220	324	324	207	207	287	287	243	243	263	263
137	135	135	217	217	146	146	243	243	133	133	191	193	140	142	218	220	324	324	207	207	287	287	243	243	263	266
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139	127	135	217	217	146	146	243	243	133	133	191	193	140	140	220	220	324	324	207	207	287	297	243	243	263	263
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143	135	135	217	217	146	146	243	243	133	133	191	191	140	142	220	220	324	324	207	207	287	287	241	243	263	266
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146	127	135	217	219	146	148	243	243	133	133	191	191	140	142	220	220	324	324	207	207	287	297	243	243	263	266
147	135	135	217	219	146	146	243	243	133	133	191	193	140	140	220	220	324	324	205	207	287	297	243	243	263	263
148	127	135	217	217	146	146	243	243	133	133	191	191	140	142	220	220	324	324	205	207	287	297	243	243	263	266
149	131	135	217	217	146	146	243	243	133	133	191	191	140	140	220	220	324	324	207	207	287	287	243	243	263	263
150	135	135	217	219	146	148	243	243	133	133	191	191	140	140	220	220	324	324	205	207	287	297	243	243	266	266
151	131	135	217	217	146	148	243	243	133	133	191	191	140	142	220	220	324	324	207	207	287	287	243	243	263	266
152	131	135	217	217	146	148	243	245	133	135	191	191	140	142	220	220	324	324	207	207	287	297	243	243	263	263

Sample ID	ISA		PV13		PV17		PV31		PV139		PV141		PV91		PV127		PV104		PV88		PV103		PV83		PV101	
153	135	135	217	217	148	148	243	243	133	133	191	191	140	140	220	220	324	324	205	207	297	297	243	243	263	263
154	135	135	217	217	146	148	243	243	133	135	191	191	140	142	220	220	324	324	207	207	287	297	243	243	263	263
155	127	131	217	219	146	146	243	243	133	133	191	191	140	142	220	220	324	324	207	207	287	287	243	243	263	263
156	131	135	217	219	146	146	243	243	133	133	191	191	140	142	220	220	324	324	207	207	287	297	243	243	266	266
157	135	135	217	217	146	146	243	243	133	133	191	191	140	142	220	220	324	324	207	207	287	287	243	243	263	266
158	135	135	217	219	146	146	243	243	133	133	191	191	140	140	220	220	324	324	207	207	287	287	243	243	266	266
159	127	135	217	217	148	148	243	243	133	133	191	191	140	140	220	220	324	324	207	207	287	287	243	243	263	263
160	135	135	217	217	148	148	243	243	133	133	191	191	140	140	220	220	324	324	205	207	287	287	243	243	263	266
161	131	135	217	217	148	148	243	243	133	133	191	191	140	142	220	220	324	324	207	207	287	287	243	243	263	266
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173	131	135	217	217	146	146	243	243	133	133	191	191	140	142	220	220	324	324	205	207	287	287	243	243	263	266
174	135	135	217	217	146	146	243	243	133	133	191	193	140	140	220	220	324	324	207	207	287	297	243	243	263	266

Sample ID	ISA		PV13		PV17		PV31		PV139		PV141		PV91		PV127		PV104		PV88		PV103		PV83		PV101	
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176	127	135	217	217	146	146	243	243	133	133	191	191	140	140	220	220	324	324	205	207	287	297	243	243	263	263
177	127	131	217	219	146	146	243	243	133	133	191	191	140	142	220	220	324	324	207	207	287	287	243	243	263	263
178	135	135	217	219	146	148	243	243	133	133	191	191	140	142	220	220	324	324	207	207	297	297	243	243	263	263
179	135	135	217	219	146	148	241	243	133	133	191	193	142	142	218	220	324	324	207	207	287	287	243	243	263	266
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181	127	135	217	217	146	146	243	243	133	133	191	193	140	140	220	220	324	324	207	207	287	297	243	243	263	263
182	135	135	217	217	146	146	243	243	133	133	191	191	142	142	220	220	324	324	207	207	287	297	243	243	263	266
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187	131	135	217	219	146	146	243	243	133	135	191	193	140	142	220	220	324	324	207	207	287	297	243	243	263	263
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192	131	135	217	217	146	148	243	243	133	133	191	191	140	142	220	220	324	324	207	207	287	287	243	243	263	266
193	127	127	217	217	146	148	243	243	133	133	191	191	140	140	220	220	324	324	205	207	287	297	243	243	263	266
194	135	135	217	217	146	146	241	243	133	133	191	193	140	142	220	220	324	324	207	207	287	297	243	243	263	263
195	135	135	217	217	146	148	243	245	133	133	191	191	140	142	218	220	324	324	207	207	287	287	243	243	263	266
196	135	135	217	217	146	146	243	243	133	133	191	191	142	142	220	220	324	324	207	207	287	287	243	243	263	266

Sample ID	ISA		PV13		PV17		PV31		PV139		PV141		PV91		PV127		PV104		PV88		PV103		PV83		PV101	
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200	127	135	217	217	146	148	243	243	133	133	191	191	140	142	220	220	324	324	205	207	287	287	243	243	263	263
201	127	135	217	217	146	148	243	243	133	135	191	193	140	140	220	220	324	324	207	207	287	287	243	243	263	266
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203	135	135	217	217	146	148	243	243	133	133	191	191	140	140	220	220	324	324	207	207	287	287	243	243	263	263
204	131	135	217	217	146	148	243	243	133	133	191	191	140	140	220	220	324	324	207	207	287	287	243	243	266	266
205	131	135	217	217	146	148	243	243	133	133	191	191	142	142	220	220	324	324	205	207	287	297	243	243	263	263
206	135	135	217	219	146	148	243	243	133	133	191	193	140	142	220	220	324	324	207	207	287	287	243	243	263	266
207	135	135	217	217	146	146	243	243	133	133	191	191	140	142	218	220	324	324	207	207	287	287	243	243	263	266
208	135	135	217	217	146	148	243	243	133	133	191	193	140	142	220	220	324	324	207	207	287	287	243	243	263	263
209	135	135	217	217	146	148	243	243	133	135	191	193	140	142	220	224	324	324	207	207	287	287	243	243	263	266
210	127	135	217	217	146	146	243	243	133	133	191	193	140	142	220	220	324	324	205	207	287	287	243	243	263	263
211	135	135	217	217	0	0	243	243	133	133	191	191	140	142	220	220	324	324	207	207	287	297	243	243	263	266
212	135	135	217	217	146	148	243	243	133	133	191	191	140	140	220	220	324	324	207	207	287	287	243	243	263	266
213	135	135	217	217	146	146	243	243	133	133	191	191	140	142	220	220	324	324	205	207	287	297	243	243	263	263
214	135	135	217	217	146	148	243	243	133	133	191	191	140	140	220	220	324	324	207	207	287	287	243	243	263	266
215	131	135	217	217	146	148	243	243	133	133	191	191	140	142	220	220	324	324	207	207	287	287	243	243	263	266
216	135	135	217	217	146	148	243	243	133	133	191	191	140	140	220	220	324	324	205	207	287	287	243	243	263	263
217	131	135	217	217	146	146	243	243	133	133	191	191	140	142	220	220	324	324	207	207	287	287	243	243	263	263
218	135	135	217	217	146	148	243	243	133	133	191	191	140	142	220	220	324	324	205	207	287	287	243	243	263	266

Sample ID	ISA		PV13		PV17		PV31		PV139		PV141		PV91		PV127		PV104		PV88		PV103		PV83		PV101	
219	127	135	217	219	146	148	243	243	133	133	191	191	140	142	220	220	324	324	207	207	287	297	243	243	263	263
220	131	135	217	217	146	148	243	243	133	133	191	193	140	142	220	220	324	324	207	207	287	297	243	243	263	266
221	135	135	217	217	146	148	243	243	133	133	191	191	140	142	220	220	324	324	207	207	287	297	243	243	263	263
222	135	135	217	217	146	148	243	243	133	133	191	193	140	142	220	220	324	324	207	207	287	297	243	243	263	266
223	135	135	217	217	146	148	243	243	133	133	191	191	140	142	220	220	324	324	207	207	287	297	243	243	263	266
224	135	135	217	217	146	148	243	243	133	133	191	193	140	142	220	220	324	324	207	207	287	287	243	243	263	263
225	135	135	217	219	146	146	243	243	133	133	191	193	140	140	220	220	324	324	207	207	287	287	243	243	263	266
226	135	135	217	217	146	148	243	243	133	133	0	0	140	142	220	220	324	324	207	207	287	287	243	243	263	263
227	135	135	217	217	146	146	241	243	133	135	0	0	140	140	220	220	324	324	207	207	287	287	243	243	263	266
228	135	135	217	217	146	146	243	243	133	133	191	193	140	140	220	220	324	324	207	207	287	287	243	243	263	266
229	135	135	217	217	146	148	243	243	133	133	191	191	140	140	220	220	324	324	207	207	287	297	243	243	263	266
230	135	135	217	217	146	148	243	243	133	133	191	191	140	142	220	220	324	324	207	207	287	297	243	243	263	263
231	135	135	217	217	146	148	243	243	133	133	191	191	140	142	220	220	324	324	207	207	287	297	243	243	263	266
232	135	135	217	217	146	148	243	243	133	133	191	193	140	142	220	220	324	324	207	207	287	297	243	243	263	266
233	135	135	217	217	146	146	243	243	133	133	191	193	140	142	220	220	324	324	207	207	287	297	243	243	263	263
234	135	135	217	217	146	146	243	243	133	133	191	191	140	140	220	220	324	324	207	207	287	287	243	243	263	266
235	135	135	217	219	146	148	243	243	133	133	191	191	140	140	220	220	324	324	207	207	287	287	243	243	263	263
236	131	131	217	219	146	146	243	243	133	133	191	191	140	142	220	220	324	324	207	207	287	287	243	243	263	263
237	135	135	217	219	146	146	243	243	133	133	191	193	140	142	220	220	324	324	207	207	287	287	243	243	263	263
238	131	131	217	219	146	146	243	243	133	133	191	193	140	142	220	220	324	324	207	207	287	287	243	243	263	263
239	131	135	217	217	146	148	243	243	133	133	191	193	140	142	220	220	324	324	207	207	287	297	243	243	263	266
240	131	135	217	217	146	146	243	243	133	133	191	191	140	142	220	220	324	324	207	207	287	287	243	243	263	266

Sample ID	ISA		PV13		PV17		PV31		PV139		PV141		PV91		PV127		PV104		PV88		PV103		PV83		PV101	
241	131	135	217	217	146	148	243	243	133	133	191	193	140	142	220	220	324	324	207	207	287	297	243	243	263	266
242	131	135	217	217	146	148	243	243	133	133	191	193	140	142	220	220	324	324	207	207	287	297	243	243	263	266
243	131	135	217	217	146	148	243	243	133	133	191	193	140	142	220	220	324	324	207	207	287	297	243	243	263	266
244	127	135	217	217	148	148	243	243	133	133	191	193	140	140	220	220	324	324	207	207	287	287	243	243	263	263
245	131	135	217	217	146	146	243	243	133	133	191	191	140	142	220	220	324	324	207	207	287	287	243	243	263	266
246	135	135	217	217	146	146	243	243	133	133	191	193	140	142	220	220	324	324	207	207	287	297	241	243	263	263
247	135	135	217	217	146	146	243	243	133	133	191	193	142	142	220	220	324	324	205	207	287	287	243	243	263	266
248	131	135	217	217	146	148	243	243	133	133	191	193	140	140	220	220	324	324	207	207	287	287	243	243	263	263
249	135	135	217	217	146	146	243	243	133	133	191	191	142	142	220	220	324	324	207	207	287	287	243	243	263	263
250	131	135	217	217	146	146	243	243	133	133	191	191	140	140	220	220	324	324	205	207	287	297	243	243	263	263
251	0	0	217	217	146	146	243	243	133	133	0	0	140	142	220	220	324	324	207	207	287	297	241	243	263	263
252	135	135	217	217	146	148	243	243	0	0	0	0	140	140	220	220	324	324	207	207	287	297	243	243	263	266
253	135	135	217	217	146	148	243	243	133	133	0	0	140	140	220	220	324	324	207	207	287	297	243	243	263	266
254	131	135	217	217	146	146	243	243	133	133	191	191	140	142	220	220	324	324	207	207	287	297	243	243	263	266
255	135	135	217	217	146	146	243	243	133	133	191	193	142	142	220	220	324	324	207	207	287	287	243	243	263	266
256	135	135	217	219	146	148	243	243	133	133	191	191	140	142	220	220	324	324	207	207	287	287	243	243	263	263
257	135	135	217	219	146	148	243	243	133	133	191	191	140	142	220	220	324	324	207	207	287	297	243	243	263	266
258	131	135	217	217	146	146	243	243	133	133	191	193	140	142	220	220	324	324	207	207	287	297	243	243	263	266
259	135	135	217	217	146	146	243	243	133	135	191	193	140	142	220	220	324	324	207	207	287	287	243	243	263	266
260	135	135	217	217	146	146	243	243	133	135	191	193	140	142	220	220	324	324	207	207	287	287	243	243	263	263
261	135	135	217	217	146	146	243	243	133	133	191	193	140	142	220	220	324	324	205	207	287	297	243	243	263	266
262	135	135	217	217	146	148	243	243	133	133	191	193	142	142	220	220	324	324	207	207	287	297	243	243	263	266

Sample ID	ISA		PV13		PV17		PV31		PV139		PV141		PV91		PV127		PV104		PV88		PV103		PV83		PV101	
263	135	135	0	0	0	0	0	0	133	133	191	191	140	142	220	220	324	324	207	207	287	297	243	243	266	266
264	135	135	217	217	146	148	243	243	133	133	191	193	140	142	220	220	324	324	207	207	297	297	243	243	263	263
265	135	135	217	217	146	148	243	243	133	133	193	193	140	140	220	220	324	324	207	207	287	287	243	243	263	266
266	131	135	217	219	146	148	243	243	133	133	191	193	140	142	220	220	324	324	207	207	287	297	243	243	263	263
267	135	135	217	217	146	146	243	243	0	0	0	0	140	142	220	220	324	324	207	207	287	297	243	243	263	266
268	135	135	217	219	146	146	241	243	0	0	0	0	140	142	220	220	324	324	207	207	287	297	243	243	263	266
269	131	135	217	217	146	148	243	243	0	0	0	0	140	142	220	220	324	324	207	207	287	297	243	243	263	266
270	135	135	217	217	146	146	243	243	133	133	0	0	140	142	220	220	324	324	207	207	287	297	243	243	263	266
271	127	135	217	217	146	148	243	243	133	133	193	193	140	140	220	220	324	324	207	207	287	287	243	243	266	266
272	135	135	217	217	146	148	243	243	133	133	191	193	140	140	220	220	324	324	207	207	287	287	243	243	263	266
273	135	135	217	217	146	148	243	243	133	133	191	193	140	142	220	220	324	324	207	207	287	297	243	243	263	266
274	135	135	217	217	146	148	243	243	133	133	191	193	140	140	220	220	324	324	207	207	287	297	243	243	263	266
275	131	135	217	219	146	148	243	243	133	133	191	193	140	142	220	220	324	324	207	207	287	297	243	243	263	266
276	131	135	217	217	146	148	243	243	133	133	191	193	140	142	220	220	324	324	207	207	287	297	243	243	263	266
277	135	135	217	217	146	146	243	243	133	133	191	193	140	140	220	220	324	324	207	207	287	287	243	243	263	266
278	131	135	217	217	146	148	243	243	133	133	191	193	140	142	220	220	324	324	207	207	287	297	243	243	263	266
279	127	135	217	219	146	146	243	243	133	133	191	193	140	140	220	220	324	324	207	207	287	287	243	243	266	266
280	135	135	217	219	146	146	243	243	133	133	191	191	140	140	220	220	324	324	207	207	287	297	243	243	263	266
281	131	135	217	217	146	148	243	243	133	133	191	193	140	142	220	220	324	324	205	207	287	287	243	243	263	263
282	135	135	217	219	146	146	243	243	133	133	191	193	140	142	220	220	324	324	207	207	287	287	243	243	266	266
283	135	135	217	217	146	146	241	243	133	133	191	191	140	140	220	220	324	324	207	207	287	287	243	243	266	266
284	135	135	217	217	146	146	243	243	133	133	191	191	140	140	220	220	324	324	207	207	287	297	243	243	263	263

Sample ID	ISA		PV13		PV17		PV31		PV139		PV141		PV91		PV127		PV104		PV88		PV103		PV83		PV101	
285	131	135	217	217	146	146	243	243	133	133	191	191	140	140	220	220	324	324	207	207	287	297	243	243	263	266
286	135	135	217	217	146	146	243	243	133	133	191	193	140	140	220	220	324	324	207	207	287	287	243	243	263	263
287	135	135	217	217	146	146	243	243	133	133	191	193	140	142	220	220	324	324	207	207	287	297	243	243	263	266
288	135	135	217	217	146	148	243	243	133	133	191	191	140	140	220	220	324	324	207	207	287	297	243	243	263	266
289	135	135	217	219	146	148	243	243	133	133	191	193	140	142	220	220	324	324	207	207	287	287	243	243	263	266
290	135	135	217	217	146	148	243	243	133	133	191	193	140	142	220	220	324	324	207	207	287	287	243	243	261	261
291	127	135	217	217	146	146	243	243	133	133	191	191	140	140	220	220	324	324	207	207	287	287	243	243	263	266
292	135	135	217	217	146	146	243	243	133	133	191	191	140	140	220	220	324	324	207	207	287	297	243	245	263	266
293	127	135	217	219	146	146	243	243	133	133	191	191	140	140	220	220	324	324	207	207	287	287	243	243	263	266
294	135	135	217	217	146	148	243	243	133	133	191	193	140	142	220	220	324	324	207	207	287	287	243	243	263	266
295	135	135	217	219	146	148	243	243	133	133	191	193	140	142	220	220	324	324	207	207	287	287	243	243	263	266
296	131	135	217	217	146	148	243	243	133	133	0	0	140	142	220	220	324	324	207	207	287	297	243	243	263	266
297	135	135	217	217	146	146	243	243	133	133	191	191	140	142	220	220	324	324	207	207	287	287	243	243	263	263
298	135	135	217	219	146	146	243	243	133	133	191	193	140	140	218	220	324	324	207	207	287	297	243	243	263	263
299	135	135	217	217	146	148	243	243	133	133	191	193	140	142	220	220	324	324	207	207	287	297	243	243	263	266
300	131	131	217	217	146	146	243	243	133	133	191	191	140	142	220	220	324	324	207	207	287	287	243	243	263	266
301	131	135	217	217	146	148	243	243	133	133	191	191	140	140	220	220	324	324	207	207	287	297	243	243	263	266
302	135	135	217	217	146	148	243	243	133	133	191	193	140	142	220	220	324	324	207	207	287	297	243	243	263	266
303	135	135	217	217	146	148	243	243	133	133	191	193	140	142	220	220	324	324	207	207	287	297	243	243	263	263
304	135	135	217	219	146	146	243	243	0	0	0	0	140	140	220	220	324	324	207	207	287	287	243	243	263	266
305	135	135	217	217	146	148	243	243	133	133	191	193	140	142	220	220	324	324	207	207	287	287	243	243	263	266
306	131	135	217	219	146	148	243	243	133	133	191	193	140	142	220	220	324	324	207	207	287	287	243	243	263	266

Sample ID	ISA		PV13		PV17		PV31		PV139		PV141		PV91		PV127		PV104		PV88		PV103		PV83		PV101	
307	135	135	217	217	146	148	243	243	133	133	0	0	140	142	220	220	324	324	207	207	287	287	243	243	263	263

Supplementary table 3 *Plasmopara viticola* samples that shared identical multilocus profile divided in subgroups.

Sample ID	Winegrowing areas	Management system	Genotype (allelic profile)	No. of sample	Genotype label
177	Mag	I	127131217219146146243243133133191191140142220220324324207207287287243243263263g	2	A
155	Mag	O+	127131217219146146243243133133191191140142220220324324207207287287243243263263g		
20	Ter	O+	127135217217146146243243133133191191140142220220324324207207297297243243263263g	2	B
19	Ter	O+	127135217217146146243243133133191191140142220220324324207207297297243243263263g		
181	Mag	I	127135217217146146243243133133191193140140220220324324207207287297243243263263g	2	C
139	Tr	O	127135217217146146243243133133191193140140220220324324207207287297243243263263g		
43	Ter	I	131135217217146146241243133133191191140140220220324324205207287297243243266266g	2	D
41	Ter	I	131135217217146146241243133133191191140140220220324324205207287297243243266266g		
245	SaM	O	131135217217146146243243133133191191140142220220324324207207287287243243263266g	2	E
240	Sam	I	131135217217146146243243133133191191140142220220324324207207287287243243263266g		
87	Tr	O+	131135217217146146243243133133191193140140220220324324207207287287243243263263g	2	F
17	Ter	O+	131135217217146146243243133133191193140140220220324324207207287287243243263263g		
124	Tr	I	131135217217146146243243133133191193140142220220324324205207287297243243263266g	2	G
14	Ter	O+	131135217217146146243243133133191193140142220220324324205207287297243243263266g		
7	Ter	0+	131135217217146148243243133133191191140140220220324324207207287287243243263263g	2	H
4	Ter	O+	131135217217146148243243133133191191140140220220324324207207287287243243263263g		
301	Rov	O	131135217217146148243243133133191191140140220220324324207207287297243243263266g	2	I
167	Mag	O+	131135217217146148243243133133191191140140220220324324207207287297243243263266g		
215	Mag	O	131135217217146148243243133133191191140142220220324324207207287287243243263266g	3	J
192	Mag	I	131135217217146148243243133133191191140142220220324324207207287287243243263266g		

Sample ID	Winegrowing areas	Management system	Genotype (allelic profile)	No. of sample	Genotype label
151	Mag	O+	1311352172171461482432431331331911911401422202203243242072072872872432432632666g		
248	SaM	O	13113521721714614824324313313319119314014022022032432420720728728724324326326 3g	2	K
171	Mag	O+	13113521721714614824324313313319119314014022022032432420720728728724324326326 3g		
109	Tr	I	1311352172171461482432431331331911931401422202203243242072072872872432432632666g	2	L
93	Tr	O+	1311352172171461482432431331331911931401422202203243242072072872872432432632666g		
278	Vdl	O	1311352172171461482432431331331911931401422202203243242072072872972432432632666g	7	M
276	Vdl	O	1311352172171461482432431331331911931401422202203243242072072872972432432632666g		
243	SaM	I	1311352172171461482432431331331911931401422202203243242072072872972432432632666g		
242	SaM	I	1311352172171461482432431331331911931401422202203243242072072872972432432632666g		
241	SaM	I	1311352172171461482432431331331911931401422202203243242072072872972432432632666g		
239	SaM	I	1311352172171461482432431331331911931401422202203243242072072872972432432632666g		
220	Mag	O	1311352172171461482432431331331911931401422202203243242072072872972432432632666g		
306	Rov	O	1311352172191461482432431331331911931401422202203243242072072872872432432632666g	2	N
12	Ter	O+	1311352172191461482432431331331911931401422202203243242072072872872432432632666g		
234	SaM	I	1351352172171461462432431331331911911401402202203243242072072872872432432632666g	6	O
202	Mag	O	1351352172171461462432431331331911911401402202203243242072072872872432432632666g		
69	Ter	O	1351352172171461462432431331331911911401402202203243242072072872872432432632666g		
66	Ter	O	1351352172171461462432431331331911911401402202203243242072072872872432432632666g		
26	Ter	I	1351352172171461462432431331331911911401402202203243242072072872872432432632666g		
16	Ter	O+	1351352172171461462432431331331911911401402202203243242072072872872432432632666g		
191	Mag	I	1351352172171461462432431331331911911401402202203243242072072872972432432632666g	2	P
60	Ter	O	1351352172171461462432431331331911911401402202203243242072072872972432432632666g		

Sample ID	Winegrowing areas	Management system	Genotype (allelic profile)	No. of sample	Genotype label
116	Tr	I	135135217217146146243243133133191191140140220220324324207207297297243243263266g	2	Q
35	Ter	I	135135217217146146243243133133191191140140220220324324207207297297243243263266g		
297	Rov	I	135135217217146146243243133133191191140142220220324324207207287287243243263263g	2	R
54	Ter	O	135135217217146146243243133133191191140142220220324324207207287287243243263263g		
157	Mag	O+	135135217217146146243243133133191191140142220220324324207207287287243243263266g	4	S
105	Tr	I	135135217217146146243243133133191191140142220220324324207207287287243243263266g		
85	Tr	O+	135135217217146146243243133133191191140142220220324324207207287287243243263266g		
18	Ter	O+	135135217217146146243243133133191191140142220220324324207207287287243243263266g		
277	Vdl	O	135135217217146146243243133133191193140140220220324324207207287287243243263266g	2	T
228	SaM	O+	135135217217146146243243133133191193140140220220324324207207287287243243263266g		
287	Rov	O+	135135217217146146243243133133191193140142220220324324207207287297243243263266g	2	U
199	Mag	O	135135217217146146243243133133191193140142220220324324207207287297243243263266g		
307	Rov	O	135135217217146148243243133133001401422202203243242072072872872432432632 63g	2	V
226	SaM	O+	135135217217146148243243133133001401422202203243242072072872872432432632 63g		
203	Mag	O	135135217217146148243243133133191191140140220220324324207207287287243243263263g	3	W
141	Tr	O	135135217217146148243243133133191191140140220220324324207207287287243243263263g		
104	Tr	I	135135217217146148243243133133191191140140220220324324207207287287243243263263g		
214	Mag	O	135135217217146148243243133133191191140140220220324324207207287287243243263266g	3	X
212	Mag	O	135135217217146148243243133133191191140140220220324324207207287287243243263266g		
118	Tr	I	135135217217146148243243133133191191140140220220324324207207287287243243263266g		
288	Rov	O+	135135217217146148243243133133191191140140220220324324207207287297243243263266g	4	Y
229	SaM	O+	135135217217146148243243133133191191140140220220324324207207287297243243263266g		

Sample ID	Winegrowing areas	Management system	Genotype (allelic profile)	No. of sample	Genotype label
27	Ter	I	13513521721714614824324313313319119114014022022032432420720728729724324326326 6g		
25	Ter	O+	13513521721714614824324313313319119114014022022032432420720728729724324326326 6g		
218	Mag	O	13513521721714614824324313313319119114014222022032432420520728728724324326326 6g	2	Z
56	Ter	O	13513521721714614824324313313319119114014222022032432420520728728724324326326 6g		
183	Mag	I	13513521721714614824324313313319119114014222022032432420720728728724324326326 6g	2	AA
123	Tr	I	13513521721714614824324313313319119114014222022032432420720728728724324326326 6g		
230	SaM	O+	13513521721714614824324313313319119114014222022032432420720728729724324326326 3g	3	BB
221	Mag	O	13513521721714614824324313313319119114014222022032432420720728729724324326326 3g		
82	Tr	B	13513521721714614824324313313319119114014222022032432420720728729724324326326 3g		
231	SaM	O+	13513521721714614824324313313319119114014222022032432420720728729724324326326 6g	3	CC
223	Mag	O	13513521721714614824324313313319119114014222022032432420720728729724324326326 6g		
96	Tr	O+	13513521721714614824324313313319119114014222022032432420720728729724324326326 6g		
274	Vdl	O	13513521721714614824324313313319119314014022022032432420720728729724324326326 6g	2	DD
22	Ter	O+	13513521721714614824324313313319119314014022022032432420720728729724324326326 6g		
142	Tr	O	13513521721714614824324313313319119314014222022032432420520728728724324326326 3g	2	EE
91	Tr	O+	13513521721714614824324313313319119314014222022032432420520728728724324326326 3g		
224	SaM	O+	13513521721714614824324313313319119314014222022032432420720728728724324326326 3g	3	FF
208	Mag	O	13513521721714614824324313313319119314014222022032432420720728728724324326326 3g		
198	Mag	I	13513521721714614824324313313319119314014222022032432420720728728724324326326 3g		
305	Rov	O	13513521721714614824324313313319119314014222022032432420720728728724324326326 6g	4	GG
294	Rov	I	13513521721714614824324313313319119314014222022032432420720728728724324326326 6g		
138	Tr	O	13513521721714614824324313313319119314014222022032432420720728728724324326326 6g		

Sample ID	Winegrowing areas	Management system	Genotype (allelic profile)	No. of sample	Genotype label
15	Ter	O+	13513521721714614824324313313319119314014222022032432420720728728724324326 3266g		
302	Rov	O	13513521721714614824324313313319119314014222022032432420720728729724324326 3266g	5	HH
299	Rov	O	13513521721714614824324313313319119314014222022032432420720728729724324326 3266g		
273	Vdl	O	13513521721714614824324313313319119314014222022032432420720728729724324326 3266g		
232	SaM	O+	13513521721714614824324313313319119314014222022032432420720728729724324326 3266g		
222	Mag	O+	13513521721714614824324313313319119314014222022032432420720728729724324326 3266g		
262	Vdl	I	13513521721714614824324313313319119314214222022032432420720728729724324326 3266g	2	II
119	Tr	I	13513521721714614824324313313319119314214222022032432420720728729724324326 3266g		
39	Ter	I	13513521721914614624124313313319119314014221822032432420720728729724324326 3263g	2	JJ
36	Ter	I	13513521721914614624124313313319119314014221822032432420720728729724324326 3263g		
70	Ter	O	13513521721914614624324313313319119114214222022032432420720728728724324326 3263g	3	KK
68	Ter	O	13513521721914614624324313313319119114214222022032432420720728728724324326 3263g		
67	Ter	O	13513521721914614624324313313319119114214222022032432420720728728724324326 3263g		
256	Vdl	O+	13513521721914614824324313313319119114014222022032432420720728728724324326 3263g	2	LL
37	Ter	I	13513521721914614824324313313319119114014222022032432420720728728724324326 3263g		
257	Vdl	O+	13513521721914614824324313313319119114014222022032432420720728729724324326 3266g	2	MM
44	Ter	I	13513521721914614824324313313319119114014222022032432420720728729724324326 3266g		
295	Rov	I	13513521721914614824324313313319119314014222022032432420720728728724324326 3266g	3	NN
289	Rov	I	13513521721914614824324313313319119314014222022032432420720728728724324326 3266g		
206	Mag	O	13513521721914614824324313313319119314014222022032432420720728728724324326 3266g		