UNIVERSITÀ
DEGLI STUDI
DI PADOVA

Sede Amministrativa: Università degli Studi di Padova

Dipartimento di Agronomia Animali Alimenti Risorse Naturali e Ambiente

---

CORSO DI DOTTORATO DI RICERCA IN: CROP SCIENCE

CICLO: 34°

ENVIRONMENTALLY SUSTAINABLE TOOLS FOR GRAPEVINE PROTECTION AGAINST FUNGAL AND OOMYCETES DISEASES

Activity of peptaibol analogs and a grape chitinase against the grape pathogens *B. cinerea* and *P. viticola*

Coordinatore: Ch.mo Prof. Claudio Bonghi

Supervisore: Ch.mo Prof. Francesco Favaron

Co-Supervisore: Ch.mo Prof. Luca Sella

Dottorando: Angela Bolzonello

Declaration

I hereby declare that this submission is my own work and that, to the best of my knowledge and belief, it contains no material previously published or written by another person nor material which to a substantial extent has been accepted for the award of any other degree or diploma of the University or other institute of higher learning, except where due acknowledgment has been made in the text.

A copy of the thesis will be available at

http://paduaresearch.cab.unipd.it/

Index

ABSTRACT **9**

CHAPTER I 11

OVERVIEW ON GRAPEVINE AND ITS MAIN PATHOGENS IN TEMPERATE CLIMATE AREAS: *BOTRYTIS CINEREA* AND *PLASMOPARA VITICOLA* ... 11

1.1. Grapevine distribution and economic relevance **13**

1.2. Grapevine pathogens: main actors **15**
1.2.1. *Botrytis cinerea* on grapevine 15
Life cycle 15
Grapevine tolerance 18
Disease control 19
1.2.2. *Plasmopara viticola* 19
Life cycle 20
Grapevine resistance 23
Disease control 24

1.3. References **27**

OBJECTIVES OF THE THESIS **35**

CHAPTER II 37

A*VITIS VINIFERA* CHITINASE IV IS CLEAVED BY *BOTRYTIS CINEREA* PROTEASE ACTIVITY AND LOOSE ITS ANTIFUNGAL ACTIVITY 37

2.1 Abstract **39**

2.2. Introduction **41**

2.3 Material and methods **45**
2.3.1. Plant materials 45
2.3.2. Fungal strains and cultures 45
2.3.3. Extraction and purification of grape proteins 45
2.3.4. Production of protease activity and protease assay 46
2.3.5. Purification of *B. cinerea* proteases 47
2.3.6. Incubation of grape chitinase with *B. cinerea*, fungal crude protease or purified protease fractions 47
2.3.7. Blotting of chitinases for N-terminal sequencing and molecular modeling 48
2.3.8. Chitinase activity 49
2.3.9. Analysis of chito-oligomers produced by chitinase activity 50
2.3.10. Characterization of *B. cinerea* protease activity against grape chitinase by inhibition assays .. 51
2.3.11. Grape proteins administration to fungal mycelium for RNA extraction and to conidia for germination assay 52
2.3.12. Infection on *V. vinifera* leaves 53
2.3.13. RNA extraction and gene expression 53

2.3.14. Statistical analysis 54

2.4. Results 57
2.4.1. Grape proteins purification 57
2.4.2. *B. cinerea* protease activity on grape chitinase 58
2.4.3. Chitinase activity decreases when the CBD is removed 61
2.4.4. Characterization of protease activity 63
2.4.5. Inhibition of chitinase cleavage in presence of *B. cinerea* 65
2.4.6. Effect of chitinase administration on expression of *B. cinerea* chitin synthase and deacetylase genes 66
2.4.7. Effect of native and cleaved chitinase on *B. cinerea* conidia germination 67
2.4.8. Transcript expression, enzymatic activity, and protein detection of chitinase in *B. cinerea* infected leaves 68

2.5. Discussion 71

2.6. References 75

CHAPTER III 81

TARGETED AMINO ACID SUBSTITUTIONS IN A *TRICHODERMA* PEPTAIBOL CONFER ACTIVITY AGAINST INFECTION OF *PLASMOPARA VITICOLA* AND *BOTRYTIS CINEREA* 81

3.1. Abstract 83

3.2. Introduction 85

3.3. Material and methods 87
3.3.1. Water-soluble analogs of trichogin 87
3.3.2. Peptides assays against *Plasmopara viticola* 88
Grapevine leaf material 88
Plasmopara viticola strain, propagation and production of zoospores 88
P. viticola infection experiments and peptides treatments 89
Microscopic observations 89
3.3.3. Peptides assays against *Botrytis cinerea* 90
Fungal strain and conidia production 90
In vitro assays 90
Microscopy analysis 90
In vivo assays 91

3.4. Results 93
3.4.1. Peptides activity against *P. viticola* 93
In vivo experiments 93
Microscopic observations 95
3.4.2. Peptides activity against *B. cinerea* 97
In vitro experiments 97
In vivo experiments 99

3.5. Discussion 101

3.6. References 105

MAIN CONCLUSIONS **109**

RINGRAZIAMENTI **111**

ABSTRACT

Grapevine productions is worldwide affected by fungal pathogens both in the field and during post-harvest storage. In addition to yield losses, even the quality of grape and wine is affected. The management of these pathogens mainly relies on chemicals and agronomical practices. The majority of the plant protection products (PPPs) distributed in the vineyard are directed to control *Botrytis cinerea* and *Plasmopara viticola*, causal agents of gray mold and downy mildew disease, respectively. However, pathogens can develop resistance to synthetic fungicides. Moreover, the high use of PPPs poses serious risks to the environment and the health of operators and consumers. In this regard, European Union (UE) policies foresee a 50% reduction in the use of chemical pesticides by 2030 and the implementation of integrated approaches for crop protection.

The research studies presented in this thesis share the final purpose to find out new alternative strategies and molecules to counteract grapevine pathogens. To this aim, a deep knowledge of the pathogenic mechanism is fundamental. Therefore, in the first part of the thesis, the interaction between *B. cinerea* and grapevine was investigated by characterizing the ability of this fungus to detoxify plant defense proteins. During ripening, grape berries accumulate a class IV chitinase, a pathogenesis related (PR) protein with antifungal activity. However, the protease activity of *B. cinerea* is able to cleave this chitinase impairing its antifungal activity. This research could represent a first step into the identification of new fungal virulence factors to be counteracted.

The second part of the thesis focused on the development of new biopesticides active against *P. viticola* and *B. cinerea*. The effectiveness of several water-soluble analogs produced by targeted amino acid substitutions of an antimicrobial peptide (peptaibol) naturally produced by *Trichoderma longibrachiatum*, was evaluated against the two pathogens. The assays allowed to identify a peptide highly effective against both pathogens that may be developed as a biopesticide.

CHAPTER I

Overview on Grapevine and its main pathogens in temperate climate areas: *Botrytis cinerea* and *Plasmopara viticola*

1.1. Grapevine distribution and economic relevance

The *Vitaceae* family includes about 60 wild *Vitis* species distributed all around the world. Among them, *Vitis vinifera* L. is the main species cultivated today while others are mostly used for rootstocks or as donors of resistance genes (This *et al.*, 2004; Terral *et al.*, 2010).

The wild grapevine is an heliophilous liana growing along river banks and in deciduous or semi-deciduous forests. It is distributed in a wide area from Western Europe to the Trans-Caucasian zone and around the Mediterranean Basin (Arnold *et al.*, 1998; Terral *et al.*, 2010; Marvelli *et al.*, 2013).

Archaeobotanical studies provided us indirect evidence about the long history of grapevine domestication and cultivation that likely began between the 7th and 4th millennium BC in the geographical area between the Black Sea and Iran (Figure 1) (McGovern *et al.*,1996; Zohary *et al.*, 2000; Terral *et al.*, 2010; Marvelli *et al.*, 2013).

Considering that grapevine is one of the major crops all around the world in relation to both grape and wine production (Pertot *et al.*, 2017), those ancient evidences suggest and confirm as *V. vinifera* acquired a significant economic interest from the beginning of its domestication to nowadays (Terral *et al.*, 2010).

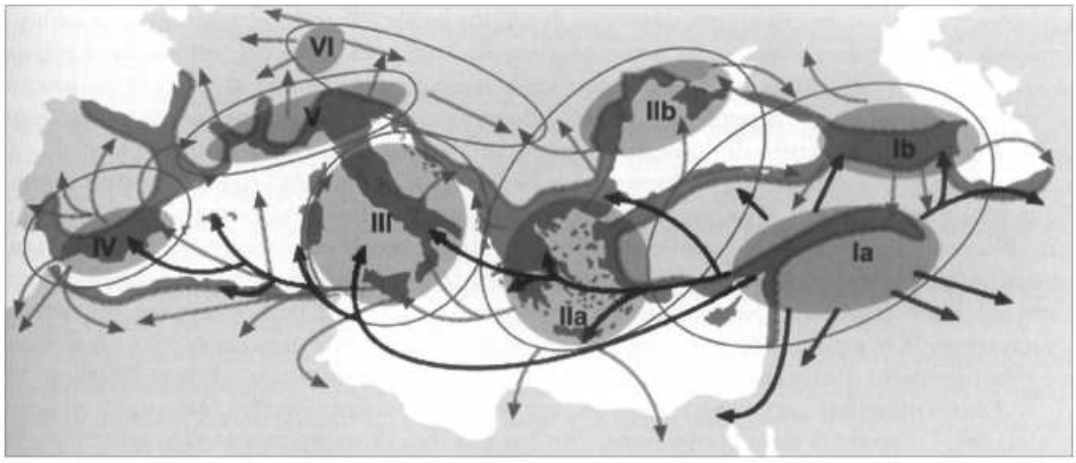

Figure 1: Grapevine domestication centers with relative diffusion phases (I-VI) (Marvelli *et al.*, 2013)

According to statistics updated in 2018 by the Food and Agriculture Organization of the United Nations (FAO), the European Union is responsible for approximately 60% and 35% of wine and grape world production, respectively (Figure 2). Despite the small size of Italy, our country is the main world producer of wine with 5.4 million tons, followed by France and Spain, and the second for grape production with 8.5 million tons of grape, following China and before South

American states (FAOSTAT, 2018). Again, those data highlight the relevance of grapevine cultivation at the national, European, and worldwide levels.

In the following chapters, different research studies will be presented concerning the interaction of grapevine with two of the major fungal and oomycetes pathogens affecting field production, namely *Botrytis cinerea* and *Plamopara viticola*. These studies were conducted on three different clones of relevance in theVeneto Region: Incrocio Manzoni 6.0.13b. (IM 6.0.13b), Glera and Pinot bianco. In particular, IM 6.0.13b., which represents the major result of the breeding research of Prof. Luigi Manzoni at the *"Regia Scuola di Viticoltura e di Enologia di Conegliano"* on white berry resulting from the breeding *Riesling renano* X *Pinot bianco* (Cancellier *et al.*, 1997), was selected because of a background of knowledge about its chitinase enzymes synthesized in berries (Vincenzi *et al.*, 2014). The clone Pinot bianco was interesting for the slight epicarp of berries and close structure of the bunch that confer higher susceptibility to pathogens. Lastly, Glera was chosen because of its high susceptibility to pathogens and its widespread in the last decades thanks to the DOC denomination assignment to the wine produced with its berries in a typical area and known all around the world (Prosecco DOC).

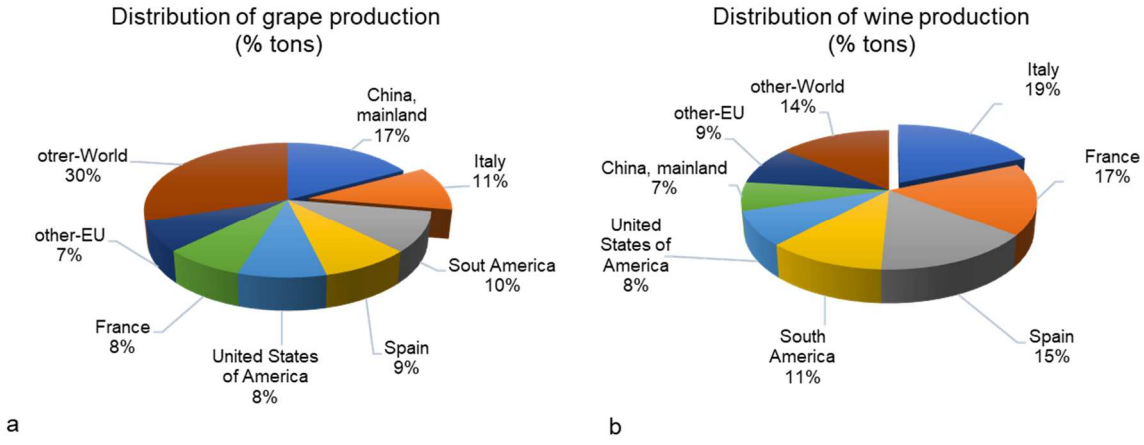

Figure 2: Pie charts representing the distribution of grape production (a) and wine production (b) all around the world in 2018 (raw data from https://www.fao.org/faostat/en/ #data).

1.2. Grapevine pathogens: main actors

All around the world, fungal diseases are considered the most serious problem for grapevine cultivation because of the extensive losses in production and quality. In temperate climates, *Plasmopara viticola* and *Botrytis cinerea* together with *Erysiphe necator* are the main pathogens affecting grapevine and imposing the largest amount of treatments in vineyards (Pertot *et al.*, 2017). *P. viticola* and *B. cinerea* are the fungal pathogens considered in this thesis.

1.2.1. *Botrytis cinerea* on grapevine

World-wide *B. cinerea* Pers. Fr. [teleomorph *Botrytinia fuckeliana* (De Bary) Whetzel] causes heavy economic losses, infecting more than 200 plant species at any plant tissue and stage of development (Elmer and Michailides, 2007; Williamson *et al.*, 2007; González-Rodriguez *et al.*, 2015), including the post-harvest stage (Dean *et al.*, 2012; Williamson *et al.*, 2007).

This necrotrophic fungus is responsible for gray mold disease. In grapevine, under mild wet weather, it causes bunch rot with accumulation of metabolites that reduce wine quality as consequence of the chemical composition of berries (Bocquet and Valade, 1995; Agudelo-Romero *et al.*, 2015). Indeed, gray mold disease has negative consequences on wine fermentation: some metabolites kill yeasts leading sometimes to an early arrest of fermentation (Hong *et al.*, 2012; Agudelo-Romero *et al.*, 2015). Another collateral damage caused by *B. cinerea* infection consists on haze formation in white wines. The infection process, in fact, stimulates the plant to accumulate a high amount of defense proteins named pathogenesis-related (PR) proteins, in particular chitinases (Sels *et al.*, 2008), which have been demonstrated to be responsible for haze formation, making wines unattractive for consumers (Waters *et al.*, 1991; Vincenzi *et al.*, 2005; Marangon *et al.*, 2011).

Despite the negative aspects above reported, under climatic conditions slowing down the infection rate *B. cinerea* can develop a "Noble rot" that can be exploited to produce relevant sweet white wines, which can be sold for prices up to 500 €/bottle (Dean *et al.*, 2012; Lovato *et al.*, 2019).

Life cycle

In vineyards, this pathogen represents a part of the environmental microflora (Keller *et al.*, 2003). *B. cinerea* overwinters as sclerotia or mycelium on dying host tissues (Williamson *et al.*, 2007) or on the soil surface (Elmer and Michailides, 2007). Conidia produced in late winter and early spring on sclerotia and over-wintering mycelium are considered the most important source

of spring inoculum (Williamson *et al.*, 2007; Elmer and Michailides, 2007); sporadic differentiation of apothecia are also reported, and insects and invertebrates may act as casual vectors of inoculum (Figure 3) (Elmer and Michailides, 2007).

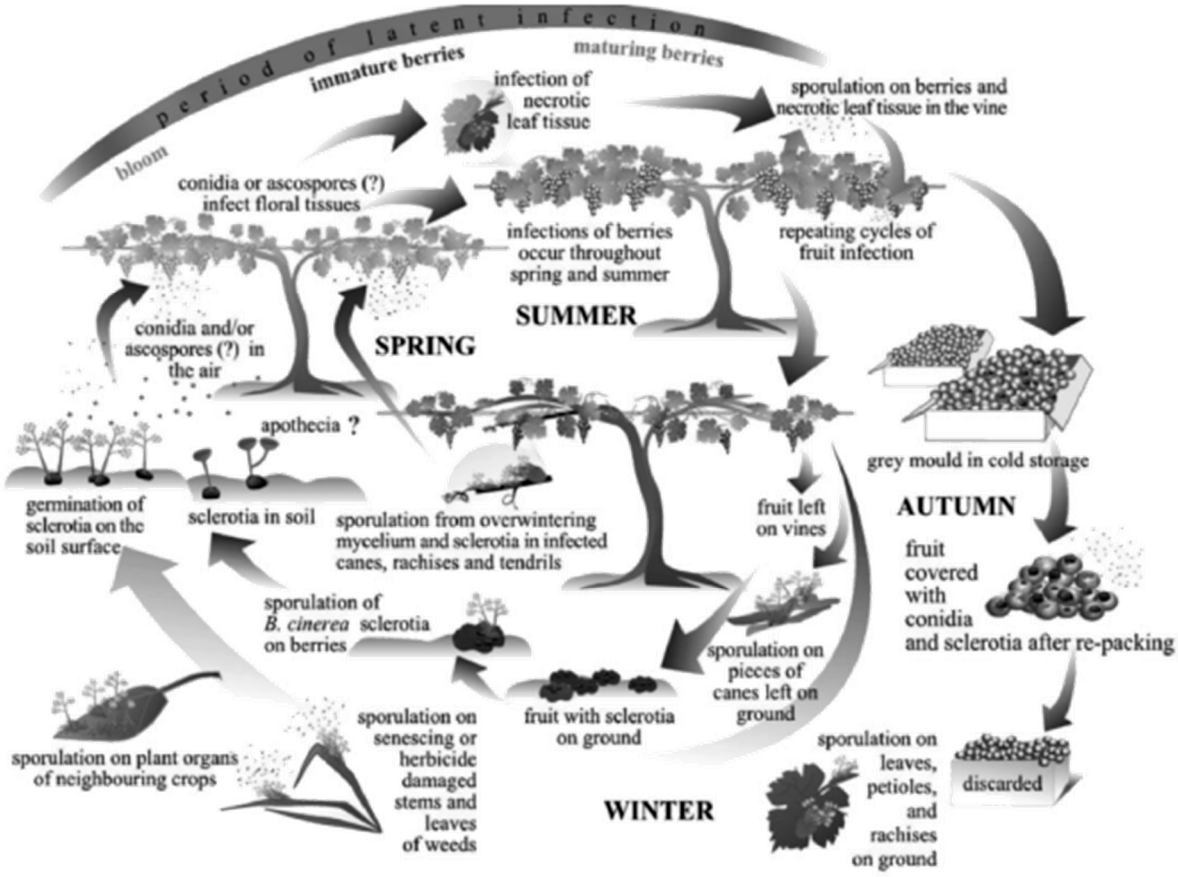

Figure 3: Proposed life cycle of *Botrytis cinerea* in wine and table grape vineyards (from Elmer and Michailides, 2007)

The primary inoculum follows a well-defined cycle regulated by temperature and humidity: even if sporulation can occur in darkness, conidia production is mainly stimulated by specific wave length (generally closed to UV); a rapid decline in humidity, simultaneously to a rise in temperature in the early morning, determines the drying of conidiophores with the consequent ejection of conidia into air currents, which appears to be the major dispersal method (Williamson *et al.*, 2007). Once on the host, with appropriate environmental conditions, *B. cinerea* spores germinate (Leroch *et al.*, 2013) and the fungus differentiates specific structures called appressoria or infection cushions, which allow direct penetration into intact tissues, stomata, or micro-fissures in the grape berry skin (Holz *et al.*, 2007; Williamson *et al.*, 2007). In grapes, floral infections are considered fundamental in the epidemiology of *B. cinerea*. Conidia mainly infect grape flower receptacle but also stigma and styles. However, host defense responses keep the pathogen into a latent phase that will end in correspondence with berry

ripening. The pathogen can also colonize flower debris remaining in the bunch; in this way, *B. cinerea* colonizes with a saprophytic life-style the developing bunch, but as soon as proper conditions occur a great amount of conidia will be spread on the bunch surfaces producing a new strong inoculum ready to infect ripe berries (Keller *et al.*, 2003; Elmer and Michailides, 2007; Williamson *et al.*, 2007).

Lateral pedicle and rachis are also susceptible to *B. cinerea* at the early stages of grape berry development and, except for the pedicle, the resistance increases as the season progresses (Holz, 2001; Elmer and Michailides, 2007). Microscopic lesions on the grape cuticle of swollen berries play a role in the later infections, offering an open door to the pathogen; those become a source of new conidia and give start to the "pre-harvest polycyclic epidemic" (Keller *et al.*, 2003; Elmer and Michailides, 2007; Williamson *et al.*, 2007). Indeed, the berry-to-berry contact when the cuticle is absent or very thin increases the susceptibility of grape berries to *B. cinerea* infection (Marois *et al.*, 1986; Rosenquist and Morrison, 1989; Elmer and Michailides, 2007), and more severe bunch rot occurs on cultivar characterized by tight clusters (Vail and Marois, 1991; Vail *et al.*, 1998; Elmer and Michailides, 2007). The tolerance of grape berries to *B. cinerea* fails during ripening due to some physiological changes. In immature berries, there is a constitutive presence of antifungal compounds such as phenolic compounds, polymeric proanthocyanidins, and tannins on the skin, which are toxic to *B. cinerea* (Pezet *et al.*, 2004; Sarig *et al.*, 1998). With ripening, sugar content inside berries increases, the thickness of the epidermal cell layer decreases due to the conversion of skin cells into pulp tissue (Kretschmer *et al.*, 2007), and in those cells, tannic content decreases. All these changes were demonstrated to be correlated with the increase of berries susceptibility to *B. cinerea* (Deytieux-Belleau *et al.*, 2009), allowing the quick berry-to-berry spread above mentioned (Figure 4).

In this epidemic scenario, environmental conditions are fundamental for the pathogen life cycle. Even if *B. cinerea* can develop in a wide range of temperatures (5-30 °C), the optimum temperatures for growth and sporulation are 20 °C and 21 °C, respectively (Matta *et al.*, 2017). However, water availability appears as the vital factor for *B. cinerea* development (Pearson and Goheen, 1996; Latorre and Rioja, 2002; Alonso-Villaverde *et al.*, 2008). In particular, relative humidity (RH) is a crucial factor for *B. cinerea*, which requires high humidity for conidial germination, infection (Harrison *et al.*, 1994; Williamson *et al.*, 2007), and sporulation (Matta *et al.*, 2017).

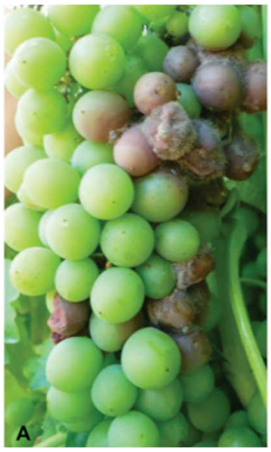

Figure 4: *B. cinerea* infection on Trincadeira berries before veraison (from Agudelo-Romero *et al.*, 2015).

Grapevine tolerance

Resistant genes against *B. cinerea* are not yet known (Pertot *et al.*, 2017). Therefore, all the high-yield grafted grapevine varieties are susceptible to this pathogen (Rosslenbroich and Stuebler, 2000), although different levels of susceptibility have been identified. Among commercial *V. vinifera* cultivars, a moderate resistance was found only in "Emperor" and "Autumn Black", whereas the highly resistant grapes belong to *V. labrusca* or hybrids (Gabler *et al.*, 2003). The different level of susceptibility to *B. cinerea* of varieties or clones is mainly due to physiochemical features, both preformed and induced upon infection, interfering with gray mold disease. Thickness of the berry skin and the number of skin cell layers (Karadimtcheva, 1981; Sarig *et al.*, 1998;), cell density in the skin (Prudet *et al.*, 1992; Sarig *et al.*, 1998), the thickness of the berry cuticle, and the wax content (Hill *et al.*, 1981; Marois *et al.*, 1986; Percival *et al.*, 1993; Commenil *et al.*, 1997), berry density within a cluster (Vail and Marois 1991; Percival *et al.*, 1993), cluster morphology (Alonso-Villaverde *et al.*, 2008), the number of pores or microscopic tears in the skin of the berry (Eibach, 1994) and antifungal constituents like tannins and phenolic compounds inside berries (Sarig *et al.*, 1998; Goetz *et al.*, 1999) were reported as related with the tolerance to *B. cinerea*. Among inducible factors, there are phytoalexins and pathogenesis-related (PR) proteins. Phytoalexins mainly induced by *B. cinerea* infection are stilbenes such as *trans*- and *cis*- resveratrol, α- and ε- viniferin and protostilbene (Langcake and McCarthy, 1979; Jeandet *et al.*, 1991; Adrian *et al.*, 2000); among PR proteins, chitinase, grape osmotin, β-1,3-glucanase, phenylalanine ammonialyase and stilbene synthase are induced after pathogen infection (Liswidowati *et al.*, 1991; Pocock *et al.*, 2000; Sarry *et al.*, 2004; da Silva *et al.*, 2005; Monteiro *et al.*, 2007). American *Vitis* spp. and interspecific hybrids with higher tolerance to *B. cinerea* show a higher capacity to synthetize stilbenes (Bavaresco and Fregoni, 2001; Keller et al., 2003). However, this ability is not sufficient to guarantee resistance to

pathogens, as it was demonstrated that *B. cinerea* is able to degrade or detoxified stilbenes during infection (Pezet *et al.*, 1991; Breuil *et al.*, 1998; Favaron *et al.*, 2009); furthermore, *B. cinerea* could neutralize also grapevine PR protein making them insoluble (Favaron *et al.*, 2009), sequestering them or affecting chitinase integrity (Marcato *et al.*, 2017).

Disease control

Since 1793, when sulphur and potassium were recommended to control this disease in vineyards (Rosslenbroich and Stuebler, 2000), chemical control of *B. cinerea* is still the main strategy adopted to reduce disease incidence in grapevine and other major crops (Leroux, 2007; Pertot *et al.*, 2017). Several synthetic botryticides, characterized by different modes of action, are currently available (Rosslenbroich and Stuebler, 2000). Botryticides have been distinguished into fungicides affecting fungal respiration (boscalid), osmoregulation (fludioxonil), microtubules formation (benzimidazoles), amino acids biosynthesis (anilinopyrimidines), and inhibiting sterol biosynthesis (fenhexamid). Resistance phenomena are associated with several botryticide families (see FRAC Code list 2021), thus alternating or mixing different groups of fungicides is a suggested strategy (Leroux, 2007). Environmental and human health aspects have pushed towards the search for new tools for disease management in the framework of Integrated Pest Management (IPM). Reduction of fungicides usage against *B. cinerea* can be achieved integrating the use of less susceptible varieties (except for wine produced in typical areas such as DOC, DOCG in Italy or OAC in France), agronomic practices to reduce risk of infections and the use of biofungicides, where the active ingredient is a botanical extract (botanicals) or a beneficial microorganism (biocontrol agent or BCA) or its derivate. Currently, *Bacillus subtilis, B. amyloliquefaciens and Aureobasidium pullulans* are authorized in Europe against *B. cinerea* on grapevine (Pertot *et al.*, 2017).

1.2.2. *Plasmopara viticola*

Plasmopara viticola is an obligate biotrophic oomycete responsible for downy mildew disease on grapevine (Göker *et al.*, 2007; Gessler *et al.*, 2011; Yin *et al.*, 2017). It is recognized as one of the most serious diseases that affects worldwide viticulture (Korterkamp, 2006; Boso and Kassemeyer, 2008; Dagostin *et al.*, 2011; Pressotti *et al.*, 2011; Yin *et al.*, 2017), mainly in warm and humid climates areas (Boso and Kassemeyer, 2008) with high annual rainfall (Emmet *et al.*, 1992; Lafon *et al.*, 1988; Kennelly *et al.*, 2007).

The first isolation of the downy mildew causal agent is dated 1834 in the northeastern USA, where *P. viticola* is endemic. First classified as *Botrytis cana* (Gregory, 1915), in 1848 Berkley

and Curtis re-classified it as *B. viticola*. De Bary (expert of *Phytophtora infestans*) placed the pathogen in the genus *Peronospora* as *P. viticola* (De Bary, 1863), while the separation of the genus in Peronospora and Plasmopara (Schröter, 1886) was subsequently used by Berlese and de Toni (1888) to rename it as *Plasmopara viticola* (Gessler *et al.*, 2011). The first attribution as causal agent of downy mildew is from 1876 (Farlow, 1876; Gessler *et al.*, 2011). It was the last grapevine disease that arrived in Europe from America, following powdery mildew and phylloxera (Gessler *et al.*, 2011). Actually, its European diffusion from 1878 appears related with phylloxera pandemic: it was probably accidentally introduced with American grape cuttings used to replace French destroyed vineyard (Boso and Kassemeyer, 2008; Gessler *et al.*, 2011; Yin *et al.*, 2017; Fontaine *et al.*, 2021).

Recently, it has been demonstrated by Fontaine *et al.* (2021) that the *P. viticola* population, invasive in Europe, was the source of worldwide diffusion of the pathogen, probably mediated by grapevine material (Figure 5).

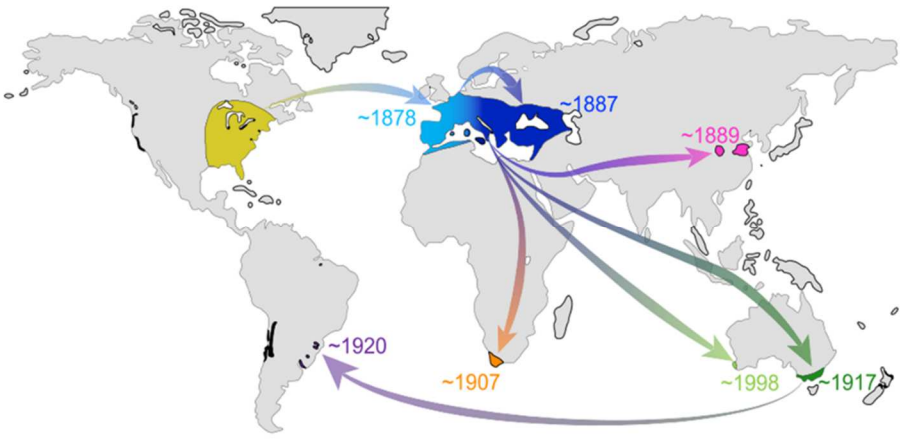

Figure 5: Geographic representation of the invasion scenario with the highest likelihood based on the approximate Bayesian computation random forest (ABC-RF) analysis; areas are shown in color, based on their population assignment. Dates represent the first report of the disease in each area of introduction. Arrows indicate the most likely invasion pathways. Vineyards in other regions of the world not included in this study are indicated in black (from Fontaine *et al.*, 2021).

Life cycle

The life cycle of this pathogen was described first by Ravaz et Verge (1913) and Gregory (1915). Later, several models have been constructed to determine infection risks (Gessler *et al.*, 2011). *P. viticola* is an heterotallic oomycete (Wong *et al.*, 2001; Kennelly *et al.*, 2007) and produces oospores under rather dry conditions or on senescent leaves (Grünzel, 1961), maturing over the winter (Gessler *et al.*, 2011). Primary infection, in spring, requires a minimum atmospheric temperature of 10 °C, at least 10 mm of rain in the previous 48 h and 10 cm shoot length

(corresponding to opened leaves measuring 6-8 cm^2) (Baldacci 1947; Baldacci and Refatti, 1956; Goidànich, 1964). During primary infection, oospores germinate producing macrosporangia, which release zoospores (Kennelly *et al.*, 2007) once exposed to a water film and in particular when the outer walls are broken as result of a light freeze and sufficient humidity (Gessler *et al.*, 2011). Macrosporangia are spread by rain splash and air turbulence onto leaves, where zoospore are released (Gessler *et al.*, 2011). Zoospores are motile thanks to two flagella (Hardham, 2007) that allow them to swim toward stomata. Zoospores settle in proximity of stomata to germinate and then penetrate leaf forming a swelling into the stomatal cavity. *P. viticola* infection occurs within 3 h at 20 °C with or without light and only through stomata (Müller-Thurgau, 1911; Gessler *et al.*, 2011); this may explain the ontogenic resistance of berries and, even if to a lesser extent, leaves (Kennelly *et al.*, 2005; Gessler *et al.*, 2011). Stomata are essential for both infection and sporulation, indeed *P. viticola* is able to alter their opening on infected leaves, probably to facilitate the emergence of sporangiophores (Allègre *et al.*, 2007). When *P. viticola* penetrates the host, the pathogen develops an intercellular network of mycelium in the mesophyll tissue and differentiates haustoria to obtain nutrients from plant cells, causing the so called 'oil spot' symptom on the adaxial leaf side (Figure 6). In presence of appropriate climate conditions, sporulation occurs within 6 days on the abaxial surface through stomata (Figure 6) (Allègre *et al.*, 2007), otherwise in the absence of sporulating condition the potential productivity of sporangia is maintained for about 20 days (Kennelly *et al.*, 2007). Sporangia produced by primary infection are responsible for the secondary infections. Repeated cycles of secondary infections are thought to determine the epidemic, but recently it has been demonstrated that also oospores, which can survive over seasons for years, can contribute to epidemic progress (Gobbin *et al.*, 2003; Kennelly *et al.*, 2004; Gobbin *et al.*, 2005; Kennelly *et al.*, 2007). Indeed, sporulation and secondary infections appear more selective in terms of climate conditions necessary to produce sporangia and cause infection: darkness and high humidity to produce and maintain living zoosporangia, rain to spread zoosporangia and, finally, presence of water film after a minimum of degree-hours of 50 °C x h for zoospores release and infection (Gessler *et al.*, 2011). Moreover, even if each lesion can sporulate several times, sporangial production declines after the third sporulation event (Kennelly *et al.*, 2007).

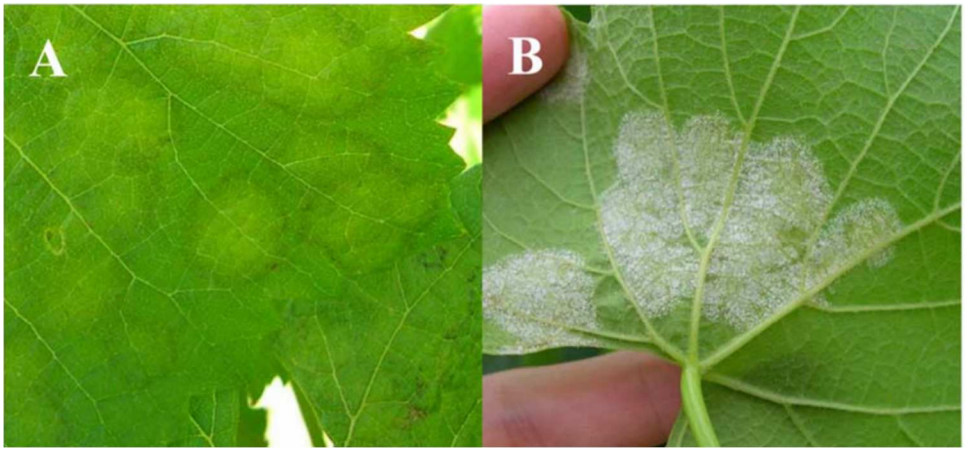

Figure 6: oil spot on the adaxial leaf surface (A) and profuse sporulation on the abaxial leaf surface (B) typical of primary infections of *P. viticola* on grape leave (from Kennelly *et al.*, 2007).

Even if leaf lesion results in reduced photosynthetic activity that may affect productivity, damages on bunches are more relevant. During a brief period before and after flowering, bunch are high susceptible and infection results in a discolored bunch with sporulation events (Figure 7) followed by losses of infected necrotic tissues. With bunch ontogenesis, stomata on the surface of berries are converted into lenticels, but berries can be still invaded through the susceptible pedicel even if the pathogen is then unable to sporulate (Figure 7), leading to yield and quality losses. Thus, the ontogenic resistance of the fruit is not sufficient to contrast the production losses due to *P. viticola* that can infect grape berries also from vegetative close tissues (pedicels and rachids) maintaining functional stomata. This makes necessary the maintenance of a healthy canopy throughout all the vegetative season (Kennelly *et al.*, 2005).

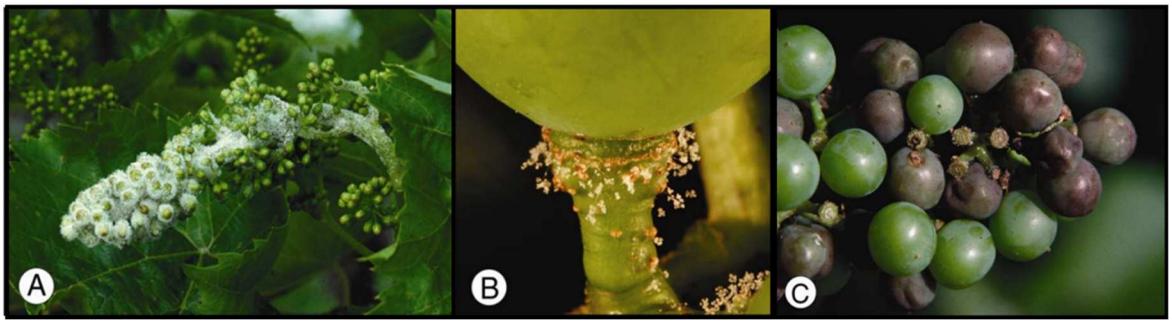

Figure 7: Symptoms of grapevine downy mildew on different bunch developmental stages. Sporulation on pre-bloom flowers, pedicels and rachis (A). Sporulation on pedicel before symptoms appearance on berry (B). Discoloration of berries with necrosis of pedicel and/or sporulation on it (C) (from Kennelly *et al.*, 2005).

Grapevine resistance

In North America, the coevolution of grapevine with *P. viticola* led to the development of resistant or immune species (Boso and Kassemeyer, 2008; Yin *et al.*, 2017) such as *V. riparia*, *V. rupestris* (Alleweldt *et al.*, 1988) or *Muscadinia rotundifolia* (Staudt and Kassemeyer, 1995). European *V. vinifera* cultivars, instead, showed high susceptibility causing huge losses in the whole continent (Milliardet, 1883; Viala, 1887; Ravaz and Verge, 1911; Boso and Kassemeyer, 2008; Gessler *et al.*, 2011; Yin *et al.*, 2017). Also in the Asiatic continent, among Chinese wild *Vitis* species, *V. pseudoreticulata* (accession 1057/1058) was found resistant to downy mildew and others display different levels of resistance (Yu *et al.*, 2012).

Several studies compared genotypes resistant and susceptible to *P. viticola* and/or non-host pathogens. Among physiochemical mechanisms involved in the resistance, an inner cuticular rim between stomata cavity and stomata (Jurges *et al.*, 2009) and less attractive factors released by open stomata seem relevant for resistance (Díez-Navajas *et al.*, 2008). Stomatal movement and reactive oxygen species (ROS) production into guard cells are also involved in protection against downy mildew, but other mechanisms are activated by recognition of pathogen elicitors (Allègre *et al.*, 2009). Indeed, infection occurs in both susceptible and resistant genotypes, but resistant genotypes can stop the hyphal growth at an early stage (Unger *et al.*, 2007) or after haustoria development (Díez-Navajas *et al.*, 2008). Gene expression analysis of resistant genotypes during infection highlighted the up-regulation of genes encoding stilbene synthases (Kortekamp, 2006; Malacarne *et al.*, 2011), receptor-like protein kinases, TIR-NBS receptors, Calcium-dependent protein kinases, ethylene synthesis and responsive factors, PR-5, PR-2 and photosynthetic proteins (Malacarne *et al.*, 2011). Differently from *B. cinerea*, several quantitative trait loci (QTL) associated with *P. viticola* resistance (*Rpv*) have been identified such as *Rpv* 1, 2 and 3 originated from North American species (Bellin *et al.*, 2009) or *Rpv* 10 from Asian species (Schwander *et al.*, 2012). These monogenic resistances, as for example the resistant allele Rpv3 in Bianca (Bellin *et al.*, 2009) can be overcome by some pathogen strains, as already reported (Peressotti *et al.*, 2010). Pyramiding resistance genes was demonstrated to ensure higher and more stable resistance, even if segregation study on hybrid genotypes highlighted that the same resistant profiles can include other resistance sources and mechanisms still unknown (Possamai *et al.*, 2020). Advanced breeding programs with Marker Assisted Selection (MAS) and genome-editing technology will allow to obtain resistant genotypes for quality wine production. However, on one side growers lack agronomical and oenological experience with new variety; on the other, the market asks for known wines produced in typical areas (Pertot *et al.*, 2017). Despite those bottlenecks, resistant cultivars should be considered an

important tool in the IMP framework, promoted also by the European Union (UE) policies (EU Directive 2009/128/CE).

Disease control

As mentioned before, as a result of its recent contact with the pathogen, the important cultivars of European grapevine (*V. vinifera*) are highly susceptible to *P. viticola* (Dagostin *et al.*, 2011). The disease is usually controlled by synthetic fungicides (Peressotti *et al.*, 2011) and copper, the latter allowed also in organic agriculture (Dagostin *et al.*, 2011). In 1882, copper was the first active substance identified as effective against downy mildew, posing the bases for the development of the Bourdeaux mixture by Millardet in 1885 (Millardet, 1885; Gessler *et al.*, 2011). Only later acupric fungicides were developed with successful results (Boubals *et al.*, 1954; Zobrist *et al.*, 1954). Since it was discovered, the amount of copper needed to counteract *P. viticola* was investigated with the aim to reduce it for economic or availability reasons (Gessler *et al.*, 2011). Furthermore, frequent applications of copper-containing fungicides to control downy mildew cause the accumulation of this heavy metal in soil and groundwater (Kortekamp, 2006), and residues in musts can result in detrimental effect on wine quality (Tromp and Klerk, 1988; Kortekamp, 2006). Nevertheless, also synthetic fungicides reveal their drawbacks in terms of toxicity (Gessler *et al.*, 2011) and also by promoting the development of resistant *P. viticola* strains (Herzog and Schüepp, 1985; Gullino *et al.*, 1997; Genet and Vincent, 1999; Fourie, 2004; Kortekamp, 2006). Currently, a number of synthetic fungicides with different mode of action is available against *P. viticola*: protectant fungicides such as Ditianon, Folpet and Fluazinam, systemic fungicides such as Fosetyl Al, Metalaxil-M and Benalaxil-M, mostly used to protect the vegetation in rapid growth, and penetrant products such as Ametoctradin, Amisulbron, Cyazofamid, Fluopicolide, Oxathiapiprolin, Zoxamide and those belonging to the Carboxylic Acid Amides (CAA) group, used to protect grape bunches having high affinity for grape skin waxes.

Nowadays, environmental and human health motivations prompt EU policies to promote the sustainable use of pesticides, with strict parameters for admitted pesticides. At this regard, EU has recently reduced the amount of copper fungicide to 28 kg/ha/7years (EU Directive 2009/128/CE; Regulation (EC) 1107/2009; Commission implementing regulation (EU) 2018/1981), and a further reduction is expected in the future.

In this scenario, several studies have been conducted with the perspective to find out effective alternatives to copper and chemicals able to protect grapevine from *P. viticola* infection. During last decades, many microorganisms have been identified as antagonists of *P. viticola* (Arnone *et al.*, 2008; Dagostin *et al.*, 2011; Gessler *et al.*, 2011), but none have been successfully developed

and used as biofungicide. Indeed, promising candidates in indoor trials demonstrated a reduced activity under field conditions due to the low fitness of these biocontrol agents (BCAs) (Guetsky *et al.*, 2001). Even the most studied fungal biocontrol agents, belonging to the *Trichoderma* genus, are effective only against wood diseases of grapevine (Pertot *et al.*, 2017). Indeed, *T. harzianum* strain T39 was demonstrated able to induce systemic resistance against downy mildew but unable to produce a direct antagonistic effect on *P. viticola* (Perazzolli *et al.*, 2008). Botanicals, such as *Salvia officinalis* extract, showed decreasing efficacy under field conditions (Dagostin *et al.*, 2011) due to its low rainfastness (Dagostin *et al.*, 2010). Despite the negative results obtained by Dagostin *et al.* (2011), commercial orange oil products have been developed against *P. viticola*.

1.3. References

- Adrian M, Jeandet P, Douillet-Breuil A C, Tesson L, Bessis R. Stilbene content of mature *Vitis vinifera* berries in response to UVC elicitation. Journal of Agricultural and Food Chemistry 48: 6103-6105 (2000).
- Agudelo-Romero P, Erban A, Rego C, Carbonell-Bejerano P, Nascimento T, Sousa L, Martínez-Zapater J M, Kopka J, Fortes1 A M. Trascriptome and metabolome reprogramming in *Vitis vinifera* cv Trincadeira berries upon infection with *Botrytis cinerea*. Journal of Experimental Botany. 66: 7: 17696-1785 (2015).
- Allègre M, Daire X, Héloir M-C, Trouvelot S, Mercier L, Adrian M, Pugin A. Stomata deregulation in *Plasmopara viticola*-infected grapevine leaves. New Phytologist 173: 832-840 (2007).
- Allègre M, Héloir M-C, Trouvelot S, Daire X, Pugin A, Wendehenne D, Adrian M. Are Grapevine Stomata Involved in the Elicitor-Induced Protection Against Downy Mildew? Molecular Plant-Microbe Interactions 22: 8: 977-986 (2009).
- Alleweldt G, Possingham J. Progress in grapevine breeding. Theoretical and Applied Genetics 75: 669–673 (1988).
- Alonso-Villaverde V, Boso S, Santiago J L, Gago P, Martínez M-C. Relationship Between Susceptibility to *Botrytis* Bunch Rot and Grape Cluster Morphology in the *Vitis vinifera* L. Cultivar Albariño. InternationaL Journal of Fruit Science 8: 4: 251-265 (2008)
- Arnold C, Gillet F, Gobat J M. Situation de la vigne sauvage *Vitis vinifera* subsp. *silvestris* en Europe. Vitis 37: 159–17 (1998).
- Arnone A., Nasini G, Panzeri W, d. Pava O V, Malpezzi L. Acremine G, dimeric metabolite from cultures of *Acremonium byssoides* A20. Journal of Natural Products 71: 146-149 (2008).
- Baldacci E and Refatti E. La lotta contro le due maggiori ampelopatie: peronospora (*Plasmopara viticola*) e oidio (*Uncinula necator*) in relazione alla conoscenza dei periodi di incubazione e dei cicli biologici. Annali della Facoltà di Agraria di Milano V: 35: 151–178 (1956).
- Baldacci E. Epifitie di *Plasmopara viticola* (1941–46) nell'Oltrepo pavese ed adozione del calendario di incubazione come istrumento di lotta. Atti Istituto Botanico Laboratorio Crittogamico di Pavia 8: 45–85 (1947).
- Bavaresco L and Fregoni C. Physiological role and molecular aspects of grapevine stilbenic compounds. Pages 153-182 in: Molecular Biology and Biotechnology of the Grapevine. Roubelakis Angelakis K A, ed. Kluwer Academic Publishers, Dordrecht, The Netherlands (2001).
- Bellin D, Peressotti E, Merdinoglu D, Wiedemann-Merdinoglu S, Adam-Blondon A-F, Cipriani G, Morgante M, Testolin R, Di Gaspero G. Resistance to *Plasmopara viticola* in grapevine 'Bianca' is controlled by a major dominant gene causing localized necrosis at the infection site. Theoretical and applied Genetics 120:163–176 (2009).
- Berlese A N, de Toni J B. In Saccardo, Sylloge Fungorum Omnium hucusque cognitorum, VII. (Pars. I.) *Phycomyceteae* auctoribus, *Peronosporaceae*, 233– 264 (1888).
- Bocquet F M D, Valade M. Etat sanitaire de la vendange et qualité des vins. Le Vigneron Champenois 7/8, 9 (1995).

- Boso S and Kassemeyer H H. Different susceptibility of European grapevine cultivars for downy mildew. *Vitis* 41:1: 39- 49 (2008).
- Boubals D, Vergnes A, Bobo H. Essais de fongicides organiques dans la lutte contre le mildiou de la Vigne effectués en 1954. Progres Agricole et Viticole 143: 64–74 (1954).
- Breuil A C, Adrian M, Pirio, N, Meunier P, Bessis R, Jeandet P. Metabolism of stilbene phytoalexins by *Botrytis cinerea*: 1. Characterization of a resveratrol dehydrodimer. Tetrahedron Letters 39: 537-540 (1998).
- Cancellier S, Roncador I. Gli incroci Manzoni. Camera di commercio industria artigianato agricoltura Treviso (1997).
- Commenil P, Brunet L, Audran J C. The development of the grape berry cuticle in relation to susceptibility to bunch rot disease. Journal of Experimental Botany 48:1599-1607 (1997).
- da Silva F G, Iandolino A, Al-Kayal F, Bohlmann M C, Cushman M A, Lim H, Ergui A, Figueroa R, Kabuloglu E K, Osborne C, Rowe J, Tattersall E, Leslie A, Xu J, Baek J M, Cramer G R, Cushman J C, Cook D R. Characterizing the grape transcriptome. Analysis of ex- pressed sequence tags from multiple *Vitis* species and development of a compendium of gene expression during berry development. Plant Physiology 139: 574-5 (2005).
- Dagostin S, Formolo T, Giovannini O, Pertot I, Schmitt A. *Salvia officinalis* extract can protect grapevine against *Plasmopara viticola*. Plant disease 94: 5: 575-580 (2010).
- Dagostin S, Schärer H-J, Pertot I, Tamm L. Are there alternative to copper for controlling grapevine downy mildew in organic viticulture? Crop protection 30: 776-788 (2011).
- De Bary A. Recherches sur le développement de quelques champignons parasites. Annales Sciences Naturelles. Paris, France Serie IV: 20: 5–148 (1863).
- Dean R, Van Kan J A L, Pretorius Z A, Hammond-Kosack K E, Di Pietro A, Spanu P D, Rudd J J, Dickman M, Kahmann R, Ellis J, Foster G D. The top 10 fungal pathogens in molecular plant pathology. Molecular Plant Pathology 12:4: 414-430 (2012).
- Deytieux- Belleau C, Geny L, Roudet J, Mayet V, Donèche B, Fermaud M. Grape berry skin features related to ontogenic resistance to *Botrytis cinerea*. European Journal of Plant Pathology 125: 551-563 (2009).
- Díez-Navajas A M, Wiedemann-Merdinoglu S, Greif C, Merdinoglu D. Nonhost versus host resistance to grapevine downy mildew, *Plasmopara viticola*, studied at the tissue level. Phytopathology 98: 776- 780 (2008).
- Eibach R. Defense mechanisms of the grapevine to fungus disease. American Vineyard 1: 8-10 (1994).
- Elmer P A G and Michailides T J. Epidemiology of *Botrytis cinerea* in orchard and vine crops. In *Botrytis*: Biology, Pathology and Control. Elad Y et al. (eds) 243-272 Springer (2007).
- Emmett R W, Wicks T J, Magarey P A. Downy mildew of grapes. Pages 90-128 in: Plant Diseases of International Importance: Diseases of Fruit Crops, edited by J. Kumar, H. Chaube, U. S. Singh, and A. Mukhopadhyay. Prentice Hall, Englewood Cliffs, NJ (1992).
- FAOSTAT dataset updated on 2018. https://www.fao.org/faostat/en#data
- Farlow W G. The American grape mildew. Bussy Institut Bulletin 1- 423 (1876).

- Favaron F, Lucchetta M, Odorizzi S, Pais da Cunha A T, Sella L. The role of grape polyphenols on trans-resveratrol activity against *Botrytis cinerea* and of fungal laccase on the solubility of putative grape pr proteins. Journal of Plant Pathology 91: 3: 579-588 (2009).
- Fontaine M C, Labbé F, Dussert Y, Delière L, Richart-Cervera S, Giraud T, Delmotte F. Europe as a bridgehead in the worldwide invasion history of grapevine downy mildew, *Plasmopara viticola*. Current Biology 31: 2155-2166 (2021).
- Fourie P H. Metalaxyl sensitivity status of downy mildew populations in western Cape vineyards. South African Journal of Enology and Viticulture 25: 19–22 (2004).
- FRAC Code list 2021 available online: https://www.frac.info
- Gabler F M, Smilanick J L, Mansour M, Ramming D W, Mackey B E. Correlations of morphological, anatomical and chemical features of grape berries with resistance to *Botrytis cinerea*. Phytopathology 93: 10 :1263–1273 (2003).
- Genet J-L and Vincent O. Sensitivity of European *Plasmopara viticola* populations to cymoxanil. Pesticide Science 55:129-136 (1999).
- Gessler C, Pertot I, Perazzolli M. Plasmopara viticola: a review of knowledge on downly mildew of grapevine and effective disease management. Phytopathologia Mediterranea 50: 3-44 (2011).
- Gobbin D, Jermini M, Loskill B, Pertot I, Raynal M, Gessler C. Importance of secondary inoculum of *Plasmopara viticola* to epidemics of grapevine downy mildew. Plant Pathology 54: 522-534 (2005).
- Gobbin D, Pertot I, Gessler C. Identification of microsatellite markers for *Plasmopara viticola* and establishment of high throughput method for SSR analysis. European Journal of Plant Pathology 109: 153-164 (2003).
- Goetz G, Fkyerat A, Metais N, Kunz M, Tabacchi R, Pezet R, Pont V. Resistance factors to grey mould in grape berries: Identification of some phenolics inhibitors of *Botrytis cinerea* stilbene oxidase. Phytochemistry 5: 759-767(1999).
- Goidànich G. Le Peronosporales. In: Manuale di Patologia Vegetale, Vol. II. Edizione agricole, Bologna, Italy, 315–346, 1283 pp (1964).
- Göker M, Voglmayr H, Riethmüller A, Oberwinkler F. How do obligate parasites evolve? A multi-gene phylogenetic analysis of downy mildews. Fungal Genetics and Biology 44: 105–122 (2007).
- González-Rodríguez V E, Liñeiro E, Colby T, Harzen A, Garrido C, Cantoral J M, Schmidt J, Fernández-Acero f J. Proteomic profiling of *Botrytis cinerea* conidial germination. Archives of Microbiology 197: 117-133 (2015).
- Gregory C. Studies on *Plasmopara viticola*. Reprint from official report of the Session of the internatioal congress of viticulture, July 12–13, San Francisco, CA, USA, 126–150 (1915).
- Grünzel H. Untersuchungen über die Oosporenbildung beim falschen Mehltau der Weinrebe (*Peronospora viticola* de Bary). Zeitschrift für Pflanzenkrankheiten, Pflanzenpathologie und Pflanzenschutz 68: 65–80 (1961).
- Guetsky R, Shtienberg D, Elad Y, Dinnor A. Combining biocontrol agents to reduce the variability of biological control. Biological control 91: 7: 621-627 (2001).
- Gullino M L, Mescalchin E, Mezzalama M, Sensitivity to cymoxanil in populations of *Plasmopara viticola* in northern Italy. Plant Pathology 46: 729–736 (1997).

- Hardham A R. Cell biology of plant-oomycete interactions. Cellular Microbiology 9:1: 31-39 (2007).
- Harrison J G, Lowe R, Williams N A. Humidity and fungal diseases of plants — Problems. In: Ecology of Plant Pathogens (Blakeman J P and Williamson B eds) 79–97. Wallingford: CAB International (1994).
- Herzog J and Schüepp H. Three types of sensitivity to Metalaxyl in *Plasmopara viticola*. Phytopathology 114: 90–93 (1985).
- Hill G K, Stellwaag-Kittler F, Huth G T, Schlosser E. Resistance of grapes in different developmental stages to *Botrytis cinerea*. Journal of Phytopathology Z. 102: 328-338 (1981).
- Holz G, Coertze S, Williamson B. The ecology of *Botrytis cinerea* on plant surfaces. in *Botrytis*: Biology, Pathology and Control. Elad Y et al. (eds) 9-27 Springer (2007).
- Holz G. The occurrence and control of *Botrytis*. Wynboer 9: 111-120 (2001).
- Hong Y, Martinez A, Liger-Belair G, Jandet P, Nuzillard J, Cilindre C. Metabolomics reveals simultaneous influences of plant defence system and fungal growth in B*otrytis cinerea*-infected *Vitis vinifera* cv. Chardonnay berries. Journal of Experimental Botany. 63:16: 5773-5785 (2012).
- Jeandet P, Bessis R, Gautheron B. The production of resveratrol (3,5,4-trihydroxystilbene) by grape berries in different developmental stages. American Journal of Enology and Viticulture 42: 41-46 (1991).
- Jürges G, Kassemeyer H H, Dürrenberger M, Düggelin M, Nick P. The mode of interaction between *Vitis* and *Plasmopara viticola* Berk. & Curt. Ex de Bary depends on the host species. Plant Biology 11: 886- 898 (2009).
- Karadimtcheva B. Characteristics of the anatomical structure of the grape skin in relation to resistance to grey mold. Gradinar. Lozar. Nauka 18: 94-99 (1981).
- Keller M, Viret O, Cole F M. *Botrytis cinerea* infection in Grape Flowers: defense reaction, latency and disease expression. Biochemistry and Cell Biology. 93: 3: 316-322 (2003).
- Kennelly M M, Eugster C, Gadoury D M, Smart C D, Seem R C, Gobbin D, Gessler C. Contributions of oosporic inoculum to epidemics of grapevine downy mildew (*Plasmopara viticola*). Phytopathology 94: S50 (2004).
- Kennelly M M, Gadoury D M, Wilcox W F, Magarey P A, Seem R C. Primary infection, lesion productivity and survival of sporangia in the grapevine downy mildew pathogen *Plasmopara viticola*. Phitopathology. Ecology and Epidemology 94: 4 512-522 (2007).
- Kennelly M M, Gadoury D M, Wilcox W F, Magarey P A, Seem R C. Seasonal development of ontogenic resistance to downy mildew in grape berries and rachises. Phytopathology 95: 1445–1452 (2005).
- Kortekamp A. Expression analysis of defence-related genes in grapevine leaves after inoculation with a host and a non-host pathogen. Plant Physiology and Biochemistry 44: 58-67 (2006).
- Kretschmer M, Kassemeyer H H, Hahn M. Age-dependent grey mold susceptibility and tissue-specific defence gene activation of grapevine berry skins after infection by *Botrytis cinerea*. Journal of Phytopathology, 155, 258–263 (2007).
- Lafon R and Clerjeau M. Downy mildew. Pages 11-13 in: Compendium of Grape Diseases, R. C. Pearson and A. C. Goheen, eds. The American Phytopathological Society, St. Paul, MN (1988).

- Langcake P and McCarthy W V. The relationship of resveratrol production to infection of grapevine leaves by *Botrytis cinerea*. *Vitis* 18: 244-253 (1979).
- Latorre B A and Rioja M E. Efecto de la temperatura y de la humedad relativa sobre la germinación de conidias de *Botrytis cinerea*. Cienci e Investigació Agraria 29: 67–72 (2002).
- Leroch M, Kleber A, Silva E, Coenen T, Koppenhöfer D, Shmaryahu A, Valenzuela P D T, Hahn M. Transcriptome profiling of *Botrytis cinerea* conidial germination reveals upregulation of infection-related genes during the prepenetration stage. Eukaryotic Cell-ASM Journals 12: 4: 614-626 (2013).
- Leroux P. *Botrytis* chemical control and resistance. in *Botrytis*: Biology, Pathology and Control. Elad Y et al. (eds) 195-222 Springer (2007).
- Liswidowati F M, Hohmann F, Schiver B, Kindl H. Induction of stilbene synthase by *Botrytis cinerea* in cultured grapevine cells. Planta 183: 307-314 (1991).
- Lovato A, Zenoni S, Tornielli G B, Colombo T, Vandelle E, Polverari A. Specific molecular interactions between *Vitis vinifera* and *Botrytis cinerea* are required for noble rot development in grape berries. Postharvest Biology and Technology 156: 110924 (2019).
- Malacarne G, Vrhovsek U, Zulini L, Cestaro A, Stefanini M, Mattivi F, Delledonne M, Velasco R, Moser C. Resistance to *Plasmopara viticola* in a grapevine segregating population is associated with stilbenoid accumulation and with specific host transcriptional responses. BMC Plant Biology 11:114 (2011).
- Marangon M, Van Sluyter S, Neilson K, Chan C, Haynes P, Waters E, Falconer R. Roles of grape thaumatin-like protein and chitinase in white wine haze formation. Journal of Agriculture and Food Chemistry 59: 2: 733-740 (2011).
- Marcato R, Sella L, Lucchetta M, Vincenzi S, Odorizzi S, Curioni A, Favaron F. Necrotrophic fungal plant pathogens display different mechanism to counteract grape chitinase and thaumathin-like protein. Physiological and Molecular Plant Pathology. 99: 7- 15 (2017).
- Marois J J, Nelson J K, Morrison J C, Lile L S, Bledsoe A M. The influence of berry contact within grape clusters on the development of *Botrytis cinerea* and epicuticular wax. American Journal of Enology and Viticulture 37: 293-296 (1986).
- Marvelli S, De' Siena S, Rizzoli E, Marchesini M. The origin of grapevine cultivation in Italy: the archeobotanical evidence. Annali di Botanica (Roma) 3: 155-163 (2013).
- Matta A, Buonaurio R, Favaron F, Scala A, Scala F. Fondamenti di patologia vegetale. Pàtron ed. Bologna (2017).
- McGovern P E, Glusker D L, Exner L J, Voigt M M. Neolithic resonated wine. Nature 381: 480–481 (1996).
- Millardet A. Sur le rôle des spores d´hiver du mildiou (*P. viticola*) dans la reinvasion par ce parasite. Mémories de la Société des Science Naturelle (1883).
- Millardet A. Traitement du mildiou par le mélange de sulphate de cuivre et chaux. Journal Agriculture Pratique 49: 2: 707–710 (1885).
- Monteiro S, Piçarra-Pereira M A, Loureiro V B, Teixeira A R, Ferreira R B. The diversity of pathogenesis-related proteins decreases during grape maturation. Phytochemistry 68: 416-425 (2007).

- Müller-Thurgau H. Die Ansteckung der Weinrebe durch *Plasmopara* (*Peronospora viticola*). Schweizerische Zeitschrift für Obst und Weinbau 20: 337–344 (1911).
- Pearson R C and Goheen A C. Plagas y enfermedades de la vid. Mundi-Prensa, Madrid (1996).
- Perazzolli M, Dagostin S, Ferrari A, Elad Y, Pertot I. Induction of systemic resistance against *Plasmopara viticola* in frapevine by *Trichoderma harzianum* T39 and benzothiadiazole. Biological control 47: 228-234 (2008).
- Percival D C, Sullivan J A, Fisher K H. Effect of cluster exposure, berry contact and cultivar on cuticular membrane formation and occurrence of bunch rot (*Botrytis cinerea* PERS.: FR.) with 3 *Vitis vinifera* L. cultivars. *Vitis* 32: 87-97 (1993).
- Peressotti E, Duchêne E, Merdinoglu D, Mestre P. A semi-automatic non-destructive method to quantify grapevine downy mildew sporulation. Journal of Microbiological Methods 84: 265-271 (2011).
- Peressotti E, Wiedemann-Merdinoglu S, Delmotte F, Bellin D, Di Gaspero G, Testolin R, Merdinoglu D, Mestre P. Breakdown of resistance to grapevine downy mildew upon limited deployment of a resistant variety. BMC Plant Biology 10:147 (2010).
- Pertot I, Caffi T, Rossi V, Mugnai L, Hoffmann C, Grando M S, Gary C, Lafond D, Duso C, Thiery D, Mazzoni V, Anfora G. A critical review of plant protection tools for reducing pesticide use on grapevine and new perspectives for the implementation of IPM in viticulture. Crop Protection 97:70-84 (2017).
- Pezet R, Pont V, Hoang-Van K. Evidence for oxidative detoxification of pterostilbene and resveratrol by a laccase-like stilbene oxidase produced by *Botrytis cinerea*. Physiological and Molecular Plant Pathology 39: 441-450 (1991).
- Pezet R, Viret O, Gindro K. Plant microbe interaction: The *Botrytis* gray mold of grapes. In Hemantaranjan A (Ed.), Advances in Plant Physiology, vol. 7 (pp. 75–120). India: Varanasi (2004).
- Pocock K F, Hayasaka Y, McCarthy M G, Waters E J. Thaumatin-like proteins and chitinases, the haze-forming proteins of wine, accumulate during ripening of grape (*Vitis vinifera*) berries and drought stress does not affect the final levels per berry at maturity. Journal of Agricultural and Food Chemistry 48: 1637-1643. (2000).
- Possamai T, Migliaro D, Gardiman M, Velasco R, De Nardi B. *Rpv* mediated defense responses in grapevine offspring resistant to *Plasmopara viticola*. Plants 9: 781 (2020).
- Prudet S, Dubos B, Le Menn R. Some characteristics of resistance of grape berries to grey mold caused by *Botrytis cinerea*. Pages 99-103 in: Recent Advances in *Botrytis* Research. K. Verheff, N. E. Malathrakis, and B. Williamson, eds. Pudoc Scientific Publishers, Wageningen, the Netherlands (1992).
- Ravaz L and Verge G. La germination des spores d'hiver de *Plasmopara viticola*. Compte rendues de l'Academie des Sciences 156: 800-802 (1913).
- Ravaz L and Verge G. Sur le Mode de Contamination de la Vigne par le *Plasmopara viticola*. Comptes rendues de l'Academie des Sciences 156 (1911).
- Rosenquist J K and Morrison JC. Some factors affecting cuticle and wax accumulation on grape berries. American Journal of Enology and Viticulture 40: 241-244 (1989).
- Rosslenbroich H-J and Stuebler D. Botrytis cinerea – history of chemical control and novel fungicides for its management. Crop Protection 19: 557- 561 (2000).

- Sarig P, Zutkhi Y, Lisker N, Shkelerman Y, Ben-Arie R. Natural and induced resistance of table grapes to bunch rots. Acta Horticulturae (ISHS) 464: 65-70 (1998).
- Sarry J-E, Sommerer N, Sauvage F-X, Bergoin A, Rossignol M, Albagnac G, Romieu C. Grape berry biochemistry revisited upon proteomic analysis of the mesocarp. Proteomics 4: 201-215 (2004).
- Schröter J. Fam. Peronosporaceae. In: Kryptogamenflora von Schlesien, Band 3, Heft 3, Pilze J.U. Kern's Verlag, Breslau, Germany, (F. Cohn, ed.), 814: 1885–1889 (1886).
- Schwander F, Eibach R, Fechter I, Hausmann L, Zyprian E, Töpfer R. *Rpv*10: A New Locus from the Asian Vitis Gene Pool for Pyramiding Downy Mildew Resistance Loci in Grapevine. Theoretical and applied Genetics. 124: 163–176 (2012).
- Sels J, Mathys J, De Coninck B M A, Cammune B P A, De Bolle M F C. Plant pathogenesis-related (PR) proteins: A focus on PR peptides. Plant Physiology and Biochemistry 46: 841-950 (2008).
- Staudt G and Kassemeyer H H. Evaluation of downy mildew resistance in various accessions of wild Vitis species. *Vitis* 34: 225–228 (1995).
- Terral J-F, Tabard E, Bouby Laurent, Ivorra S, Pastrol T, Figueiral I, Picq S, Chevance J-B, Jung C, Fabre L, Tardy C, Compan M, Bacilieri R, Lacombe T, This P. Evolution and history of grapevine (*Vitis vinifera*) under domestication: new morphometric perspectives to understand seed domestication syndrome and reveal origins of ancient European cultivars. Annals of Botany 105: 443-455 (2010).
- This P, Jung A, Boccacci P, Borrego J, Botta R, Costantini L, Crespan M, Dangl G S, Eisenheld C, Ferreira-Monteiro F, Grando S, Ibáñez J, Lacombe T, Laucou V, Magalhãnez R, Meredith C P, Milani N, Peterlunger E, Regner F, Zulini L, Manul E. Development of a standard set of microsatellite reference alleles for identification of grapevine cultivars. Theoretical and Applied Genetics 109: 1448-1458 (2004).
- Tromp A and De Klerk C A. Effect of copperoxychloride on the fermentation of must and on wine quality. South African Journal of Enology and Viticulture 9: 31–36 (1988).
- Unger S, Büche C, Boso S, Kassemeyer H H. The course of colonization of two different *Vitis* genotypes by *Plasmopara viticola* indicates compatible and incompatible host–pathogen interactions. Phytopathology 97: 780- 786 (2007).
- Vail M E and Marois J J. Grape cluster architecture and the susceptibility of berries to *Botrytis cinerea*. Phytopathology 81: 188-191 (1991).
- Vail M E, Wolpert J A, Gubler W D, Rademacher M R. Effect of cluster tightness on *Botrytis* bunch rot in six Chardonnay clones. Plant Disease 82: 107-109 (1998).
- Viala P. Les Maladies de la Vigne. Bibliothèque du Progrès Agricole et Viticole. Montpellier, Paris (1887).
- Vincenzi S, Bierma J, Wickramasekara S I, Curioni A, Gazzola D, Bakalinsky A T. Characterization of a grape class IV chitinase. Journal of Agricultural and Food Chemistry 32: 5660-5668 (2014).
- Vincenzi S, Polesani M, Curioni A. Removal of specific protein components by chitin enhances protein stability in white wine. Journal of Enology and Viticulture 56: 246-254 (2005).
- Waters E J, Wallace W, Williams P. Heat haze characteristics of Fractioned wine proteins. American Journal of Enology and Viticulture 42: 2: 123-127 (1991).

- Williamson B, Tudzynski B, Tudzynski P, Van Kan J A L. *Botrytis cinerea*: the cause of grey mould disease. Molecular Plant Pathology 8: 5: 561-580 (2007).
- Wong F P, Burr H N, Wilcox W F. Heterothallism in Plasmopara viticola. Plant Pathology 50: 427-432(2001).
- Yin X, Liu R-Q, Su L, Guo Y-R, Wang Z-J, Du W, Li M-J, Zhang X, Whang Y-J, Liu G-T, Xu Y. Pathogen development and host responses to *Plasmopara viticola* in resistant and susceptible grapevines: an ultrastructural study. Horticulture Research 4: 17033 1-10 (2017).
- Yu Y, Zhang y, Yin L, Lu J. The mode of Host Resistnace to *Plasmopara viticola* infection of grapevines. Phytopathology 102: 1094- 1101 (2012).
- Zobrist L. L'efficacité des fongicides organiques en viticulture. Proceedings III int. Congress on Phyto-pharmacologie 2: 682–688 (1954).
- Zohary D, Hopf M. Domestication of plants in the Old World, 3rd ed New York: Oxford University Press, 151–159 (2000).

OBJECTIVES OF THE THESIS

Given the high use of chemical fungicides to protect grapevine and the increasing attention on human health and environmental safety, in the last decades researchers have been working to find and develop new strategies for crop protection even in the contest of the IPM. This thesis aimed to contribute to this framework through two independent researches. In the first part (chapter II), *B. cinerea* protease activity on grapevine class IV chitinase, a PR-protein highly expressed in berries, was investigated to verify whether the fungal protease activity could be a mechanism adopted by the pathogen to favor grape berry infection. The demonstration of the importance of this activity could suggest possible targets for the future development of novel effective control strategies.

In the second part (chapter III) of the thesis, the effectiveness of several water-soluble peptide analogs of a natural occurring peptaibol of *Trichoderma longibrachiatum*, belonging to the most studied fungal biocontrol agent genus, was evaluated against *B. cinerea* and *P. viticola*. The identification of peptides able to inhibit symptoms caused by these two pathogens could allow the development of new biofungicides.

CHAPTER II

A*Vitis vinifera* chitinase IV is cleaved by *Botrytis cinerea* protease activity and loose its antifungal activity

2.1 Abstract

Plant-pathogen interaction is a complex and crucial phenomenon which determines the outcome of pathogen infections. To counteract the plant defense tools, pathogens evolved strategies to escape them and successfully infect the host.

Botrytis cinerea is a necrotrophic fungal pathogen able to overcome constitutive and induced physical and chemical barriers of the hosts thanks to a wide spectrum of mechanisms such as cell-wall degrading enzymes (CWDEs), secretion of phytotoxic compounds, detoxification of phytoalexins, inactivation of plant pathogenesis-related proteins (PRs).

In this research, the ability of *B. cinerea* to counteract a grapevine class IV chitinase through protease activity was investigated. This protein is abundantly present in berries during ripening as a pre-formed defense mechanism. Despite the abundance of this plant defense protein, *B. cinerea* successfully infects grape berries. Indeed, the fungus was demonstrated to inactivate the detrimental effect of this PR-protein by cleaving in two steps its chitin-binding domain (CBD), responsible for the interaction with the chitin substrate. This cleavage occurs already during conidia germination, determining a significant reduction of chitinase activity and preventing its harmful effects on spore germination and fungal growth. These effects are likely due to a reduced affinity for the substrate but also to a likely toxic effect. The pattern of chito-oligomers of the cleaved chitinase did not change, excluding the activation of PTI in plant tissues. Enzymatic assays in the presence of protease inhibitors allowed us to verify that a metalloprotease activity contributes to the cleavage of the CBD domain. Other protease activities are likely involved and are not yet identified.

Expression analysis of class IV chitinase encoding genes on grape leaves infected by *B. cinerea* showed that this PR protein is up regulated by 16 h post infection (hpi). These data were confirmed by an SDS-PAGE, showing that the class IV chitinase is very abundant following pathogen infection. However, a gel activity assay demonstrated that the enzymatic activity of this protein was very low. Interestingly, other two active chitinase were highlighted by the assay.

In conclusion, the CBD cleavage by the *B. cinerea* protease activity seems crucial to significantly reduce the chitinase enzymatic activity and to contrast is antimicrobial effect. The characterization of this mechanism could be useful to develop in future new biotechnology-based control strategies against *B. cinerea*.

2.2. Introduction

Plant-pathogen interaction is a complex phenomenon comparable to an open warfare in which the outcome of each battle results in the establishment of resistance or pathogenesis (Ferreira *et al.*, 2006 and 2007). On one side plants evolved defensive tools, on the other side pathogens developed strategies to overcome defense mechanisms; this coevolution began millions of years ago and is still ongoing (Keen, 1999; Ferreira *et al.*, 2007). Thus, studying the pathogenetic mechanisms of pathogens responsible for their adaptation to the host is fundamental to providing the basis for biotechnologically based control measures.

To obstacle pathogens infection, plants own physical barriers (pre-formed or inducible) considered the first line of plant defenses and a two-tiered innate immune system: a more general pattern-triggered immunity (PTI), and a specific effector-triggered immunity (ETI) (Jones and Dangl, 2006; Ferreira *et al.*, 2007;). Necrotrophic fungal pathogens such as *Botrytis cinerea* overcome constitutive and induced physical and chemical barriers of the host. To successfully infect the plant, this fungus exploits a wide spectrum of mechanisms such as cell-wall degrading enzymes (CWDEs), secretion of phytotoxic compounds, detoxification of phytoalexins (Choquer *et al.*, 2007; Williamson *et al.*, 2007; Zhang *et al.*, 2019; Haile *et al.*, 2020). Moreover, there is evidence of a slight but efficient adaptation strategy adopted by this widespread necrotrophic pathogen to specific hosts (Cotoras and Silva, 2005; Choquer *et al.*, 2007).

Chitinase and glucanase are plant pathogenesis-related proteins (PRs) present in the apoplast and able to degrade the hyphae cell wall, mainly constituted of chitin and β-glucans (Fink *et al.*, 1988; Karimi Jashni *et al.*, 2015b). Besides their enzymatic activity, the fragmentation of the fungal cell wall also generates oligosaccharides that, recognized by the plant immune system, induce defense responses that interfere with fungal infection (Ferreira *et al.*, 2007; Brulé *et al.*, 2019).

Plant chitinases, depending on the catalytic domain, belong to glycoside hydrolase family 18 (GH-18) and 19 (GH-19) and are classified according to their amino acid sequences (Collinge *et al.*, 1993; Taira, 2010; Zheng *et al.*, 2020). Plant chitinase expression can be constitutive, induced by biotic or abiotic stresses, or by ontogenesis and tissue or organ-specific (Robinson *et al.*,1997; Kasprzewska, 2003; Taira, 2010; Zheng *et al.*, 2020). In grapevine, chitinase genes belong to classes I, III, IV and V (Zheng *et al.*, 2020). All of them are related to plant defense, class I and IV belonging to PR-3, while classes III and V to PR-8 and PR-11, respectively (van Loon *et al.*, 1994; Sels *et al.*, 2008; Taira *et al.*, 2010). Class I, III and IV also show antifungal activity (Derckel *et al.*, 1996; Busam *et al.*, 1997; Saito *et al.*, 2011). Chitinase function and

expression are still under investigation. Class I and III were found induced by pathogens in leaves and even in berries in particular conditions (Robert *et al.*, 2002). Class I and V were recently found involved in response to both biotic and abiotic stresses (Zheng *et al.*, 2020). The highest chitinase activity has been found in berries (Derckel *et al.*, 1996), and it is mainly due to a Class IV chitinase highly expressed in berries during ripening (Robinson *et al.*, 1997; Derckel *et al.*, 1998; Robert *et al.*, 2002; Van Sluyter *et al.*, 2009) as a preformed defense against pathogens (Martinez-Esteso *et al.*, 2009). This chitinase is present in the skin (Deytieux *et al.*, 2007), mesocarp (Sarry *et al.*, 2004) and grape juice (Tattersall *et al.*, 1997; Vincenzi *et al.*, 2014; Marcato *et al.*, 2017). Class IV chitinase seems involved also in the response to biotic (Dadakova *et al.*, 2015; Zheng *et al.*, 2020) or abiotic stresses (Zheng *et al.*, 2020). Recently, even the expression of class I chitinase has been detected in grape berries skin (Zheng *et al.*, 2020). These different expression results may depend on more sensitive techniques used but also on the different grape varieties analyzed (Robinson *et al.*, 1997; Zheng *et al.*, 2020). Class IV chitinase has homology with class I and both seem evolutionarily derived from the class II nowadays missing in grape (Zheng *et al.*, 2020). These chitinases belong to the GH-19 family and own a chitin-binding domain (CBD). The CBD seems to contribute to the chitinase antifungal ability, making it accessible to the enzymatic activity also lateral walls and septa (Taira, 2010); moreover, the CBD belongs to the carbohydrate-binding module family 18 (CBM-18), sharing homology to hevein and chitin-binding lectins such as wheat germ agglutin (WGA) (Taira, 2010), with demonstrated antifungal properties (Parijs *et al.*, 1991; Ciopraga *et al.*, 1999). Thus, the class IV chitinases have both lectin and enzymatic properties to exert their antifungal activity.

Despite the abundance of class IV chitinases and antifungal secondary metabolites such as *trans*-resveratrol in grape berries, *B. cinerea* has evolved mechanisms to overcome these plant defenses. It has been previously demonstrated that *B. cinerea* laccase activity can detoxify trans-resveratrol and modify the grape protein solubility (Favaron *et al.*, 2009). Moreover, the glucan matrix of the fungal cell wall seems able to sequester chitinases, possibly escaping their detrimental effects (Marcato *et al.*, 2017). Interestingly, a reduction of grape chitinase size has been also observed during incubation with *B. cinerea* (Favaron *et al.*, 2009; Marcato *et al.*, 2017). A serin and a metalloprotease of *Fusarium oxysporum* have been reported to cleave tomato chitinase reducing its detrimental effect (Karimi Jashni *et al.*, 2015a), but the same mechanism cannot be easily transferred to the *B. cinerea*-grape chitinase system due to the lack of orthologous genes of the *F. oxysporum* serin-protease in the *B. cinerea* genome. Besides, it is not clear if the *B. cinerea* most expressed proteases are in some way involved in this process (Van

Sluyter *et al.*, 2013; Karimi Jashni *et al.*, 2015a; Marcato *et al.*, 2017). Thus, the *B. cinerea* protease mechanism affecting grape chitinase remains to be clarified.

This work aimed to investigate the type of *B. cinerea* protease activity involved in the cleavage of grape chitinases and whether the cleavage affects its detrimental effect on fungal growth or the pattern of chitooligomers produced. Besides, expression analysis of genes encoding chitin synthase and deacetylase has been performed on the fungus after treatment with the native and cleaved chitinase to verify whether the fungal perception of these proteins by hyphal tips may modify the synthesis and properties of the chitin.

2.3 Material and methods

2.3.1. Plant materials

Grape proteins were extracted and purified from ripe berries of the IM 6.0.13b cultivar harvested in a vineyard in Conegliano (North East of Italy). Fungal infections, RNA transcription and protein analyses were carried out on leaves of cv Glera grown in a climate chamber.

2.3.2. Fungal strains and cultures

The fungal pathogen *B. cinerea* (strain B05.10) and the BcΔAcp1 mutant, were obtained by coutesy of Nathalie Pousserau laboratory (INSA, Lyon).
The strains were grown on Petri dishes on potato dextrose agar (PDA, BD, Difco, USA) at 25°C. Mycelium inoculum consisted of PDA plugs taken from the marginal zone of actively growing colonies. Conidia inoculum was obtained from sporulating plates exposed to light on a laboratory bench after growing the plate in the dark for 10-15 days. Conidia were collected by washing the plate surface with sterile water and stored at -80°C in 10% glycerol until used.

2.3.3. Extraction and purification of grape proteins

Grape proteins were extracted from about 2 liters of grapes juice obtained by hand crushing about 3 kg of ripe berries at 4°C. The grape juice was immediately filtered through a nylon gauze and cysteine was added to a final concentration of 4 mM to prevent polyphenolic oxidation (Favaron *et al.*, 2009), and stored at -20°C until processed. All the subsequent operations were performed at 4°C. The juice was centrifuged at 2,000 g for 10 min and the supernatant was filtered two times through grade glass microfiber filters (MGA) (Sartorius, Germany). Ammonium sulfate ($(NH_4)_2SO_4$) at 20% saturation was added and after 16 h the precipitated material was removed by centrifugation at 2,000 g for 10 min. The supernatant was filtered in succession through MGA and cellulose acetate filter 0.8 μm (Sartorius, Germany). The filtrate was concentrated twice with polyethylene glycol (PEG) 35000 (Merk kGaA, Darmstadt, Germany) using a dialysis membrane (cut off = 12 kDa). The juice was filtered in succession through MGA and 0.8, 0.45 and 0.2 μm cellulose acetate filters and then concentrated 3 times with a VivaFlow 5000 apparatus (Millipore Corporation, Bedford, USA) and dialyzed twice against 5 liters of ultrapure water (upH_2O). Seventy ml of the concentrated material were loaded onto an LKB electrophoresis column (B101, Sweden) to perform a liquid-phase

isoelectric focusing (IEF) in the pH range 3-10. At the end of the run, 2 ml fractions were collected and the fractions with pH between 3.5 and 5 were analyzed through SDS-PAGE according to Laemmli (1970) with some modification: the samples were diluted in sample buffer containing dithiothreitol (DTT) as reducing reagent. Samples were boiled for 4 minutes and loaded on electrophoresis gel containing 12% (w/v) polyacrylamide and 1% sodium dodecyl sulfate. The gel was stained with the colloidal Coomassie G-250 blue silver method (Candiano *et al.*, 2004). Fractions containing thaumatin-like proteins (TLP) or chitinase bands were separately buffer exchanged by Sephadex G-25 column (PD-10, GE Healthcare, UK) in 20 mM Tris-HCl pH 8.5 (Sigma-Aldrich, USA). TLP and chitinase were further purified by a Resource Q anion exchange chromatography column (1ml, Pharmacia Biotech, Sweden) connected to an AKTA purifier (GE Healthcare, UK). The column was equilibrated with 20 mM Tris-HCl pH 8.5 (buffer A) and the proteins were eluted with a linear gradient of 1M NaCl in buffer A, from 0 % to 50% in 80 min for chitinase and 120 min for TLP.

Peaks corresponding to TLP and chitinase were collected and checked on SDS-PAGE as reported above.

Protein concentration was measured spectrophotometrically at 280 nm and estimated as albumin equivalents.

Grape proteins were also extracted from infected leaves at different times from the inoculation with *B. cinerea* (1 - 3 days). Leaf material was ground with liquid nitrogen and repeatedly extracted with cold acetone. The acetonic powder was extracted with 2.5 ml g^{-1} of a 0.35 M Tris-acetate buffer pH 8 containing 20 Mm ethylene diamine tetra acetate (EDTA), 15 mM Cysteine hydrochloride (Cys-HCl), 0.5 M NaCl, 6% PEG 4000 (Sigma-Aldrich, Steinheim, Germany), 2.5 mM DTT and added with 0.3 g g^{-1} of polyvinylpolypyrrolidone (PVPP). The mixtures were crushed for 5 min then left at 4°C crushing every 10 min for 2 h.

The mixtures were cleared by centrifugation 18,400 g 10 min at 4°C and the supernatants were used in the experiments of chitinase activity (subchapter 2.3.8). The detectable band through SDS-PAGE (above reported) was also analyzed by MALDI-TOF.

2.3.4. Production of protease activity and protease assay

The fungal protease was obtained by growing *B. cinerea* (B05.10) in wells of 24 wells microtiter plates each containing 2 ml of a modified Czapek-Dox medium (6.7 mM KCl, 2 mM $MgSO_4 \cdot 7H_2O$, 7.3 mM $K_2HPO_4 \cdot 3H_2O$, 0.036 mM $FeSO_4 \cdot 7H_2O$ and 111 mM glucose), containing 10 mM citric acid, 0.5% (w/v) of enzymatically hydrolyzed casein (N-Z-Amine A, Sigma-Aldrich, USA) and adjusted to pH 3.5 with 1M tartaric acid. Each well was inoculated with a

mycelium-colonized PDA disk (0.7 cm in diameter). After three days of growth at 25°C in the darkness, the broth was removed from each well and 2 ml of fresh medium were added. After three days of further growth, the cultural broth from several wells was collected, filtered in succession through MGA and cellulose acetate (0.8 and 0.45 µm) membranes. This crude protease preparation was assayed for protease activity and used for protease purification.

Protease activity was assayed on 225 µl of hemoglobin 1% (w/v) by adding 50 µl of the fungal filtrate. The mixture was adjusted to pH 3.5 with 1M HCl and incubated at 37°C in an orbital shaker at 230 rpm. After 90 min, the reaction was stopped by adding 225 µl of 20 % (w/v) trichloroacetic acid (TCA), and the samples were centrifuged at 18400 g for 10 min at 4°C. Then, 300 µl of the supernatant were mixed with the same volume of 0.5 M NaOH and the A_{280} of the samples was measured spectrophotometrically. The A_{280} value carried out by adding TCA to the assay mixture at zero time was subtracted. One enzyme unit (U) was defined as the amount yielding 1AU min^{-1} and the enzymatic activity was expressed as U ml^{-1}.

2.3.5. Purification of *B. cinerea* proteases

About 30 ml of the crude protease preparation were dialyzed (membrane cut off = 12 kDa) against sodium succinate pH 3.2, concentrated with PEG 35000 (Merk kGaA, Darmstadt, Germany) and filtered through 0.45 µm cellulose acetate membrane (Sartorius, Germany). This concentrated crude preparation was loaded on a Resource S cation exchange chromatography column (1ml, Pharmacia Biotech, Sweden) connected to the AKTA purifier (GE Healthcare, UK). The column was equilibrated with 50 Mm sodium succinate at pH 3.2 (buffer A) and eluted with a linear gradient of 1M NaCl in buffer A, from 0 % to 50% in 40 min. Proteins peaks were collected, subjected to SDS-PAGE, and the proteins were stained with Silver Nitrate (http://www.ucdavis.edu, 2010). The activity of the major protease fractions (F17-F18) was measured as reported in subchapter 2.3.4

2.3.6. Incubation of grape chitinase with *B. cinerea*, fungal crude protease or purified protease fractions

An aliquot of *B. cinerea* crude protease and an aliquot of the purified chitinase were separately buffer exchanged through a Sephadex G-25 column (PD-10, GE Healthcare, UK) and G-25 M DNA Grade column (Nap-5 column, Pharmacia Biotech, Sweden) respectively, equilibrated with sodium acetate 20 mM at pH 4 and eluted in the same buffer. Different amounts of crude fungal protease preparation (from 0 to 0.004 U) were incubated with 4 µg of purified chitinase for 24 h at 25°C in sodium acetate 20 mM at pH 4. The samples were then precipitated with

four volumes of cold absolute ethanol for 2 h at -20°C and the pellet obtained after centrifugation at 10,400 g for 10 min was washed with cold 70% ethanol, resuspended in the sample buffer and analyzed through SDS-PAGE as reported in sub-chapter 2.3.3. A control sample of 4 μg of chitinase incubated at 25°C for 24 h in the same buffer was also loaded into the gel.

To produce a stock of cleaved chitinase, 3 mg of the purified chitinase were incubated with fungal crude protease preparation (0.0005 U protease activity/μg of chitinase) at 25°C for 24 h at pH 4. The reaction outcome was checked through SDS-PAGE as indicated above and the purification of cleaved chitinase was performed as reported for the native chitinases (sub-chapter 2.3.1).

Purified chitinase was also incubated with the main fractions obtained from protease purification (F17 and F18, subchapter 2.3.5), and with the culture filtrate obtained from the 6-day-old culture of the *B. cinerea* mutant BcΔAcp1 grown in modified Czapeck medium containing hydrolyzed casein as reported in sub-chapter 2.3.4. The results of incubations were analyzed through SDS-PAGE as mentioned in sub-chapter 2.3.3 and stained with the Silver Nitrate method (sub-chapter 2.3.5).

Native and cleaved grape chitinases were administered also to *B. cinerea* culture. To this aim, single PDA plugs (0.7 cm) colonized with *B. cinerea* (B05.10) were inoculated on wells of microtiter plates (one plug/well) containing 1 ml of a Czapek medium (23.5 mM $NaNO_3$, 6.7 mM KCl, 2 mM $MgSO_4 \cdot 7H_2O$, 6.1 mM $K_2HPO_4 \cdot 3H_2O$, 0.036 mM $FeSO_4 \cdot 7H_2O$, 34.7 mM ZnSO4, 20 mM CuSO4 and 111 mM glucose). After three days of growth at 25°C in the dark, the medium was replaced with the Czapek medium containing or not 50 μg ml^{-1} of chitinase protein (native or cleaved). The growth continued at 25°C with gentle shaking in the dark and the cultural broth was collected after 6, 12 and 24 h. The broth of three wells for each time and treatment was mixed and 80 μl of each mixture (corresponding to a theoretical amount of 4 μg of protein) was precipitated with ethanol and loaded into SDS-PAGE as above reported.

2.3.7. Blotting of chitinases for N-terminal sequencing and molecular modeling

An SDS-PAGE was performed (as reported in sub-chapter 2.3.3) loading 4 μg of each of the following chitinases: purified native chitinase, purified cleaved chitinase and native chitinase incubated for 6 hours with *B. cinerea*. After electrophoresis, the gel was blotted on an Immobilon-E Transfer Membrane (cat Num.IEVH00005 0.45 μm, Merk KGaA, Darmstadt, Germany) and the N-terminal sequencing of the proteins was performed. Briefly, the SDS-PAGE gel and the membrane were equilibrated with the transfer buffer (10 mM 3-

(Cyclohexylamino)-1-propane sulfonic acid (CAPS) in 10% (w/v) methanol adjusted to pH 11 with NaOH). The blotting was carried out with a semi-dry transfer system (TRANS-BLOT SD, Biorad) at a maximum voltage (V) and power (W) and the amperage was adjusted according to the size of the gel (mA = cm^2 · 2.5). After 35 min, the transfer stack was disassembled and the membrane was stained for 2 min with 0.1% (w/v) brilliant Blue R-250 (Sigma-Aldrich, USA) in 40% (w/v) methanol and 1% (w/v) acetic acid, and destained for 10 min (50% (w/v) methanol and 7% (w/v) acetic acid. The membrane was washed 10 min with upH_2O and air-dried for 60 min. A sterile blade was used to excide the membrane portions corresponding to each digested protein. The samples were sent to the Molecular Structure Facility, of the University of California, Davis to perform the N-terminal sequencing.

The outcome of N terminal sequencing was blasted (DNAMAN software) with the chitinase class IV sequences 024530, 024531 and Q7XAU6 downloaded from UniProt database: (https://www.uniprot.org/uniprot) to identify the site of the cleavage.

The native chitinase was identified by Maldi-TOF analysis. The amino acid sequences corresponding to native and cleaved chitinase were loaded into the SWISS-MODEL server (https://swissmodel.expasy.org/interactive) to identify the structural properties of this grapevine chitinase.

2.3.8. Chitinase activity

Chitinase activity of native and cleaved chitinases was measured as reported by Burzyński *et al.* (2000) with some modifications to provide the most favorable conditions for enzymatic activity as characterized in Vincenzi *et al.* (2014). Briefly, the reaction was performed in a 0.4 ml volume containing 0.25% (w/v) Chitin Azure (Sigma Aldrich, USA) and 40 µg of enzyme in 75 mM sodium acetate, pH 6. The mixture (pH 5.6) was incubated for 8 h at 37°C and 300 rpm. The reaction was stopped by adding 0.4 ml of 0.3 M HCl. Samples were cooled on ice for 10 min, then the undigested substrate was removed by centrifugation for 10 min at 10,400 g and 4°C. The absorbance of the supernatant was read spectrophotometrically at 550 nm (A_{550}) against the blank samples which were processed in the same way but without the addition of enzyme. One unit of enzymatic activity was calculated as an increase of 0.01 A_{550} under the experimental conditions and expressed as U mg_{eq}^{-1} of protein. The experiment was repeated four times with three technical replications each.

Chitinase activity assays were performed also using colloidal chitin as a substrate to allow the analysis of High-Performance Liquid Chromatography (HPLC) degradation profiles of chitin (sub-chapter 2.3.9). Colloidal chitin was obtained similarly to what was reported by Li *et al.*

(2002). Briefly, 10 g of chitin powder (practical grade from crab shells, Sigma-Aldrich, USA) were slowly added to 180 ml of 37% HCl (w/v) at 25°C under vigorous stirring for 2 h. The suspension was poured into 1 liter of ice-cold 95% ethanol under vigorous stirring for 30 min and stored at -20°C until use. An aliquot of 10 ml of suspension was centrifuged at 3,000 g for 5 min at 4°C, the pellet was washed once with 50 ml of upH_2O and twice with 50 mM ammonium acetate at pH 7 after each centrifugation step. Finally, the pellet was dissolved in 18 ml of 50 mM ammonium acetate (pH 7) obtaining a 5% colloidal chitin solution.

The enzymatic reaction was performed in 0.3 ml containing 2.5% colloidal chitin and 40 µg of enzyme in 50 mM ammonium acetate. The pH was adjusted to 6 with 0.5 M acetic acid and the mixture was incubated for 8 h at 37°C and stirred in an orbital shaker at 300 rpm. After incubation, samples were centrifuged at 18,400 g for 5 min at 4°C and the supernatant was filtered through 0.45 µm cellulose acetate filter. As a blank, a similar reaction mixture without enzyme addition was performed. The enzymatic reaction was repeated twice.

An enzyme gel assay was performed to detect the chitinase activity in grapevine leaves inoculated with *B. cinerea* (subchapter 2.3.3). Chitinase bands were detected after polyacrylamide gel electrophoresis adapting the method proposed by Trudel and Asselin (1989). Briefly, 10 µl of each protein leaf extract and 7 µg of purified chitinase were suspended in SDS sample buffer without reducing reagent and loaded on SDS-polyacrylamide gel copolymerized which 0.01% glycol chitin. Glycol chitin prepared as reported by Molano *et al.* (1979) was a gift of S. Vincenzi (University of Padova, Italy). The electrophoretic separation was set on a constant 200 V until the tracking dye bromophenol blue run off the gel. After electrophoresis the gel was incubated at 37°C in 0.1 M sodium acetate (pH 5.5), containing 1% (w/v) Triton X-100 (Sigma-Aldrich, Steinheim, Germany) under gentle shaking. After 2 h of incubation, the buffer was replaced and the incubation continued overnight. To detect chitinase activity, the gel was stained with 0.01% calcofluor white (M2R, Sigma-Aldrich, USA) in 0.5 M Tris-HCl pH 8.9 for 10 min under gentle shaking, and de-stained with upH2O for 2 h under gentle shaking replacing the water every 60 min. Lytic bands were observed and photographed under UV light. The gel, after the chitinase staining, was also stained for protein detection with the colloidal Coomassie G-250 blue silver method (Candiano *et al.*, 2004).

2.3.9. Analysis of chito-oligomers produced by chitinase activity

The analysis of degradation profiles of chitin obtained with native and cleaved chitinases was performed by HPLC (Agilent, USA) equipped with an HiPlex H column (8 µm 30 x 0.65 cm) equilibrated with 5 mM H_2SO_4. Ten microliters of the reaction mixture were loaded and an

isocratic elution was conducted for 40 min with a 0.35 ml min-1 flow rate of 5 mM H_2SO_4. To identify the chito-oligomers produced by enzymatic activity. Ten µl of N-Acetyl glucosamine (0.1 µg μl^{-1}) (GlNac, Sigma-Aldrich, USA) and 10 µl of a chito-oligomers mixture (0.4 µg μl^{-1}) (Chito I HPLC Standard, IsoSep, Sweden) were also loaded as standards. The HPLC analysis was performed two times.

2.3.10. Characterization of *B. cinerea* protease activity against grape chitinase by inhibition assays

The purified chitinase and protease culture filtrate were incubated as reported in sub-chapter 2.3.6 with the addition of protease inhibitors reported in table 1 to a final reaction mixture of 0.1 ml.

Table 1: protease inhibitors and usage doses adopted in protease inhibitory assays.

Inhibitor	Inhibitory activity	Usage dosage
Pepstatin A	Aspartic and acidic protease	22 µM
AEBSF	Serin protease	1 mM
1,10-phenanthroline	Metalloprotease	5 mM
E-64	Cysteine protease	14 µM
Diazoacetyl-DL-norleucine (DAN)	Specific for *B. cinerea* acidic protease (Acp1)	15 µM + 1Mm cofactor (Cu II acetate)

After 24 hours of incubation at 25 °C, the mixtures were analyzed by SDS-PAGE (sub-chapter 2.3.3) and stained with the nitrate silver method (sub-chapter 2.3.5). The inhibition of metalloprotease activity was analyzed also in presence of the fungus. *B. cinerea* was grown for three days as reported in sub-chapter 2.3.6, then the medium was replaced with a fresh medium containing 50 µg ml^{-1} of chitinase in combination with 1,10-phenanthroline (5, 10 mM) in a 0.5 ml final volume buffered at pH 6.5 with 3-Morpholinopropanesulfonic acid (MOPS). After 6 h under gentle shaking an aliquot corresponding to 4 µg of chitinase was precipitated with ethanol and analyzed by SDS-PAGE (sub-chapter 2.3.3).

The metalloprotease inhibitor (10 mM) was administered also on germinating conidia ($1x10^5$ conidia ml^{-1}) in 0.2 ml final volume containing a quarter PDB buffered at pH 6.5 with MOPS maintained under gentle shaking for 16 h. These last two experiments provided conditions suitable for avoiding chitinase cleavage during fungal growth.

2.3.11. Grape proteins administration to fungal mycelium for RNA extraction and to conidia for germination assay

Petri dishes (3.5 cm diameter) were equipped with 2 ml PDA and a sterile cellophane sheet. Fourteen droplets with about 100 conidia each were spotted on the cellophane sheet and the plates were maintained at 25°C in the dark for 90 hours. Colonized cellophane sheets were then put, the bottom side down, on Petri dishes containing different treatment mixtures. The treatments consisted of 1.5 ml final volume of Czapeck medium (sub-chapter 2.3.6) with 1,10-phenanthroline (10 mM) and 50 μg ml^{-1} of grape proteins (native or cleaved chitinases and TLP). The pH was adjusted at 6.5 with MOPS. Control was performed with the buffer solution only. After 6 h under gentle shaking, the mycelium colonized cellophane sheets were washed in sterile water and let dry for a few minutes on sterile paper. The mycelium was then detached from the cellophane, nitrogen frozen and stored at -80°C until RNA extraction was performed. The full experiment was repeated three times.

To establish the effect of native and cleaved chitinases on conidia germination, conidia ($1 \cdot 10^5$ ml^{-1}) were suspended in a quarter PDB medium buffered at pH 6.5 with MOPS in presence of 100 μg ml^{-1} of chitinase (native or cleaved) in a final volume of 0.2 ml in 96 wells microtiter plates (three wells for each treatment). After 16 h at 25°C in the dark and under gentle shaking, the germination and hyphal development were analyzed by microscopy (Leica DM750). The control thesis consisted of the same reaction mixture without the grape proteins. Ten microliters of suspension were collected from each well (three times) and placed on microscope slides; a total of 6 optical fields, random distributed, were analyzed at 20 magnifications. Germinated and not germinated conidia were counted and a picture was taken for each observed field (Leica EC3 apparatus connected to the microscope). The pictures were later analyzed and the length of germinating hyphae was measured. The full experiment was repeated three times.

At the end of each experiment, an aliquot of the treatment mixture was loaded into SDS-PAGE (as reported in sub-chapter 2.3.5) to detect the presence of the chitinases.

2.3.12. Infection on *V. vinifera* leaves

To investigate the expression of chitinase IV and TLP genes on grape leaves in presence of *B. cinerea*, leaf inoculations of grapevine (cv Glera) were performed. Briefly, 4 leaves were harvested from a single plant and placed on a glass box equipped with a tray and moistened sterile paper to maintain a high HR. The adaxial surface of two leaves was inoculated each with 6 PDA plugs colonized by *B. cinerea* mycelium (0.3 cm). Two control leaves were inoculated with non-colonized PDA plugs. To favor the adhesion of the plugs, 10 µl of sterile water were placed beneath each plug. The box was closed with plastic film and maintained at 22 °C with 16/8 h of light/dark period. At 16 and 24 hpi, the plugs were removed and leaf disks of 1 cm of diameter around the inoculated area were collected. The infected or control leaf disks were nitrogen frozen and stored at -80°C until RNA extraction. The disks collected from each leaf at each time were considered as a biological replicate and four replicates were performed.

The chitinase protein accumulation during fungal infection was determined on ten leaves from different plants, inoculated with 6-8 PDA plugs colonized by *B. cinerea*. In the following 3 days leaf disks (diameter of 1cm) excised around the plug were collected and separated into three categories according to the symtom size: less than 50%, comprised between 50% and 100% and completely (100%) infected. Disks from not inoculated leaves were collected as control samples. Infection and sampling procedure were repeated 3 times, the leaf disks were nitrogen frozen and stored at -80°C until the protein extraction as described in subchapter 2.3.3.

2.3.13. RNA extraction and gene expression

About 100 mg of the mycelium grown on the cellophane surface and treated for 6 h with grape proteins (subchapter 2.3.11), were ground with liquid nitrogen and RNA was extracted with the RNeasy Plant Mini Kit (Qiagen GmbH, USA) according to the manufacturer's instructions. About 100 mg of grape leaves (subchapter 2.3.12) were ground to a fine powder by nitrogen and RNA was extracted according to Reid *et al.* (2006).

Extractions were repeated on three and four different biological samples, respectivelly. The amount and purity of RNA was estimated spectrophotometrically (A_{260} and A_{280}) and by 1 % (w/v) agarose gel electrophoresis. After DNase treatment (RQ1 RNase-free DNase, Promega), 500 ng of RNA and 0.5 µg of an oligo-dT reverse primer (15/18thymine) were heated at 70°C for 5 min. Then, the ImPromII reverse transcriptase (Promega, Milano, Italy) was added according to the manufacturer's instructions. Reverse transcription was performed on a thermocycler at 25°C for 5 min, 42°C for 1 h and 70°C for 17 min.

Expression analysis was performed by qPCR. Five chitin synthase genes and four chitin deacetylase genes of *B. cinerea* wer analyzed together with the fungal β-tubulin and actin used as house-keeping genes, then β-tubulin was selected as house-keeping.

Expression of *V. vinifera* chitinase class IV and TLP genes were analyzed with grapevine actin and gliceraldeid-3-phosphate dehydrogenase as house-keeping genes, actin was selected as house-keeping. For each gene, primers were designed by using Primer3 (https://primer3.ut.ee) (https://www.ncbi.nlm.nih.gov/genbank/) (Table 2). The qPCR was performed by the Rotor-Gene Q 2plex (Qiagen GmbH, USA). The qPCR mixture contained 10 µl of 2X Rotor-Gene SYBR Green PCR MasterMix (Qiagen GmbH, USA), 0.25 µM of each primer, 3 µl of cDNA template and sterile upwater to a final volume of 20 µl. The amplification was performed by repeating 40 times the following cycle: 20 s at 94°C, 20 s at 52°C, 30 s at 72°C. Relative expression results were analysed by the Rotor-Gene 2.3.5 Software (Qiagen GmbH, USA).

2.3.14. Statistical analysis

Data were subjected to ANOVA and means were compared by Tukey's HSD test or Student's t-test according to experimental design.

Table 2: sequences accession of genes analyzed by qPCR and primers designed with Primer3 (https://primer3.ut.ee)

Sequence accession	Protein	Forward	Reverse
XM_024694912.1	Chitin synthase class I	CTTACTACCAACCTCCATACCA	TACCGTCCCGTCATAACCA
AF494188.1	Chitin synthase class IIIa	AACAACTTATCCGCCCAAC	TTAGGTCCTGGCTTCGTACC
XM_001548495.2	Chitin synthase class IIIb	GTGAATCTGAAGAAGTCCAGGT	CTGTCGTCTCCTTTCCATCC
XM_001545464.2	Chitin synthase class V	GTAACGACAGACCTACTCCAC	TACCAACTTTCACAACGACCA
XM_024696504.1	Chitin synthase class VI	GCAGACGACTTCAGAGCATC	AGCAGACCTCCAACATCCTC
XM_024696034.1	Chitin deacetylase class I	GTCCTTACATCTACACCTCCC	AATGCCATCTCGTTCTTCCA
XM_001560817.2	Chtiin deacetylase class II	AACAGCCGTTCAAGACTACC	TACTTTGAGATGAGACTGCGA
XM_001554936.2	Chtiin deacetylase class III	ATCATCCACATCCTCAACCAG	CAAAGCCCTTCTGACATCCA
XM_001552625.2	Chtiin deacetylase class IV	TAGACGTCTCTTCTGCGGAT	CTCAACGACCGGAACAGTAG
XM_024690731.1	B-tubulin	GTCTCAAGATGTCCTCCACC	AGGTATGGACGAGATGGAGT
XM_024697950.1	actin	TGGACTTTGGGTTGATTGGG	TGGACTTTGGGTTGATTGGG
NM_001281146.1	Class IV chitinase	CCAATCGGGTCCTTGTGATT	GAAGAAAGCTGCAATCTCGC
AF003007.1	TLP-1	AACAAATGCACCTACACCGT	CATTGCAGTCACCAGTTTCG
XM_0022824800.4	actin	TGTGCTGGATTCTGGTGATG	ACAATTTCCCGTTCAGCAGT
XM_002263109.3	GAPDH	AAGCCAGTCACCGTCTTTG	GGGCTGAGATGACAACCTTC

2.4. Results

2.4.1. Grape proteins purification

Protein purification from grape juice was performed by preparative isoelectrophocusing and anion-exchange chromatography as reported in Materials and Methods. The two principle protein fractions separated by isoelectrofocusing were subjected to several chromatographic steps. Two main peaks were obtained from the final chromathographic purification step (resource Q column) (Figure1). After SDS-PAGE (Figure 2) the band were excised and subjected to MALDI-TOF analysis, which allowed the identification of a class IV grape chitinase (UniProtKB: Q7XAU6) and a *V. vinifera* thaumatin-like protein (UniProtKB: F6HUH1). These protein preparations were used in the following experiments.

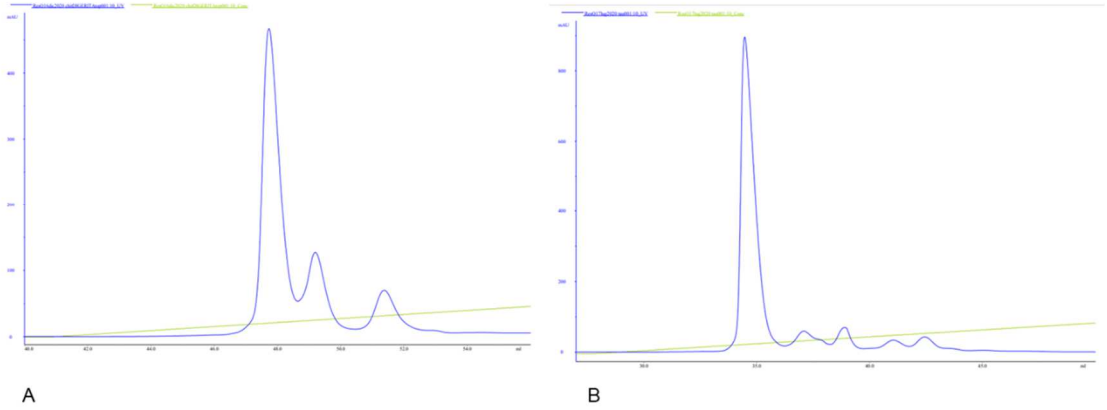

Figure 1: Resource Q patterns of grape proteins: chitinase (A) and TLP (B). Elution conditions are reported in Material and Methods.

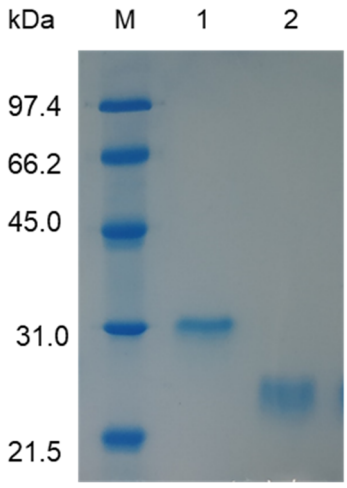

Figure 2: SDS-PAGE of the purified grape chitinase (lane 1) and TLP (lane 2). Two µg of each protein were loaded and the gel was stained with a colloidal Coomassie G250 protocol. M: Molecular weight marker (Low range, Bio-Rad Laboratories, Milano, Italy).

2.4.2. *B. cinerea* protease activity on grape chitinase

In a first experiment, protease activity was obtained from a culture of *B. cinerea* grown for 6 days in a liquid broth containing hydrolyzed casein as a protease inducer. The protease activity contained in the culture filtrate was quantified at 0.1629 U ml^{-1}. Then, 4 µg of grape chitinase were incubated with different amounts of casein-induced protease for 24 h. Results showed that a minimum protease activity of 0.001 U was necessary to decrease the size of the chitinase from about 31 kDa to about 26 kDa (Figure 3) and no further reduction of the protein size was noticed by increasing the amount of protease activity.

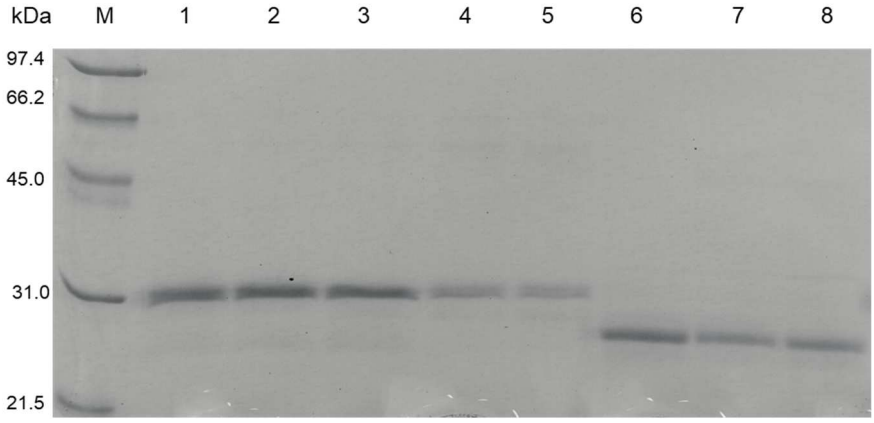

Figure 3: SDS-PAGE of the mixture containing 4 µg of grape chitinase and increasing units of *B. cinerea* culture filtrate containing protease activity after 24 h of incubation at 25 °C. Lanes 1-8: 4 µg grape chitinase with 0, 0.000063, 0.000125, 0.00025, 0.00050, 0.001, 0.002, 0.004 U of protease activity, respectively. Lane M: Molecular weight marker (Low range, Bio-Rad Laboratories, Milano, Italy).

The protein obtained after protease digestion (hereafter named cleaved chitinase) was purified as reported for the chitinase purification and used in the following experiments (Figure 4).

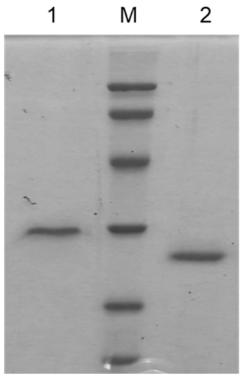

Figure 4: SDS-PAGE of native (lane 1) and protease cleaved chitinase (lane 2), at about 31 and 26 kDa, respectively. The gel was stained with a colloidal Coomassie G250 protocol. Molecular weight marker (Low range, Bio-Rad Laboratories, Milano, Italy) was loaded on lane M, the fourth band correspond to 31 kDa.

In a second experiment, *B. cinerea* was grown for 24 h in a medium containing the chitinase or the cleaved chitinase obtained in the previous experiment. Results showed that in presence of the fungus the chitinase was reduced in size. However, in the 24 h time interval, the product of chitinase digestion obtained in presence of *B. cinerea* has a size slightly higher than the cleaved chitinase obtained in the first experiment (Figure 5). Besides, the cleaved chitinase appeared proteolytically stable since no further reduction of the size was obtained in the presence of the fungus.

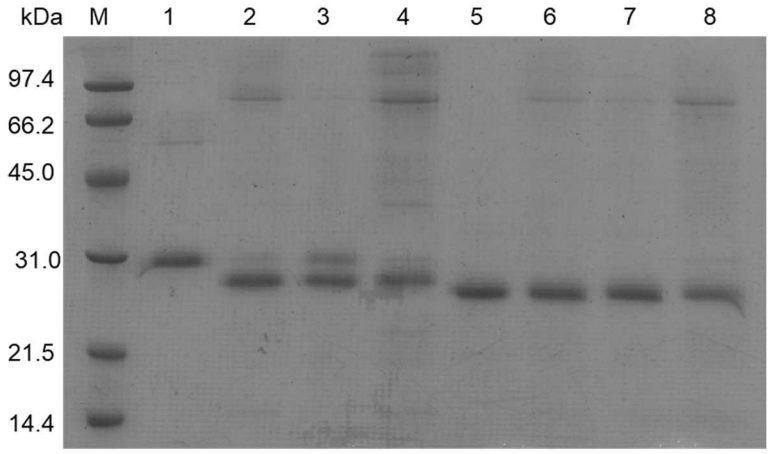

Figure 5: SDS-PAGE of native (lanes 1-4) and cleaved chitinase (lanes 5-8) added to *B. cinerea* mycelium grown in Czapek medium and incubated for different times. Native chitinase (lane 1). Cleaved chitinase (lane 5). Lanes 2 and 5: 6 hours of incubation. Lanes 3 and 6: 12 hours of incubation. Lanes 4 and 8: 24 hours of incubation. The gel was stained with a colloidal Coomassie G250 protocol. Molecular weight markers (Low range, Bio-Rad Laboratories, Milano, Italy) was loaded on lane M.

Thus, it appears that *B. cinerea* can cleave the chitinase producing two main products of degradation depending on the conditions of growth of the fungus. The N-terminal sequencing of the two cleaved products (Table 3) was aligned with the aminoacidic sequences of class IV chitinase annotated in the *V. vinifera* genome database: 024530_VITVI, 024531_VITVI and Q7XAU6-1. Despite a mismatch in the 024530_VITVI sequence (not shown), the alignment allowed to identify the two cleavage sites, that are conserved in the three sequences (Figure 6). Thus, the partially cleaved chitinase lacks 26 residues, while the completely cleaved chitinase lacks 37 residues. The 37 residues removed correspond to the chitin-binding domain (CBD). The 3-D structural models of the native and cleaved chitinases built by the SWISS-MODEL server (https://swissmodel.expasy.org/interactive) (Guex *et al.*, 2009; Bertoni *et al.*, 2017; Bienert *et al.*, 2017; Waterhouse *et al.*, 2018; Studer *et al.*, 2020) show the topology of the CBD (Figure 7). In the native protein, the CBD appears separated from the catalytic domain of the protein, containing the glutamic acid (E) residue (Figure 6).

Table 3: N-terminal sequences of the "partially-cleaved" and "cleaved" grape chitinase

Protein	N-terminal sequence
Partially-cleaved chitinase	GCQSGP
Cleaved chitinase	GSGSSV

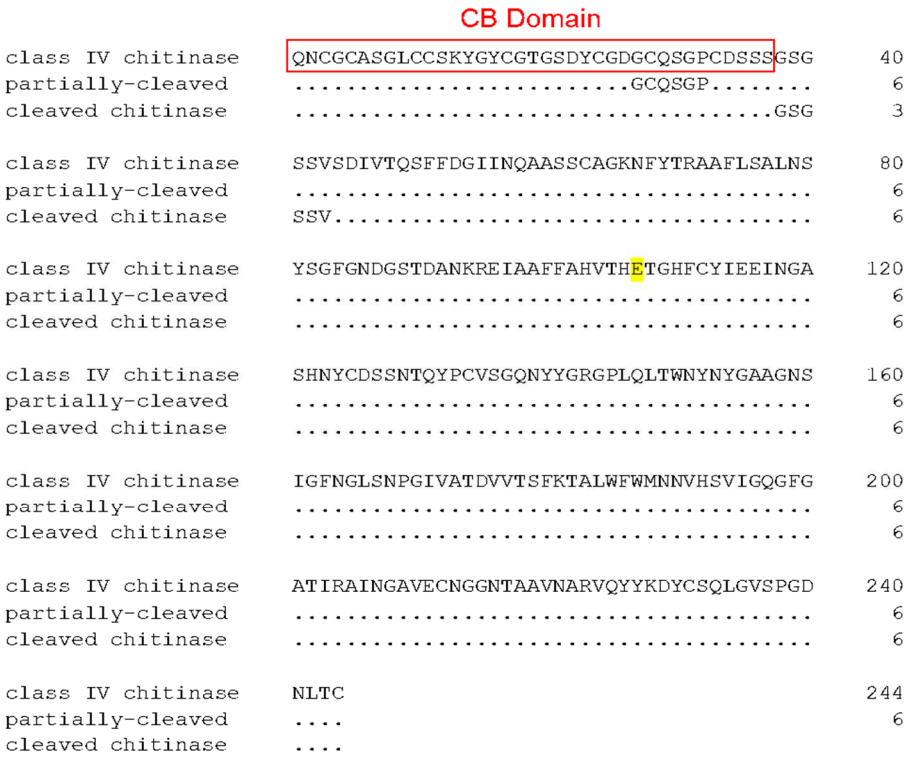

Figure 6: Alignment of the N-terminal sequences with the full-length class IV chitinase (Q7XAU6) obtained by DNAMAN software (Lynnon Biosoft). The glutamic acid residue (E) at the active site is highlighted in yellow

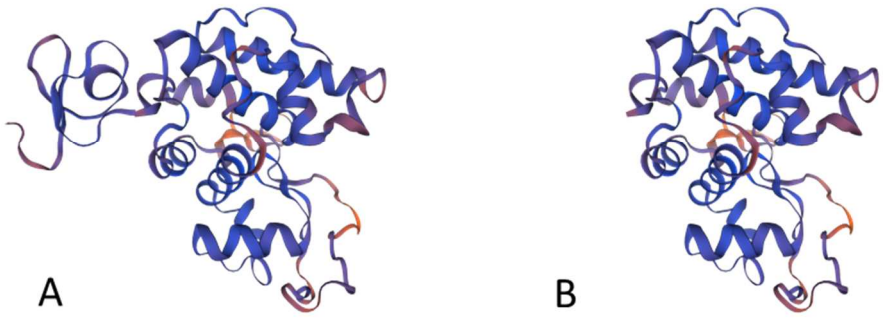

Figure 7: 3-D structural models of the class IV chitinase obtained by submitting the native chitinase sequence (Q7ZAU6) (A) or the cleaved chitinase sequence (B) to the model platform https://swissmodel.expasy.org/interactive.

2.4.3. Chitinase activity decreases when the CBD is removed

To investigate the consequences of the fungal proteolytic removal of the CBD on grape chitinase activity, we compared the chitinase activity of native and cleaved chitinase on the chitin azure substrate. While the native chitinase showed an average of 270 U mg_{eq}^{-1} of enzymatic activity/mg of protein, the cleaved chitinase showed about a 45% reduction of the chitinase activity (Figure 8).

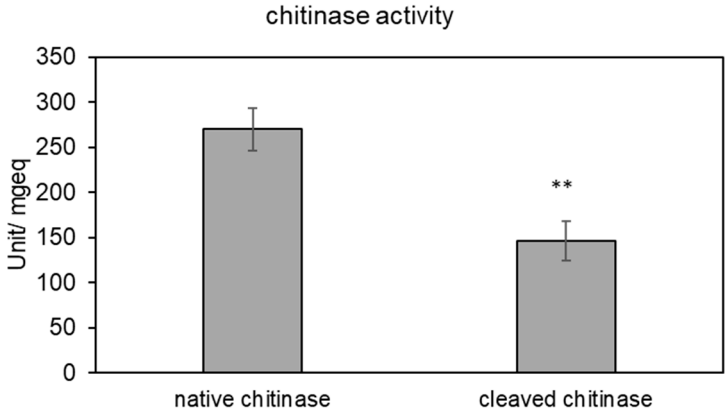

Figure 8: Chitinase activity measured on Chitin Azure substrate by incubating 40 µg of native and cleaved grape chitinase for 8 h at 37 °C and pH 6. The activity is expressed in U mg_{eq}^{-1} of protein and is reported on the y-axis. The values are the average of four experiment ±SD. Statistically significant differences according to Tukey's HDS test are reported (** = $p<0.01$).

In a successive experiment, it was verified if the CBD, which is responsible for the substrate binding, could affect the size of the oligomers produced.

To this aim, colloidal chitin was used as a substrate, and the chito-oligomers released were analyzed by HPLC. The oligomers identified as separated peaks were the monomer GlcNac, chitobiose and chitotriose, and the patterns obtained with the native and cleaved chitinase overlapped. Thus, we concluded that the CBD removal does not modify the chito-oligomers pattern released by chitinase activity (Figure 9). The reduction by about 50% of the amount of each oligomer in the chitin colloidal mixture obtained with the cleaved chitinase confirmed the above reported activity obtained on the chitin azure substrate (Figure 9). The average amounts of each chito-oligomer released are reported in Table 4.

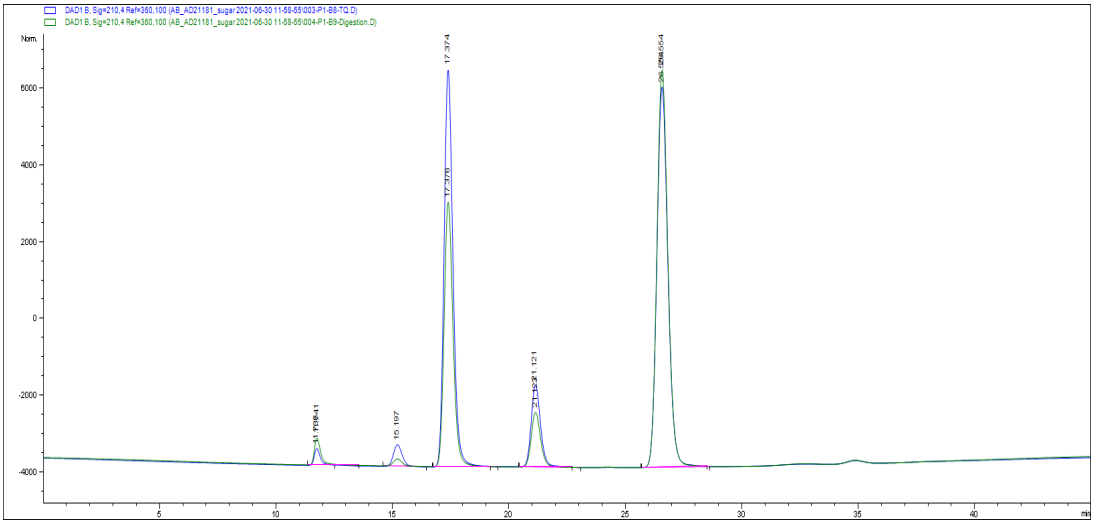

Figure 9: Overlapping patterns of chito-oligomers released by native (blue) and cleaved chitinase (green). Analysis was performed with a HiPlex H column (Agilent). Two different samples were analyzed for each chitinase, obtaining similar results. From left to the right: peak 2-3-4 correspond respectively to chitotriose, chitbiose and GlcNac; peaks 1 and 5 are a background noises.

Table 4: Chito-oligomers released by the native and cleaved chitinases expressed in µg. Areas of the samples' peaks were compared with areas of known standards loaded in a HiPlexH column (Agilent). Data are indicated as the average of two independent experiments ± SE.

Enzyme	**GlcNac**	**Chitobiose**	**Chitotriose**
Native chitinase	0.835 ± 0.40	3.801 ± 1.73	0.054 ± 0.05
Cleaved chitinase	0.437 ± 0.16	2.004 ± 0.69	0.014 ± 0.01
Reduction percentage (%)	52.33	52.72	25.9

2.4.4. Characterization of protease activity

The culture filtrate of *B. cinerea* grown on casein and containing protease activity was incubated with the grape chitinase in the presence of specific protease inhibitors. The inhibitor pepstatin, AEBSF and E-64, inhibiting respectively aspartyl, serin and cysteine protease activity, were unable to inhibit the cleavage of the chitinase (Figure 10, lanes 3, 5 and 6). Only 1-10 phenanthroline, an inhibitor of metalloprotease activity, was effective in apparently inhibiting the second step of chitinase cleavage (Figure 10, lane 4). DAN, a molecule reported as capable to inhibit the acidic protease activity, partially decrease the rate of the chitinase cleavage (Figure 10, lane 7).

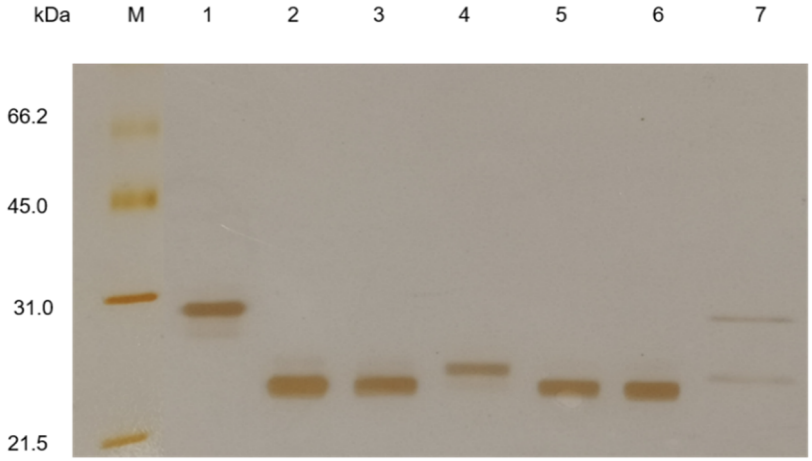

Figure 10: SDS-PAGE of 2 µg of native chitinase incubated for 24 h at 25 °C with *B. cinerea* culture filtrate with protease activity in the presence of different protease inhibitors: Lane 1: native chitinase kept at 25 °C for 24 h. Lane 2: chitinase incubated with culture filtrate containing protease activity without any protease inhibitor. Lanes 3-7: incubation conducted in presence of pepstatin A (22 µM), 1-10 phenanthroline (5 mM), AEBSF (1 mM), E-64 (14 µM) and DAN (15 µM). Gel was stained with a Silver nitrate protocol. Molecular weight marker (Low range, Bio-Rad Laboratories, Milano, Italy) was loaded on lane M.

The purification of the proteases proteins contained in the fungus culture filtrate allowed us to identify an Aspartyl (Bcap8) and a tripeptidyl peptidase as the main proteases produced by *B. cinerea* grown in casein (protein labeled with "a" and "b" in Figure 11A) confirming also previous results by Pais da Cunha (2011). The two purified proteases were incubated with the chitinase and both were unable to cleave the grape chitinase (Figure 11B), excluding the involvement of these proteases in the cleavage process of the chitinase and confirming the results obtained in the experiment with protease inhibitors.

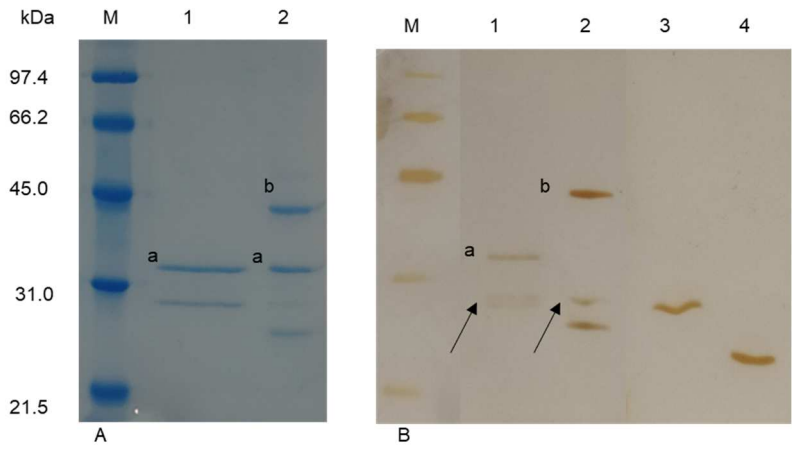

Figure 11: A) SDS-PAGE of the two main fractions obtained with protein purification of the culture filtrate containing protease activity (Lanes 1-2: F17 and F18 respectively) containing Bcap8 (a) and tripeptidyl protease (b). The gel was stained with a colloidal Comomassie G250 protocol. Molecular weight markers (Low range, Bio-Rad Laboratories, Milano, Italy) was loaded on lane M. B) SDS-PAGE of native chitinase incubates 24 h at 25°C with the main protease fractions (Lanes 1-2) native and cleaved chitinases were loaded as control on the gel (lane 3-4 respectively). Gel was stained with a Silver nitrate protocol. Molecular weight markers (Low range, Bio-Rad Laboratories, Milano, Italy) was loaded on lane M. Native chitinase remain uncleaved during incubation with these proteases as indicated by the arrow. Different intensity of Bcap8 and tripeptidyl bands in gel A and B depends on the different staining method used.

The hypothesis of an involvement of the *B. cinerea* Acidic protease Acp1 was further investigated by incubating the chitinase with the 6-days culture filtrate produced by the *B. cinerea* BcΔAcp1 gene knock-out mutant. After 24 h of incubation, the chitinase was cleaved excluding the involvement of Acp1 in the cleavage process (Figure 12, lane 3).

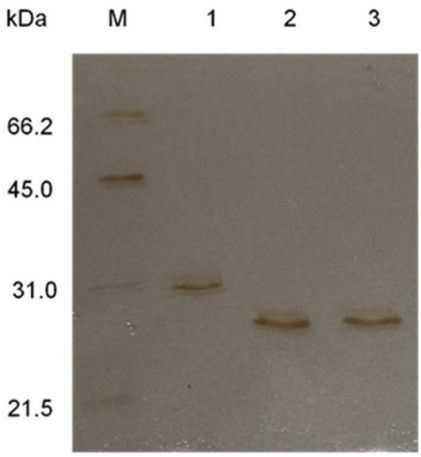

Figure 12: SDS-PAGE of native chitinase incubated for 24 h at 25 °C with protease culture filtrate containing protease activity produced by *B. cinerea* WT and ΔAcp1 mutant (lanes 2 and 3, respectively). Native chitinase was loaded on lane 1. Gel was stained with a Silver nitrate protocol. Molecular weight marker (Low range, Bio-Rad Laboratories, Milano, Italy) was loaded on lane M.

2.4.5. Inhibition of chitinase cleavage in presence of *B. cinerea*

Since, as shown above, *B. cinerea* during its growth in the Czapeck medium cleaves the native chitinase (Figure 5), an experiment was performed for tuning the cultural conditions capable to reduce the cleavage rate of chitinase by *B. cinerea*. To this aim, colonized PDA plugs were suspended in wells with 50 µg ml^{-1} of chitinase dissolved in the Czapeck medium but, in this case, the pH was maintained at 6.5 with MOPS. In separated wells, the inhibitor of metalloproteases 1-10 phenanthroline was added to the buffered broth. After 6 hours of growth of the fungus, chitinase remained uncleaved only in presence of 1-10 phenanthroline (10 mM) (data not shown). Since, as shown above, by the inhibition assay (Figure 10), the 1-10 phenanthroline blocks the second cleavage step, this experiment highlights the importance of pH close to neutrality (6.5) to prevent the first cleavage step. A similar experiment was performed in PDB buffered at pH 6.5 with conidia for 16 h. As shown in Figure 13 (E, lanes 4-6), either pH 6.5 or metalloprotease inhibitor hampered the chitinase cleavage. In comparison, a control performed in absence of MOPS buffer clearly showed that the chitinase cleavage was proceeding in the 16 h timeframe (Figure 13 E, lane 3). The microscopical observation after 16 h, confirmed that buffering the medium at pH 6.5 prevents the chitinase cleavage while the metalloprotease inhibitor prevents the conidia germination (Figure 13 D).

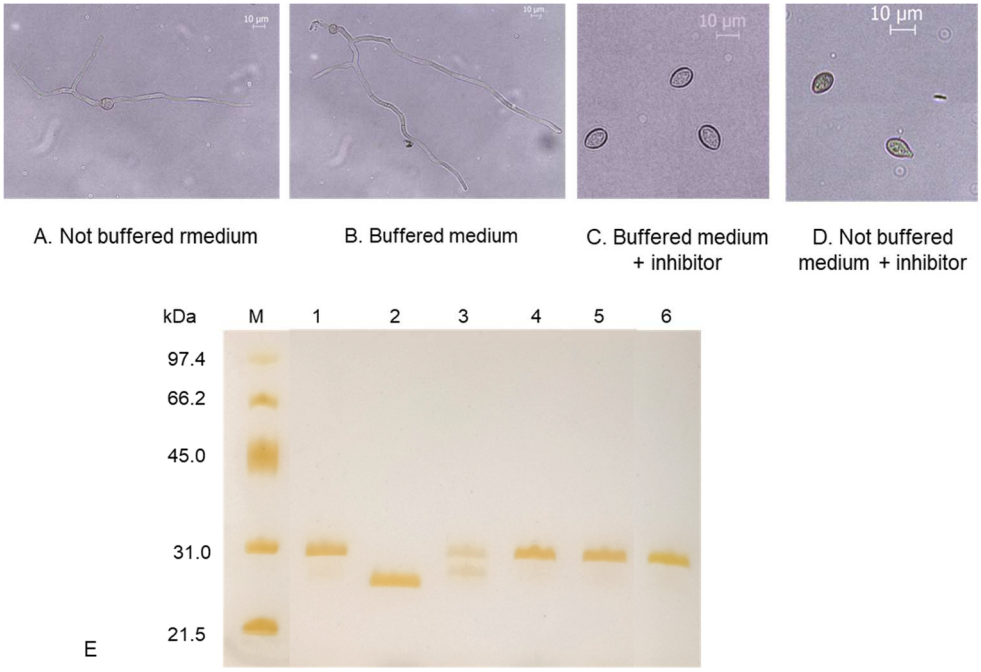

Figure 13: Conidia (10^5 ml^{-1}) germination in (A) standard medium (1/4 PDB), (B) medium buffered at pH 6.5, (C) PDB buffered medium supplemented with 10 mM of 1-10 phenanthroline or (D) not buffered medium supplemented with 10 mM of 1-10 phenanthroline. Observations were made at 16 h of incubation with a light microscope with magnification (40X). (E) SDS-PAGE of 4 µg of chitinase incubated for 16 h in the presence of germinating conidia in mixture A, B, C and D (lane 3, 4, 5 and 6, respectively). Native (lane 1) and cleaved (lane 2) chitinases were also loaded as internal control. Molecular weight marker (Low range, Bio-Rad Laboratories, Milano, Italy) was loaded on lane M. Gel was stained with a Silver nitrate protocol.

2.4.6. Effect of chitinase administration on expression of *B. cinerea* chitin synthase and deacetylase genes

To ascertain if the native or cleaved chitinase could affect the synthesis of chitin and the construction of the fungal cell wall, expression analysis of *B. cinerea* chitin synthase and chitin deacetylase encoding genes was performed by RT-qPCR. RNA was extracted from mycelia 6 h after treatment with native or cleaved chitinase (50 μg ml^{-1}). As a comparison, the expression analysis was performed also by treating *B. cinerea* with TLP. All data were compared with the expression values of untreated control and β-tubulin and actin were used as housekeeping genes. Results show that the expression level of chitin synthase genes in the treated samples does not change in comparison to the untreated control and the expression is comparable among all the treatments. The only exception occurred with the *Bcin01g02520* gene encoding for a class IIIb chitin synthase, which appears down-regulated only in presence of the native chitinase (Figure 14 A).

Three out of four chitin deacetylase genes are slightly down regulated compared to the untreated control by chitinase treatments. Instead, only two chitin deacetylase genes appeared slightly down-regulated after the treatment with TLP (Figure 14 B).

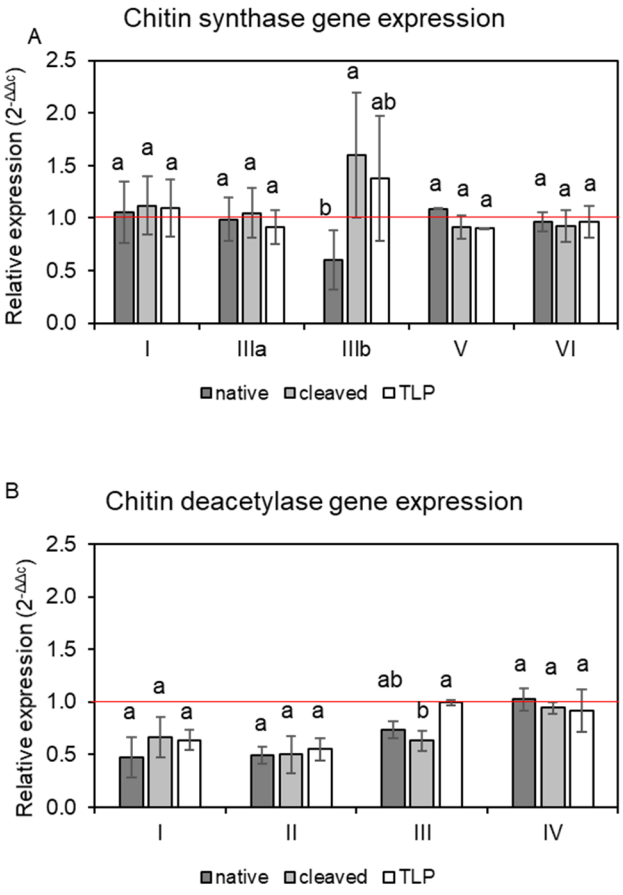

Figure 14: Relative expression level of chitin synthase genes (A, class I, IIIa, IIIb, V, VI) and chitin deacetylase genes (B, class I, II, III, IV) of *B. cinerea* after 6 h from the administration of 50 μg ml^{-1} of native chitinase (dark gray), cleaved chitinase (light gray) or TLP (white). Expression data have been normalized with the housekeeping β-tubulin by using the Rotor-Gene 2.3.5 Software version (Qiagen GmbH, USA) *by using the* $2^{-\Delta\Delta ct}$ method. Error bars indicate SE. Statistically significant differences according to Tukey's HDS test are reported ($p<0.05$). The red line indicates the fold change 1 of untreated control. The same result was obtained with the actin gene as housekeeping (data not reported). The full experiment was repeated three times, with three technical repetitions for each sample.

2.4.7. Effect of native and cleaved chitinase on *B. cinerea* conidia germination

To investigate whether the native and cleaved chitinases affect the germination of *B. cinerea* conidia, a microscopy analysis was conducted on conidia 16 h after the administration of 100 μg ml^{-1} chitinase proteins. The native chitinase significantly affected both the germination and germination tube elongation (Figure 15 A, B and C). In particular, compared to the untreated control, the percentage of germinated conidia was reduced by 50% (Figure 15 A), and also the tube length was reduced by about 50% (Figure 15 B). Conversely, the administration of the cleaved chitinase did not significantly affect the germination percentage nor the average length of the developed hyphae compared to the control (Figure 15 A, B and C).

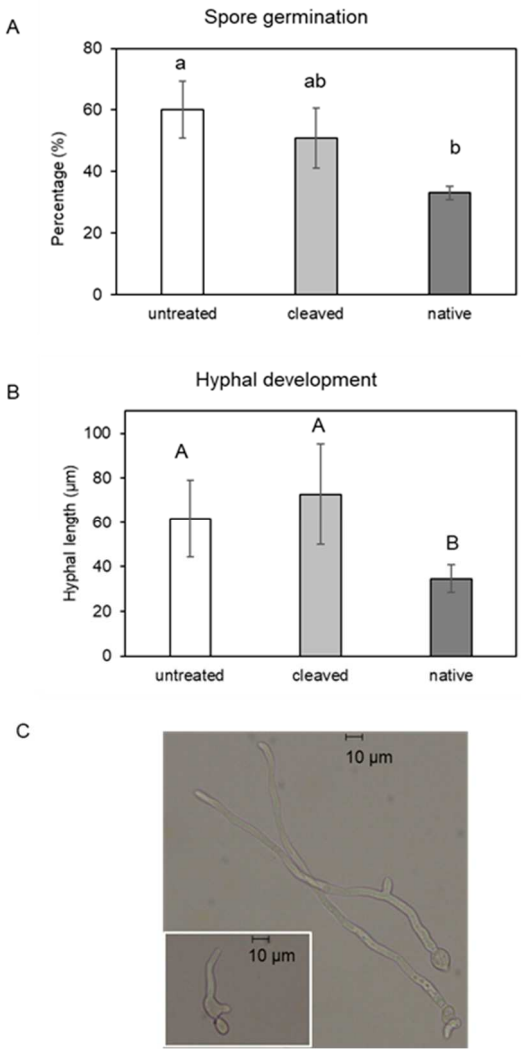

Figure 15: Percentage of germination (A) and hyphal length (B) recorded after 16 h from the administration of 100 µg ml^{-1} of native (dark gray) or cleaved chitinase (light gray). An untreated control was included in the experiment (white). Values are the average of three independent experiments ± SD. Statistically significant differences according to Tukey's HDS test are reported (lowercase letter for $p<0.05$, uppercase letter for $p<0.01$). Representative hyphal development recorded under treatment with native chitinase (white square) or cleaved chitinase (black square) is also reported (C). Observations were made by light microscope with magnification (20X) and pictures were taken with Leica camera.

2.4.8. Transcript expression, enzymatic activity, and protein detection of chitinase in *B. cinerea* infected leaves

To ascertain if the expression of *V. vinifera* class IV chitinase is induced when *B. cinerea* infects leaf tissues, expression analysis was performed by RT-qPCR on RNA extracted from grapevine leaves disks at 16 and 24 h after inoculation with mycelium of *B. cinerea*.

Expression of class IV chitinases, obtained with a primer pair designed on the conserved region of the three sequences available in NCBI, was up-regulated at 16 hpi, with a further increase at 24 hpi. To assess whether the induction involves other PR-proteins, the expression of the gene

encoding for TPL1 was also analyzed. The expression of TLP-1 was weakly up-regulated by *B. cinerea* infection only at 24 hpi (Figure 16).

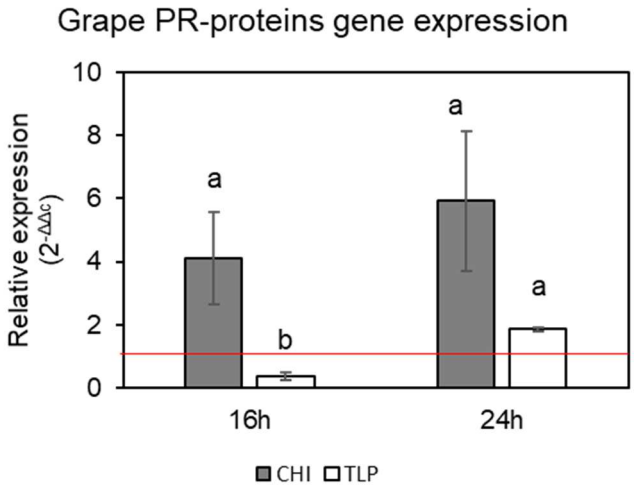

Figure 16: Relative expression level of *V. vinifera* class IV chitinase genes (grey) and TLP-1 (white) after 16 and 24 h from inoculation with *B. cinerea* agar plugs containing actively growing mycelium. RNA extraction was performed from grapevine leak disks (1 cm diameter) surrounding the inoculation point. Expression data have been normalized with the housekeeping actin by using the Rotor-Gene 2.3.5 Software version (Qiagen GmbH, USA) by using the $2^{-\Delta\Delta ct}$ method. Error bars indicate SE. Statistically significant differences according to the Student's t-test are reported ($p<0.05$). The red line indicates the fold change 1 of untreated control. The same result was obtained with the GAPDH gene as housekeeping (data not reported). The full experiment was repeated four times, with three technical repetition for each sample.

To detect protease activity in leaf tissue, proteins from *B. cinerea* infected leaf disks were extracted at different times after the inoculation (Figure 17).

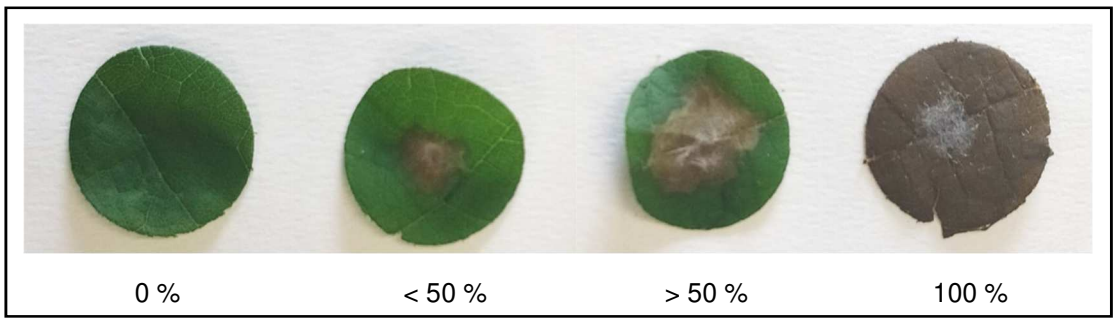

Figure 17: Grapevine leaf disks with representative disease symptoms used for proteins extraction.

The chitinase activity was revealed by a gel activity assay performed on the polyacrylamide gel containing 0.01% of glycol chitin as a substrate. After electrophoresis, and overnight incubation, three different zones of chitinolytic activity were identified after staining with calcofluor white (Figure 18A). The lowest band (L) has the same level of intensity in the non-inoculated leaf sample and infected samples except in the fully infected leaf sample, where the intensity decreased. The upper band (U) was weakly present in healthy tissue, its intensity increased with the progression of the disease and the band was not visible at all in the completely infected tissue. Finally, the intermediate band (M) was undetectable in the healthy tissue, weakly visible in the partially infected samples, and, again, was undetectable in the completely infected tissues (Figure 18A).

The intermediate band has a size comparable to that of the IV class chitinase, as revealed after the staining of the gel with Blu Coomassie. The MALDI-TOF analysis confirmed the identity of this band as a class IV chitinase (Figure 18B).

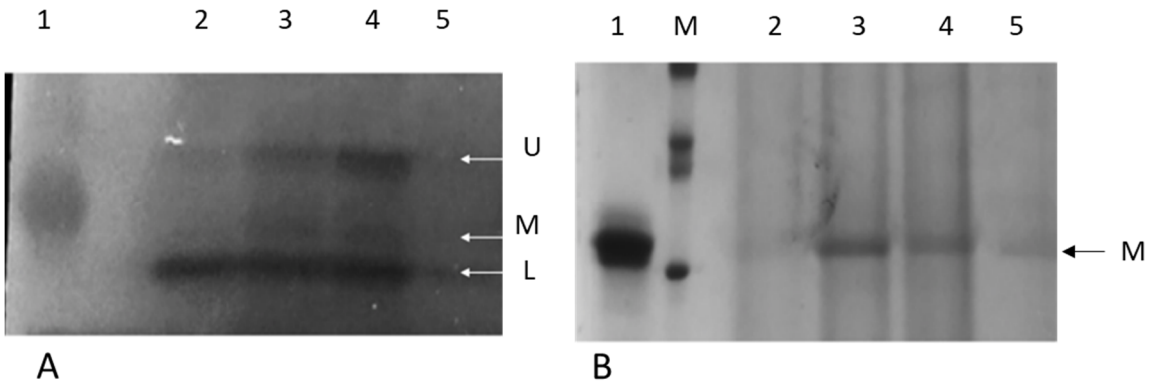

Figure 18: A) Zymogram of the chitinase activity contained in leaf protein extracts. Lane 1: purified chitinase (7 µg); lanes 2-5: 12 µl of crude extract from (2) healthy tissue; (3) leaf with < 50% of symptomatic area; (4) leaf with > 50% of symptomatic area; (5) (3) leaf with 100% of symptomatic area. B) Blu Commassie staining performed on the gel reported in (A). L: lower zone; U: upper zone; M: the middle zone.

2.5. Discussion

Botrytis cinerea is one of the main pathogens of grapevine. As a necrotrophic and polyphagous pathogen, *B. cinerea* has evolved several mechanisms to overcome plant defenses (Choquer *et al.*, 2007; Williamson *et al.*, 2007; Zhang *et al.*, 2019; Haile *et al.*, 2020) and to kill host cells causing leaf, flower and berry rot. Large bunch rot has also negative consequences to wine quality (Bocquet and Valade, 1995; Agudelo-Romero *et al.*, 2015).

Previous studies claimed that *B. cinerea,* encountering a class IV chitinases abundantly contained in grape (Favaron *et al.*, 2009; Marcato *et al.*, 2017), can sequester it with its mycelium glucan matrix or partially degrade it (Marcato *et al.*, 2017). The mechanism responsible for chitinase degradation and the importance of this activity in reducing the harmful effect of chitinase has not yet been ascertained. In this work, the putative involvement of the *B. cinerea* protease activity on the grapevine chitinases has been investigated.

We highlight that a crude protease preparation and even germinating conidia of *B. cinerea* can cleave the chitinase. The cleavage occurred slowly in presence of conidia, a process that may be explained by the necessity of the germinating conidia to recognize the chitinase and activate the protease synthesis.

The N-terminal sequence of the cleaved chitinase provided evidence that the fungal protease activity removes the chitinase CBD. The linking sequence between CBD and the enzymatic core domain (Figure 7) of the chitinase was the target of the protease activity. This process occurs in two steps as deduced by the N-terminal sequence of two partially digested protein fragments. In the first step, 26 amino acids are removed, nine further amino acids are removed in the second one. The cleavage of the CBD domain causes a significant reduction of chitinase activity. A possible explanation for the reduced catalytic activity could be that, in absence of the hydrophobic CBD, this grape acidic IV chitinase loses affinity for the neutral chitin polymer. In contrast, the removal of the CBD in the basic chitinases does not reduce the enzyme activity (Taira, 2010), but reduces the interaction of the chitinases with chitin located in the lateral cell wall where this polymer is less exposed.

We hypothesized that the lower affinity for the substrate of the grape cleaved chitinase could modify the pattern of chitooligomers produced by enzymatic activity. It is known that chitooligomers' length has a role in PTI during fungal infection (Wan *et al.*, 2008; Geoghegan *et al.*, 2017). Six to eight residues length is the most effective for PTI induction in rice and *Arabidopsis* (Miya *et al.*, 2007; Petutschnig *et al.*, 2010) and Brulé *et al.* (2019) showed that chito-oligomers with a degree of polymerization (DP) of 6 strongly induce class IV acidic chitinase expression in grapevine and allow the reduction of infection by *B. cinerea* and *P. viticola*. In our

experiments, we found that native class IV chitinase and cleaved chitinase produce the same chito-oligomers profiles in presence of colloidal chitin as a substrate. Chitobiose in both cases is the most abundant oligomer, a molecule with a negligible role in PTI. However, it is not excluded that when the substrate is the fungal cell wall the chitinase may release a different pattern of oligomers.

To investigate the protease activity responsible for the CBD cleavage three approaches were adopted: i) use of protease inhibitors, ii) purification and characterization of the proteases secreted by *B. cinerea* in the culture medium and iii) use of a *B. cinerea* mutant strain deleted for the gene encoding for the acidic protease Acp1. The most remarkable result was that Bcap8, which is the most abundant aspartyl protease secreted in the culture medium and *in planta* (Li *et al.*, 2012; ten Have *et al.*, 2010), is not active against the grape chitinase. This finding contrast with that of Van Sluyter *et al.* (2013) who showed that Bcap8 can degrade class IV grape chitinases, a result already questioned by Marcato *et al.* (2017) A tripeptidyl protease secreted in culture was also unable to cleave the chitinase. Finally, the involvement of the Acp1 was also excluded after an experiment with the BcΔacp1 mutant which maintained the ability to cleave the chitinase. Only a metalloprotease activity was somehow involved in the chitinase degradation as shown by the use of the 1-10 phenanthroline metalloprotease inhibitor. Precisely, this inhibitor was able to interfere with the second step of the chitinase degradation. This finding is partially in agreement with Karimi Jashni *et al.* (2015a), who found that the cleavage of the CBD of a tomato chitinase requires the synergic activity of a serine protease and a metalloprotease of the fungalysin family. However, in our system the inhibitor of serin protease activity failed to stop the removal of the CBD and the in silico analysis of the *B. cinerea* genome did not show any orthologous fungalysin family (Maracato *et al.*, 2017). In conclusion, the detection of the proteases activity involved in the chitinase degradation remains to be clarified.

An interesting result was that, in presence of the *B. cinerea* mycelium, the removal of the CBD was drastically slowed down when the pH was maintained close to neutrality. This observation permitted us to investigate the effect of treatment on spore germination providing conditions (pH 6.5) that prevented the removal of the chitinase CBD. At pH 6.5, the native chitinase reduced significantly both the germination rate of conidia and the average germination tube elongation in comparison to the untreated control. In contrast, the cleaved chitinase did not affect either spore germination or tube growth. A similar inhibitory effect on spore germination was recorded on *Fusarium oxysporum* spores (Karimi Jashni *et al.*, 2015a) treated with a tomato chitinase IV. However, *F. oxysporum* spores were treated with a very high amount of chitinase (1000 µg /ml^{-1}) while we used only 100 µg ml^{-1}. This difference can be explained by our growing

conditions that maintained the chitinase uncleaved during the entire experiment, while in the experiment with *F. oxysporum* (Karimi Jashni *et al.*, 2015a) a progressive removal of the chitinase CBD may have occurred during spore germination.

The same pH conditions and 10 mM metalloprotease inhibitor were applied for studying the expression of chitin synthase and chitin deacetylase encoding genes after treating the *B. cinerea* mycelium with the native and cleaved chitinase. These genes are likely involved in the synthesis and remodeling of the fungal cell wall following environmental stimuli. Results showed that both native or cleaved chitinase did not alter dramatically the expression of these genes in comparison to the untreated control. Further insights are necessary to detect subtle alteration of the fungal cell wall.

The actual involvement of the *V. vinifera* class IV chitinase during the infection of *B. cinerea* grapevine leaf tissues was investigated both at the level of gene expression, protein and enzyme activity. Indeed, class IV chitinase expression is induced after leaf inoculation with *B. cinerea* and those results agree with other studies that found class IV chitinases as induced following biotic stress (Dadakova *et al.*, 2015; Zheng *et al.*, 2020). This chitinase was also identified in the leaf tissue after *B. cinerea* inoculation. The protein is absent in healthy leaves, it is present in a high amount when the infection covers less than 50% of the leaf area, then decreases and its presence is weakly detectable when the tissue is completely rotted. Remarkable is the observation that despite the chitinase being the most represented protein during the progression of the infection, the corresponding enzymatic activity was very low. Instead, a couple of bands with chitinase activity are undetectable as proteins. This result supports the hypothesis that chitinase IV has significant importance as a defense response to *B. cinerea* infection and that the enzymatic activity is not the most important feature of this chitinase.

In conclusion, the results obtained support the importance of the proteolytic activity secreted by *B. cinerea* as a further mechanism for contrasting the detrimental effects of a grapevine chitinase IV. The removal of the CBD seems sufficient to contrast the fungitoxic effect of the chitinase against the fungal cells. Further studies are needed to identify the fungal proteases responsible for the removal of CBD and to clarify their role in the *B. cinerea* pathogenesis.

The chitinases IV are widespread in plants as a constitutive and inducible defense mechanism (Collinge *et al.*, 1993; Robinson *et al.*, 1997; Martinez-Esteso *et al.*, 2009; Dadakova *et al.*, 2015; Zheng *et al.*, 2020). The cleavage of chitinase IV has been reported also in other plant-pathosystems (Naumann *et al.*, 2011; Naumann and Price, 2012; Karimi Jashni et al., 2015a;) corroborating the results obtained from studies on the *B. cinerea* secretome which highlighted the role of proteases as virulence factors (González-Fernández and Jorrin-Novo, 2012; González-Fernández *et al.*, 2014), strictly regulated by host infected tissues and pH conditions (Li *et al.*,

2012). Thus, the identification of the *B. cinerea* proteases involved in the removal of the CBD from grapevine and other host chitinases, and the disruption of the corresponding genes may allow identifying new potential pathogenicity/virulence factors that may become targets (for example plant engineering with protease inhibitors) for biotechnological approaches in crop protection.

2.6. References

- Agudelo-Romero P, Erban A, Rego C, Carbonell-Bejerano P, Nascimento T, Sousa L, Martínez-Zapater J M, Kopka J, Fortes1 A M. Trascriptome and metabolome reprogramming in *Vitis vinifera* cv Trincadeira berries upon infection with *Botrytis cinerea*. Journal of Experimental Botany. 66: 7: 17696-1785 (2015).
- Bertoni, M., Kiefer, F., Biasini, M., Bordoli, L., Schwede, T. Modeling protein quaternary structure of homo- and hetero-oligomers beyond binary interactions by homology. Scientific Reports 7 (2017).
- Bienert, S., Waterhouse, A., de Beer, T.A.P., Tauriello, G., Studer, G., Bordoli, L., Schwede, T. The SWISS-MODEL Repository - new features and functionality. Nucleic Acids Res. 45, D313-D319 (2017).
- Bocquet F MD, Valade M. Etat sanitaire de la vendange et qualité des vins. Le Vigneron Champenois 7/8, 9 (1995).
- Brulé D, Villano C, Davies L J, Trdá L, Claverie J, Héloir M-C, Chlitz A, Adrian M, Darblade B, Tornero P, Stransfeld L, Boutrot F, Zipfel C, Dry I B, Poinssot B. The grapevine (*Vitis vinifera*) LysM receptor kinases VvLYK1-1 and VvLYK1-2 mediate chitooligosaccharide triggered immunity. Plant Biotechnology Journal 17:812-825 (2019).
- Burzytiski A, Pislewska M, Wojtaszek P. Active chitinases in the apoplastic fluids of healthy white lupin (*Lupinus albus* L.) plants. Acta Physiologiae Plantarum 22: 1: 31-38 (2000).
- Busam G and Matern K U. Diferential expression of chitinases in *Vitis vinifera* L. responding to systemic acquired resistance activators or fungal challenge. Plant Physiol 115:1029–1038 (1997).
- Candiano G, Bruschi M, Musante L, Santucci L, Ghiggeri G M, Carnemolla B, Orecchia P, Zardi L, Righetti P G. Blue silver: a very sensitive colloidal Coomassie G-25 staining for proteome analysis. Electrophoresis 25: 1327-1333 (2004).
- Choquer M, Fournier E, Kunz C, Levis C, Pradier J-M, Simon A, Viaud M. *Botrytis cinerea* virulence factors: new insights into a necrotrophic and polyphageous pathogen. FEMS Microbiology Letters 227:1-10 (2007)
- Ciopraga J, Gozia O, Tudor R, Brezuica L, Doyle R J. *Fusarium* sp growth inhibition by wheat germ agglutin. Biochemica et Biophysica Acta 1428: 424- 432 (1999).
- Collinge D B, Kragh K M, Mikkelsen J D, Nielsen K K, Rasmussen U, Vad K. Plant chitinase. The Plant Journal 3:1: 31-40 (1993).
- Cotoras M and Silva E. Differences in the initial events of infection of *Botrytis cinerea* strains isolated from tomato andgrape. Mycologia 97: 485–492 (2005).
- Dadakova K, Havelkova M, Kurkova B, Tlolkova I, Kasparoscky T, Zdrahal Z, Lochman J. Proteome and transcript analysis of *Vitis vinifera* cell cultures subjected to *Botrytis cinerea* infection. Journal of Proteomics 119: 143-143 (2015).
- Derckel J P, Legendre L, Audran J C, Haye B, Lambert B. Chitinases of the grapevine (*Vitis vinifera* L.): five isoforms induced in leaves by salicylic acid are constitutively expressed in other tissues. Plant Science 119: 31-37 (1996).

- Derckel J-P, Audran J-C, Haye B, Lambert B, Legendre L. Characterization, induction by wounding and salicylic acid, and activity against *Botrytis cinerea* of chitinase and β-1,3-glucanases of ripening grape berries. Physiologia Plantarum 104: 56-64 (1998).
- Deytieux C, Geny L, Lapaillerie D, Claveror S, Bonneu M, Donèche B. Proteome analysis of grape skin during ripening. Journal of Experimental Botany 58: 1851- 1862 (2007).
- Favaron F, Lucchetta M, Odorizzi S, Pais da Cunha A T, Sella L. The role of grape polyphenols on *trans*-resveratrol activity against *Botrytis cinerea* and of fungal laccase on the solubility of putative grape PR proteins. Journal of Plant Pathology 91: 3: 579-588 (2009).
- Ferreira R B, Monteiro S, Freitas R, Santos C N, Chen Z, Batista L M, Duarte J, Borges A, Teixeira A R. Fungal pathogens: the battle for plant infection. Critical Reviews in Plant Sciences 25: 505–524 (2006).
- Ferreira R B, Monteiro S, Freitas R, Santos C N, Chen Z, Batista1 L M, Duarte J, Borges A, Teixeira A R. The role of plant defence proteins in fungal pathogenesis. Molecular Plant Pathology 8:5: 677-700 (2007).
- Fink W, Liefland M, Mendgen K. Chitinases and, B-1,3-Glucanases in the Apoplastic Compartmentof Oat Leaves (*Avena sativa* L.). Plant Physiology 88:270-275 (1988).
- Geoghegan I, Steinberg G, Gurr S. The role of the fungal cell wall in the infection of plants. Trend in Microbiology 25: 12: 957- 967 (2017).
- González-Fernández R, Aloria K, Valero-Galván J, Redondo I, Arizmendi J M, Jorrin-Novo J V. Proteomic analysis of mycelium and secretome of different Botrytis cinerea wild-type strains. Journal of Proteomics 97:195-221 (2014).
- González-Fernández R, Jorrin-Novo J V. Contribution of proteomics to the study of plant pathogenic fungi. Journal of proteome 11:3-16 (2012).
- Guex, N., Peitsch, M.C., Schwede, T. Automated comparative protein structure modeling with SWISS-MODEL and Swiss-PdbViewer: A historical perspective. Electrophoresis 30, S162-S173 (2009).
- Haile Z M, Malacarne G, Pilati S, Sonego P, Moretto M, Masuero D, Vrhovsek U, Engelen K, Baraldi E, Moser, C. Dual transcriptome and metabolic analysis of *Vitis vinifera* cv. Pinot Noir berry and *Botrytis cinerea* during quiescence and egressed infection. Frontiers in Plant Science, 10:1704 (2020).
- http://www.ucdavis.edu, 2010
- Jones J D G and Dangl J L. The plant immune system. Nature 444: 323-329 (2006).
- Karimi Jashni M, Dols I H M, Iida Y, Boeren S, Beenen H G, Mehrabi R, Collemare J, de Wit P J G M. Synergistic action of a metalloprotease and a serine protease from *Fusarium oxysporum* f. sp. *lycopersici* cleaves chitin-bindinng tomato chitinases, reduces their antifungal activity, and enhances fungal virulence. Molecular Plant-Microbe Interactions 28: 9: 996- 1008 (2015a).
- Karimi Jashni M, Mehrabi R, Collemare J, Mesarich C H, de Wit P JGM. The battle in the apoplast: further insights into the roles of proteases and their inhibitors in plant-pathogen interactions. Frrontiers in Plant Science 6: 584 (2015b).
- Kasprzewska A. Plant chitinases: regulation and function. Cellular & Molecular Biology Letters. 8: 809-824 (2003).

- Keen N T. Plant disease resistance: Progress in basic understanding and practical application. Advanced in Botanical Research incorporating Advanced in Plant Pathology. 30: 291–328 (1999).
- Laemmli U K. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227: 680-685 (1970).
- Li B, Wang W, Zong Y, Qin G, Tian S. Exploring pathogenic mechanisms of *Botrytis cinerea* secretome under different ambient pH based on comparative proteomic analysis. Journal of Proteomic Research 11: 4249- 4260 (2012).
- Li S, Zhao ZA, Li M, Gu Z R, Bai C, Huang W D. Purification and characterization of a novel chitinase from *Bacillus brevis*. Sheng Wu Hua Xue Yu Sheng Wu Wu Li Xue Bao (Shanghai) Acta Biochimica et Biophysica Sinica PMID: 12417908, 34: 6: 690-696. (2002).
- Marcato R, Sella L, Lucchetta M, Vincenzi S, Odorizzi S, Curioni A, Favaron F. Necrotrophic fungal plant pathogens display different mechanisms to counteract grape chitinase and thaumatin-like protein. Physiological and Molecular Plant Pathology 99: 7-15 (2017).
- Martinez-Esteso M J, Sellés-Marchart S, Vera-Urbina J C, Pedreño M A, Bru-Martinez R. Change of defense proteins in the extracellular proteome of grapevine (*Vitis vinifera* cv Gamay) cell cultures in response to elicitors. Journal of proteomics 73: 331-341 (2009).
- Miya A, Albert P, Shìnya T, Desakì Y, Ichìmura K, Shìrasu K, Narusaka Y, Kawakami N, Kaku H, Shibuya N. CERK1, a LysM receptor kinase is essential for chitin elicitor signaling in *Arabidopsis*. Proceedings of the National Academy of Science USA 104: 19613-19618 (2007).
- Molano J, Polachech I, Duran A, Cabib E. An endochitinase from wheat germ Activity on nascent and preformed chitin. The Journal of Biological Chemistry 254: 11: 4901-4907 (1979).
- Naumann T A and Price N P J. Truncation of class IV chitinases from Arabidopsis by secreted fungal proteases. Moleculat Plant Pathology 13:9_1135-1139 (2012).
- Naumann T A, Wicklow D T, Price N P J. Identification of a Chitinase-modifying ptrotein from *Fusarium verticillioides*. Truncation of a n host resistance protein by a fungalysin metalloprotease. The Journal of Biological Chemistry 286: 41: 35358-35366 (2011).
- Pais da Cunha A T. Overcome of grape chemical barriers by the fungal pathogen *Botrytis cinerea*. [PhD's thesis] Padova: University of Padova (2011).
- Parijs J V, Broekaert W F, Goldstein I J, Peumans W J. Hevein: an antifungal protein from rubber-tree (*Hevea-brasiliensis*) latex. Planta 183: 258-264 (1991).
- Petutschnig E, Jones A, Serazetdinova L, Lipka U, Lipka V. The lisin motif receptor-like-kinase (LysM-RLK) CERK1 is a major chitin-binding protein in *Arabidopsis thaliana* and subjected to chitin-induced phosphorylation. Journal of Biological Chemistry 285: 28902-28911 (2010).
- Reid K E, Olsson N, Schlosser J, Peng F, Lund S T. An optimized grapevine RNA isolation procedure and statistical determination of reference genes for real-time RT-PCR during berry development. BMC Plant Biology 6: 27: 1-11 (2006).

- Robert N, Roche K, Labeau Y, Breda C, Boulay M, Esnault R, Buffard D. Expression of grapevine chitinase genes in berries and leaves infected by fungal or bacterial pathogens. Plant Science 162: 389-400 (2002).
- Robinson S P, Jacobs A K, Dry I B. A class IV chitinase is highly expressed in grape berries during ripening. Plant Physiology 114: 771-778 (1997).
- Saito S, Odagiri M, Furuya S, Suzuki S, Takayanagi T. Inhibitory effect of chitinases isolated from Semillon grapes (*Vitis vinifera*) on growth of grapevine pathogens. Journal of Plant Biochemistry and Biotechnology 20:47–54 (2011).
- Sarry J-E, Sommerer N, Sauvage F-X, Bergoin A, Rossignol M, Albagnac G, Romieu C. Grape berry biochemistry revisited upon proteomic analysis of the mesocarp. Proteomics 4: 201-215 (2004).
- Sels J, Mathys J, De Coninck B M A, Cammune B P A, De Bolle M F C. Plant pathogenesis-related (PR) proteins: A focus on PR peptides. Plant Physiology and Biochemistry 46: 841-950 (2008).
- Studer, G., Rempfer, C., Waterhouse, A.M., Gumienny, G., Haas, J., Schwede, T. QMEANDisCo - distance constraints applied on model quality estimation. Bioinformatics 36, 1765-1771 (2020).
- Taira T. Structure and antifungal activity of plant chitinases. Journal of Applied Glycoscience 57: 167-176 (2010).
- Tattersall D B, van Heeswijck R, Hoj P B. Identification and characterization of a fruit-specific, thaumatin-like protein that accumulates at very high level in conjunction with the onset of sugar accumulation and berry softening in grapes. Plant Physiology 114: 759-769 (1997).
- ten Have A, Espino J J, Dekkers E, Van Sluyter S C, Brito N, Kay J, Gonzalez C, van Kan J A L. The *Botrytis cinerea* aspartic protease family. Fungal Genetics and Biology 47: 53-65 (2010).
- Trudel J and Asselin A. Detection of chitinase activity after polyacrylamide gel electrophoresis. Analitical Biochemistry 178: 362-366 (1989).
- van Loon L C, Pierpoint W S, Boller Th Conejero V. Recommendations for naming plant pathogenesis-related proteins. Plant Molecular Biology Reporter 12: 3: 245-264 (1994).
- Van Sluyter S C, Marangon M, Stranks S D, Neilson K A, Hayasaka Y, Haynes P A, Menz R I, Waters E. Two-step purification of pathogenesis-related proteins from grape juice and crystallization of thaumatin-like proteins. Journal of Agricultural and Food Chemistry 57: 11376-11382 (2009).
- Van Sluyter S C, Warnock N I, Schmidt S, Anderson P, Van Kan J A L, Bacic A, Waters E J. Aspartic acid protease from *Botrytis cinerea* removes haze-forming proteins during white winemaking. Journal of Agriculture and Food Chemistry 61: 9705- 9711 (2013).
- Vincenzi S, Bierma J, Wickramasekara S I, Curioni A, Gazzola D, Bakalinsky A T. Characterization of a grape class IV chitinase. Journal of Agricultural and food chemistry 32: 5660-5668 (2014).
- Wan J, Zhang X C, Neece D, Ramonell K M, Clough S, Kim S Y, Stacey M G, Stacey G. A LysM receptor-like kinase plays a critical role in chitin signaling and fungal resistance in Arabidopsis. Plant Cell 20: 4171-481 (2008).
- Waterhouse, A., Bertoni, M., Bienert, S., Studer, G., Tauriello, G., Gumienny, R., Heer, F.T., de Beer, T.A.P., Rempfer, C., Bordoli, L., Lepore, R., Schwede, T. SWISS-

MODEL: homology modelling of protein structures and complexes. Nucleic Acids Res. 46(W1), W296-W303 (2018).

- Williamson B, Tudzynski B, Tudzynski P, Van Kan J A L. *Botrytis cinerea*: the cause of grey mould disease. Molecular Plant Pathology 8: 5: 561-580 (2007).
- Zhang W, Corwin J A, Copeland D H, Feusier J, Eshbaugh R, Cook D E, Atwell S, Kliebenstein D J. Plant–necrotroph co-transcriptome networks illuminate a metabolic battlefield. eLife 8: e44279 (2019).
- Zheng T, Zang K, Sadeghnezhad R, Jiu S, Zhu X, Dong T, Liu Z, Guan L, Jia H, Fang J. Chitinase family genes in grape differentially expressed in a manner specific to fruit species in response to *Botrytis cinerea*. Molecular Biology Reports 4:7349-7363 (2020).

CHAPTER III

Targeted amino acid substitutions in a *Trichoderma* peptaibol confer activity against infection of *Plasmopara viticola* and *Botrytis cinerea*

A part of data reported has been published in: Targeted amino acid substitutions in a trichoderma peptaibol confer activity against fungal plant pathogens and protect host tissues from *Botrytis cinerea* infection. De Zotti *et al.* Int. J. Mol. Sci. 2020,21,7521.

3.1. Abstract

Grapevine (*Vitis vinifera*) is one of the main crops worldwide. *Plasmopara viticola*, the causal agent of downy mildew, and *Botrytis cinerea*, the causal agent of gray mold, cause enormous economic damages in terms of yield, quality and productivity to this crop. Diseases control is largely based on the use of synthetic fungicides. In recent decades, environmental and human health concerns have prompted European Union (UE) policies to promote the reduction of the reliance on synthetic plant protection products (PPPs) and to implement integrated approaches to crop protection. Biocontrol agents (BCA) constitute a great resource for the development of biopesticides. The fungal genus *Trichoderma* includes different species commonly used as BCAs against several crop pathogens. Among *Trichoderma* antifungal strategies, there are secondary metabolites such as peptaibols, which are helical antimicrobial peptides structurally stable even under extreme pH and temperature conditions. The promise of peptaibols as agrochemicals is, however, hampered by their poor water solubility, inhibiting an efficient delivery for practical use in crop protection. In this work, the naturally occurring peptaibol trichogin and some synthesized water-soluble analogs of trichogin were analyzed for their antimicrobial activity against *P. viticola* and *B. cinerea*. Although natural trichogin was inactive, effective peptides for each pathogen were identified; in particular, peptide 4 was active against both pathogens. The effectiveness of the most active peptide was further investigated by microscopy analysis. Furthermore, assays on grapevine tissues demonstrated a reduction of disease symptoms without phytotoxic effects. A 3D-structure–activity relationship study indicated that the relative spatial position of cationic residues is crucial for increasing peptide fungicidal activity; the position of cationic residues on the chain can be also related to the specific antifungal activity. Future field trials will help to confirm the effectiveness of this peptide to counteract both *P. viticola* and *B. cinerea*.

3.2. Introduction

Grapevine (*Vitis vinifera*) is one of the major crops worldwide (Kanellis *et al.*, 1993; Kortekamp, 2006; Merz *et al.*, 2015; Pertot *et al.*, 2017). It acquired economic interest from its domestication (about 8.000 years ago) till nowadays (Terral *et al.*, 2010). According to Gessler *et al.* (2011), the European grapevine cultivation era can be divided into three periods: a first lucky period characterized by the absence of the major phytosanitary problems, a second period (1845- 1900) during which the crop faced three previously unknown diseases – powdery mildew, phylloxera and downy mildew, all coming from America – and a third period, still ongoing, characterized by intensive use of chemicals to protect the crop from these and other diseases. Nowadays, the European Union (UE) policies foresee the reduction of the reliance on synthetic plant protection products (PPPs) and the implementation of integrated approaches to crop protection (EU Directive 2009/128/CE). Indeed, the control of downy and powdery mildew and of gray mould, which are the main diseases responsible for huge losses in grape yield and quality (Merz *et al.*, 2015; Pertot *et al.*, 2017), is largely based on the use of synthetic fungicides. The management of these diseases accounts for the greater amount of treatments per year in the vineyard (Pertot *et al.*, 2017), with occasioning costs and a potential negative impact on the environment and human health (Muthmann, 2007; Merz *et al.*, 2015;).

Integrated disease management exploits the so-called biocontrol agents (BCA), namely fungi and other microorganisms that can antagonize phytopathogenic microorganisms. Fungi of *Trichoderma* spp. are among the most widely used and effective biocontrol agents (Fraceto *et al.*, 2018; Verma *et al.*, 2007). The antagonistic action of *Trichoderma* spp. concerns competition for space and nutrients, mycoparasitism through the secretion of degrading enzymes and antibiosis by secreted secondary metabolites (Marra *et al.*, 2019). Under environmental conditions, the vitality of *Trichoderma* population is contingent (Pertot *et al.*, 2017). Thus, the effectiveness of *Trichoderma* preparations is variable and sometimes unreliable under open field conditions; this determines their use mostly restricted against soil or seed-borne plant pathogens (Fraceto *et al.*, 2018; Tamandegani *et al.*, 2020). T*richoderma* spp. produce a huge amount of secondary metabolites; their use as target-specific biofungicides for controlling phytopathogens in substitution of available *Trichoderma* formulations was proposed as a tool for a next-generation fungicide era (Zeilinger *et al.*, 2016). Peptide antibiotics are the most abundant category of *Trichoderma* secondary metabolites, of which peptaibols constitute the largest group (Stoppacher *et al.*, 2013; Degenkolb *et al.*, 2015; Marik *et al.*, 2019). Peptaibols are 5-20 residue-long nonribosomal peptides synthesized by fungi and containing several noncoded α-aminobutyric acid (Aib) residues and a C-terminal 2-aminoalcohol; the antifungal-activity of

peptaibols has been attributed to their ability to induce membrane permeability (De la Fuente-Núñez *et al.*, 2013). The Aib residue confers to the peptaibols resistance to proteolytic enzymes (Yamaguchi *et al.*, 2003) and a well-defined helical structure (Toniolo *et al.*, 2001), resulting in the characteristic stability at extreme pH and temperatures. Few peptaibols have been assayed against plant pathogens. In particular, the long-chain trichokonins from *T. pseudokoningi* have been characterized as inducers of programmed cell death against *B. cinerea* (Zhao *et al.*, 2018). Despite these promising features, some limitations reduce the practical application of extracted peptides. Peptaibol biosynthesis occurs under particular environmental conditions and in presence of the antagonized species (Vinale *et al.*, 2009) difficult to reproduce *in vitro* (Speckbacher and Zeilinger, 2018). In addition, the purification is complicated by the heterogeneity of the mixtures in which they are secreted. Another trait that makes natural peptaibol unattractive is the poor water solubility due to their low polar residue content.

The naturally occurring peptaibol trichogin GA IV (hereafter called trichogin) produced by *Trichoderma longibrachiatum* (Auvin-Guette *et al.*, 1992) possesses antimicrobial activity (De Zotti *et al.*, 2009) and a well defined helical structure (Toniolo *et al.*, 1994), but is poorly water soluble. In this work, some water-soluble analogs of the natural trichogin have been assayed for their fungicidal activity against *P. viticola* and *B. cinerea*, two of the main pathogens affecting *V. vinifera*.

3.3. Material and methods

3.3.1. Water-soluble analogs of trichogin

Trichogin and its water-soluble analogs have been synthesized by the chemistry department of the University of Padova as reported in De Zotti *et al.* (2020). Trichogin amphiphilic helical conformation (Figure 1) presents a less hydrophilic face rich in Gly residues.

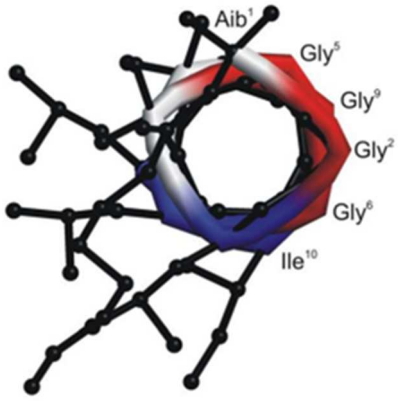

Figure 1: Helical structure of the native peptaibol tricogin (view along the helical axis)

To improve the amphiphilicity of the trichogen analogs, one or more Gly residues were replaced with Lys (peptides 1-5 and 7). The minimal peptide length needed for bioactivity was investigated through shorter peptides (peptides 4c, 4c1 and 4c2). An analog was modified also in the helical structure rigidity replacing the Gly^6 with an Aib residue (peptide 6). Because of the possible industrial production of those analogs as PPPs, the same analogs were synthesized with Rink Amide resin ("r") resulting in a replacement of the C-terminal Lol moiety with a -Leu-NH_2 (leucine amide) residue.

In Table 1 all the peptide sequences used in this study are reported in comparison with the sequence of Trichogin GA-IV and grouped according to their main characteristics.

Table 1: Sequence of the synthesized trichogin analogs. Modified residues are highlighted in bold

Ppeptide ID	Sequences[a]
Full lenght peptides	
Trichogin GA IV	nOct – Aib – Gly – Leu – Aib – Gly – Gly – Leu – Aib – Gly – Ile - Lol
1	nOct – Aib – **Lys** – Leu –Aib – **Lys** – Gly – Leu – Aib – Gly – Ile - Lol
2	nOct – Aib – **Lys** – Leu – Aib – Gly – Gly – Leu – Aib – Gly – Ile - Lol
3	nOct – Aib – Gly – Leu – Aib – **Lys** – Gly – Leu –Aib – Gly – Ile - Lol
4	nOct – Aib – Gly – Leu – Aib – **Lys** – **Lys** – Leu – Aib – Gly – Ile - Lol
5	nOct – Aib – Gly – Leu – Aib – Gly – **Lys** – Leu – Aib – Gly – Ile - Lol
6	nOct – Aib – Gly – Leu – Aib – **Lys** – **Aib** – Leu – Aib – Gly – Ile - Lol
7	nOct – Aib – **Lys** – Leu – Aib – Gly – **Lys** – Leu – Aib – Gly – Ile - Lol
Shorter analogs	
4c	nOct – Aib – – – – **Lys** – **Lys** – Leu – Aib – Gly – Ile - Lol
4c1	nOct – Aib – Gly – Leu – Aib – **Lys** – **Lys**– – – – – Leu - NH_2
4c2	nOct - Aib – – – – **Lys** – **Lys** – Leu – Aib – Gly – Ile – Leu - NH_2
Rink Amide resin	
4r	nOct – Aib - Gly - Leu – Aib – **Lys** – **Lys** – Leu – Aib – Gly – Ile – Leu - NH_2

[a] ***n*Oct, *n*-octanoyl; Aib, α-aminoisobutyric acid; Lol, leucinol;**

3.3.2. Peptides assays against *Plasmopara viticola*

Grapevine leaf material

Leaves of a white grapevine variety (*Vitis vinifera*, cv. Glera) were collected from vine cuttings grown in a climatic chamber (20-22 °C, 16 h photoperiod) for about one month after potting.

Plasmopara viticola strain, propagation and production of zoospores

Sporangia of *P. viticola* were collected from infected grapevine leaves harvested in a vineyard in the municipality of Nervesa della Battaglia in the North East of Italy (*V. vinifera*, cv. Glera). The mildew was maintained on grape leaves by inoculating sporangia on the abaxial surface of detached fresh leaves. Leaves were arranged on moist towels in plastic trays and maintained at 22-25°C in the dark under saturating humid conditions. After 6-9 days from leaf inoculation, sporangia inoculum was collected by water washing. For leaf disk inoculation, sporangia were diluted to obtain 3-5x10^5 sporangia ml^{-1}. For microscopy analysis, sporangia suspension (between 5x10^5 and 3x10^6 sporangia ml^{-1}) was directly used or incubated in the dark at room temperature (23 °C) for 6 hours to release zoospore (Islam *et al.*, 2016).

P. viticola infection experiments and peptides treatments

Infection assays were performed on grapevine leaf disks of 1 cm diameter. Twenty leaf disks cut from several leaves were placed on a moistened sterile filter in Petri plates (15 cm in diameter) with the abaxial surface up.

A synthesized trichogin GA-IV or their derivatives (Table 1) were dissolved in water at 50 μM and about 0.1 ml of each peptide solution were sprayed on each leaf disk. After drying for 10 min under a biosafety hood the leaf disks were sprayed with a sporangia suspension (about 0.75 ml of suspension per plate) and the plates were incubated in the dark at room temperature (22-23°C).

The incidence of downy mildew was determined 12 days post-inoculation (dpi). The incidence was calculated as the ratio between the number of sporulating disks on the total number of disks. Control plates contained disks treated with water only. The disease incidence on the leaf treated with peptides and trichogin was expressed as a percentage of incidence value measured on control leaves. Two or three plates were carried out for each treatment. For the most promising peptides, the same experiment was performed also with lower concentrations: 30, 20 and 15 μM. For comparison, a commercial fungicide (Tricopperland, ISAGRO S.P.A.) was assayed against the pathogen at 50, 200 and 730 μM of Cu. Data were statistically analyzed by applying the one-way Anova and Bonferroni-Holms test.

Microscopic observations

Aliquots of 200 μl of suspension containing $5x10^5$ sporangia were treated with peptide 4 at 50 μM and, after 15, 30 and 60 min, sporangia were examined by optical microscope (Laborlux 12). At least four optical fields each containing 50-100 sporangia were analyzed and sporangia with shape alterations were counted. At the same time, untreated sporangia (negative control) were also examined and counted for morphological alteration. The experiment was repeated three times.

A suspension containing $3x10^6$ sporangia was incubated for 6 h in sterile deionized water to permit the release of zoospores (Islam *et al.*, 2016). Afterward, peptide 4 was added and the zoospores were examined after 15 and 30 min. At least four optical fields containing 40-80 zoospores were analyzed and the lysed zoospores were counted. Untreated zoospores (negative control) were also analyzed. The experiment was repeated three times. Data were statistically analyzed by applying a one-way Anova Tukey-Kramer test.

3.3.3. Peptides assays against *Botrytis cinerea*

Fungal strain and conidia production

Botrytis cinerea strain PM10 (Favaron *et al.*, 2009), was cultured at 25 ◦C on potato dextrose agar (PDA, Difco Laboratories, Detroit, MI, USA). Conidia were recovered from 15 days-old, completely colonized plates by gently scraping with a glass rod in the presence of 6 ml of sterile water supplemented with glycerol (10% v/v). Conidia were filtered through sterilized gauze and counted using a hemocytometer

In vitro assays

The antimicrobial activity of the water-soluble peptides was initially evaluated against *B. cinerea* with the resazurin vital dye assay (Sella *et al.* 2014). In light of the poor water solubility of native trichogin, we solubilized it in ethanol before diluting it in water to obtain a final ethanol concentration of 5% (v/v). Wells of microtiter plates were filled with 200 µl of potato dextrose broth (PDB; Difco Laboratories) at pH 6.9 supplemented with the peptides at 15 µM, previously resuspended in water, and with *B. cinerea* conidia at a final concentration of 5×10^5 spores ml^{-1}. Controls without peptides were also prepared. To each well, 68 µg ml^{-1} of resazurin dye (Sigma-Aldrich, Milano, Italy) were added. The microtiter plates were then incubated in the dark at 25 °C and monitored every 12 h for resazurin dye color change. The time at which control wells, without peptides added, turned into pink was fixed as time zero to record the delay of growth caused by the peptides. Each peptide was tested in three independent replicates. The most active peptides were then assayed at 1, 5, and 15 µM against *B. cinerea*. Wells of microtiter plates were filled as reported above, supplemented with different concentrations of peptides, without the addition of resazurin, and incubated in the dark at 25 ◦C. After 96 h of incubation, the fungal biomass in the wells was estimated by reading the absorbance at 450 nm (Granade *et al.*, 1985), and it was expressed as a percentage of the maximum values measured in control wells containing the same conidia concentration not treated with the peptides. Each data point is the mean of 3 independent replicates. The minimal inhibitory concentration (MIC) values were also calculated.

Microscopy analysis

A cell viability assay was performed by treating 5×10^5 ml^{-1} conidia of *B. cinerea* suspended in PDB with peptide 4 at 15 µM. At 0 h and after 24 h, Evan's Blue staining was performed to label fungal protoplasts of dead cells. Evan's blue staining protocol was adapted from Martinez *et al.* (2017) and Semighini *et al.* (2006) to enhance the staining. Briefly, 100 µl of the conidia

incubation mixture were concentrated by centrifugation at 3000× g for 10 min. Evan's Blue dye was added to a final concentration of 0.05% (w/v), and the mixture was incubated for 15 min at room temperature. After incubation, fungal spores were abundantly washed with phosphate-buffered saline (PBS), centrifuged as above reported, visually analyzed for vitality by optical microscopy (Laborlux 12), and photographed (Sony 20.1 Megapixel AVCHD camera, Tokyo, Japan). Conidia suspended in PDB were used as a negative control. Aliquots of the suspensions treated with peptide 4 for 24 h were abundantly rinsed with sterile distilled water, centrifuged, and plated on PDA to check conidia viability.

In vivo assays

The most effective peptides against *B. cinerea in vitro* were assayed against *B. cinerea* infection of grapevine leaves and berries. Leaves of a white grapevine variety (*Vitis vinifera*, cv. Glera) were collected from vine cuttings grown in the climatic chamber for about one month after potting. Grape berries (cv. Pinot bianco) were harvested at maturity (by mid-August) from a typical vineyard included in the municipality of Conegliano (North-East Italy). Ripe grape berries were detached from the bunch, surface-sterilized with 0.115% (v/v) sodium hypochlorite for 15 min, rinsed with sterile water, and separately arranged on a plastic tray. Treatments were performed by spraying the peptides at 50 μM on leaves and berries. Water spraying was performed as a negative control. Leaves were arranged on moist towels in plastic trays and inoculated with a suspension of 2.5×10^6 ml^{-1} conidia of *B. cinerea* in PDB. Ten microliters of the conidia suspension were dropped on two or three points apart on the leaf surface of grapevine leaves. Berries were sprayed with *B. cinerea* conidia at a concentration of 1.85×10^6 ml^{-1}. Inoculated leaves and berries were incubated in the dark at 25 °C in plastic bags to maintain humidity. Seven days post-inoculation (dpi), the size of the symptomatic leaf area was measured from pictures taken with a Panasonic Lumix DMC-FZ2000 by using the graphic software "Fiji ImageJ" (GNU General Public License). For grape berries, symptoms were assessed at 4 dpi. As the *B. cinerea* infection produces a visible browning spot, each infected berry was indexed based on symptoms development using a Disease Index (DI) modified from Cabanne *et al.* (2002). Specifically, the DI ranged from 0 to 4, where 0 = healthy berries without lesion spots, 1 = infected berries with small spots covering <5% of the berry surface, 2 = infected berries with spots covering <25% of the berry surface, 3 = infected berries with spots covering >25% of the berry surface, and 4 = infected berries with spots covering >50% of the berry surface. The experiment was repeated three times with leaves and four times with berries. Each treatment was performed with three grapevine leaves. Twenty grape berries were used for each

treatment. Data obtained in the infection experiments performed on grapevine leaves and berries were statistically analyzed by applying the two-tailed Student's t-test.

3.4. Results

3.4.1. Peptides activity against *P. viticola*

In vivo experiments

The full-length peptides and their shorter or "r" analogs were assayed for their ability to counteract *P. viticola*. To this aim, due to the nature of this obligate biotrophic oomycete pathogen, the effectiveness of peptides was evaluated by a leaf disc assay. Leaf discs were sprayed with peptide solution at 50 µM and then inoculated with sporangia suspension. Twelve days post-inoculation (dpi) the number of infected leaf discs per treatment was recorded. The disease incidence of the leaves treated with peptides and trichogin was expressed as a percentage of incidence value measured on control leaves treated with water only. The average control incidence was 95.44 ± 8.03. Trichogin did not prevent sporangia development on leaf disc, whereas peptides 1, 4 and 6 showed a significant reduction of *P. viticola* incidence (Figure 2). No phytotoxic effects were noticed on leaves after treatment with the peptides (data not shown).

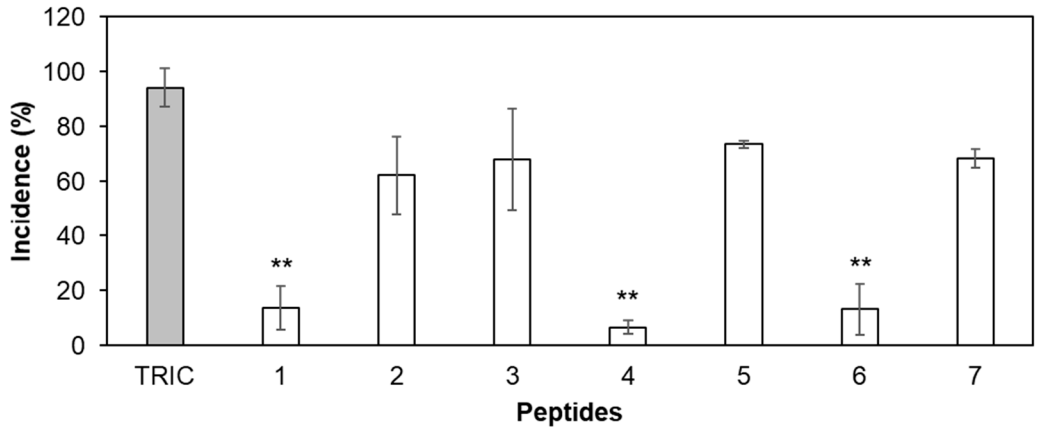

Figure 2: Incidence of sporulating grapevine leaf discs cv. Glera treated with peptides at 50 µM and inoculated with *P. viticola* sporangia. Sympthomatic discs were recorded 12 dpi and incidence was expressed as percentage of peptide treated symptomatic leaf discs respective to control symptomatic leaf discs (treated with water). TRIC = Trichogin; number-letter IDs correspond to peptides of Table 1. Bars indicate standard errors. Two or three independent infection experiment were performed for each treatment. Data were statistically analyzed by Bonferroni Holm test: only pairs relative to trichogin were simultaneously compared. ** indicates significant differences at $p < 0.01$.

For the active peptide 4, cheaper analogs were also screened for their activity against *P. viticola*. Peptides 4r, 4c, 4c2 confirmed the effectiveness showed by the respective original analog. Instead, 4c1 was ineffective against pathogen infection (Figure 3).

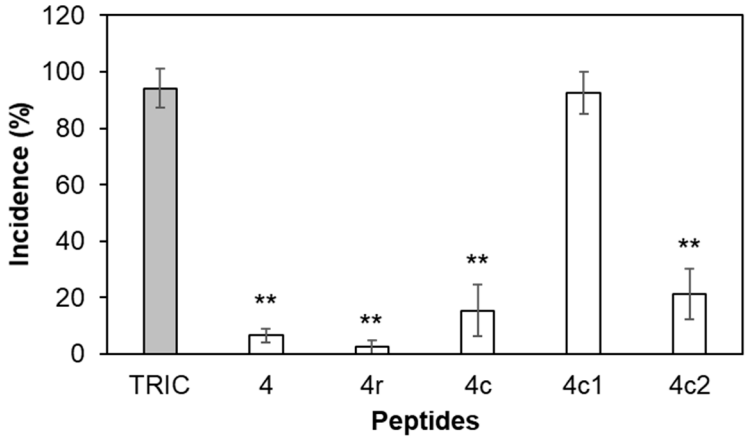

Figure 3: Incidence of sporulating grapevine leaf discs cv. Glera treated with peptides at 50 μM and inoculated with *P. viticola*. Symptomatic discs were recorded 12 dpi. The incidence was expressed as percentage of peptide treated symptomatic leaf disks respective to control symptomatic leaf discs (treated with water). TRIC = Trichogin; number-letter IDs correspond to peptides of Table 1. Bars indicate standard errors. Two or three independent infection experiment were performed for each treatment. Data were statistically analyzed by one-way Anova Bonferroni Holm test: only pairs relative to trichogin were simultaneously compared. ** indicates significant differences at $p < 0.01$.

The effective peptide 4 and its analog 4r were assayed at doses lower than 50 μM and compared to copper used as a positive control (Figure 4) starting from one-tenth of the field dose used for grapevine protection against *P. viticola* (730 μM). Treatment with trichogin (50 μM) was used as a negative control. In comparison to the control, the peptides 4 and 4r significantly prevented sporangia production at 30 μM ($p<0.01$), even if less efficiently than at 50 μM. Peptide 4 was still effective at 20 μM, reducing by 50% the disease incidence of the control. Copper was found to significantly reduce the disease incidence at 730 μM ($p<0.01$), while at 200 μM the reduction was about 50% ($p<0.05$).

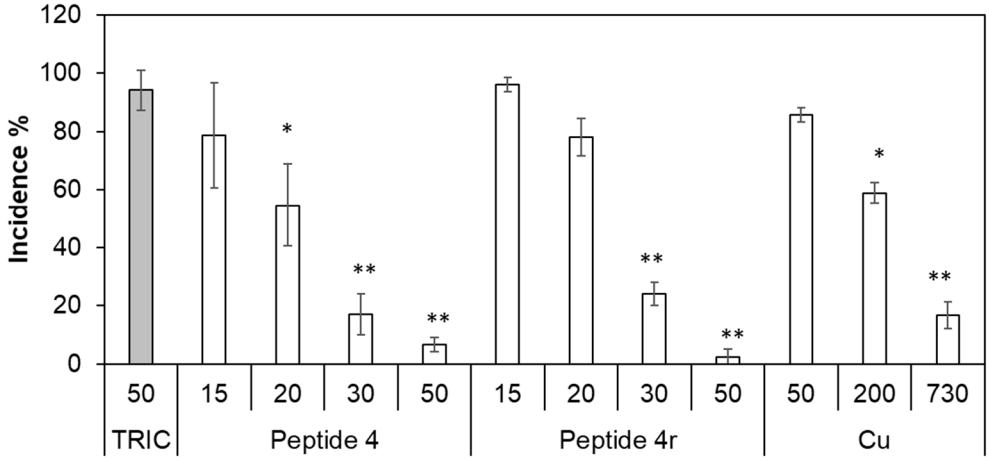

Figure 4: Incidence of sporulating grapevine leaf discs cv. Glera treated with peptides 4, 4r and copper (Cu) at different concentrations against the oomycete pathogen *P. viticola*. Peptide treatments were administered at 15, 20, 30 or 50 µM concentration. Copper treatments were administered at 50, 200 or 730 µM. Treatment with the ineffective trichogin 50 µM (TRIC) was also performed and used as a second negative control. Symptomatic discs were recorded 12 dpi and incidence was expressed as percentage of peptide treated symptomatic leaf disks respective to control symptomatic leaf discs (treated with water). Bars indicate standard errors. Three or four independent infection experiment were performed for each treatment. Data were statistically analyzed by one-way Anova Bonferroni Holm test: only pairs relative to trichogin were simultaneously compared. ** indicates significant differences at $p < 0.01$. * indicate significant differences at $p<0.05$.

Microscopic observations

Microscopic observations were carried out on zoosporangia after treatment with peptide 4. Compared to untreated sporangia, the treated ones lost their integrity and leaked cytoplasmatic material already after 15 min from the treatment (Figures 5 and 6). This effect was much evident after 30 min (Figures 5 and 6; Table 2), and 1 hour after the treatment all sporangia appeared damaged (Figure 6).

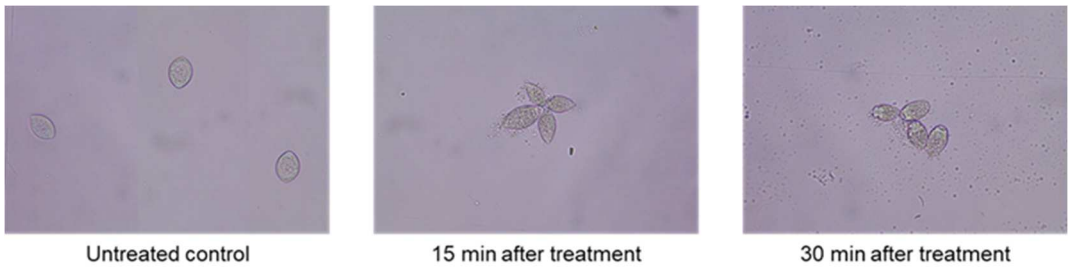

Figure 5: *P. viticola* zoosporangia after 15 and 30 minutes from the treatment with peptide 4 at 50 µM compared to the untreated control.

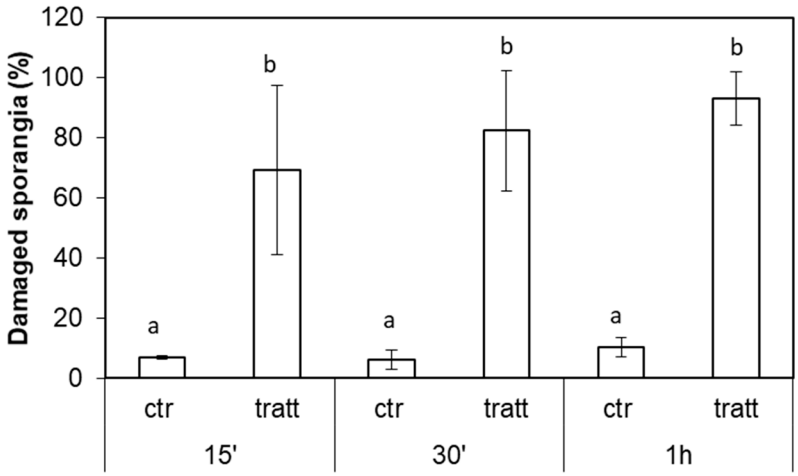

Figure 6: Percentage of damaged sporangia treated with peptide 4 at 50 µM recorded after 15, 30 and 60 minutes. Each data point is the mean of 3 independent experiments ± standard deviation (SD). Data were statistically analyzed by one-way Anova Tukey-Kramer test (minimum significant difference). Letters indicate significant differences at $p < 0.05$.

The same treatment administered on released zoospores confirmed the detrimental effect of peptide 4. The membrane of most treated zoospores appeared completely disrupted after 15 min from the treatment (Figure 7 and Table 2).

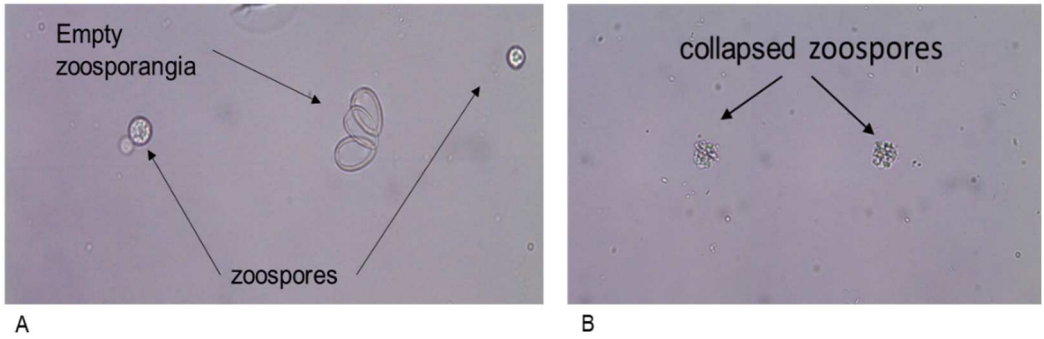

Figure 7: Empty zoosporangia and released zoospores of an untreated sample (A) and collapsed zoospores detected 15 minutes from treatment with peptide 4 at 50 µM (B).

Table 2: Percent of collapsed zoosporangia and zoospores of *P. viticola* after treatment with peptide 4 at 50 µM (30 and 15 min respectively).

	Collapsed zoosporangia (%) after 30 min	**Collapsed zoospores (%) after 15 min**
Control	6.2 ± 3.2 a	7.2 ± 1.96 a
Treated	82.4 ± 19.9 b	87.3 ± 5.15 b

Each value is the mean ± DS of counting carried out on 4 microscopic fields each containing about 20 propagules

3.4.2. Peptides activity against *B. cinerea*

In vitro experiments

The eight full-length peptides (Table 1) were assayed for their ability to inhibit the growth of *B. cinerea*. To this aim, conidia were incubated in microtiter wells supplied with the peptides at a concentration of 15 µM in the presence of the resazurin vital dye. During the following seven days, the plates were monitored every 12 h for fungal growth, as revealed by the color change of the resazurin dye. Without treatment, the fungus began to grow in the first 12 h. Similarly, fungal growth was observed after 12 h in the presence of peptide 3 and trichogin. A delayed growth was observed in wells containing 1, 2, 6, and 7. No growth occurred in wells containing conidia treated with peptides 4 or 5 (Table 3). The analogs of peptide 4 (4r, 4c, 4c1, and 4c2), with shorter and modified amino acid sequences, also exhibited activity against *B. cinerea* and were effective at 15 µM, inhibiting mycelial growth (Table 3).

Table 3: *B. cinerea* growth inhibition in microtiter wells containing potato dextrose broth (PDB) medium supplemented with the peptides at 15 µM.

Peptide	Efficacy[a]
trichogin	0 h
1	12-24 h
2	36-48 h
3	0 h
4	> 7 days
4r	> 7 days
5	> 7 days
6	12-24 h
7	72-84 h
Shorter analogs	
4c	> 7 days
4c1	> 7 days
4c2	> 7 days

[a] Cultures were monitored for seven days in the presence of the resazurin vital dye. The color change of the dye indicated the start of growth. The growth delay is reported in comparison to the untreated control. The experiment was repeated three times obtaining similar results.

A cell viability assay was performed by incubating *B. cinerea* conidia for 24 h with peptide 4, which was one of the three most active peptides. The microscopic examination showed that conidia did not germinate. Evans blue vital dye labeled the protoplasts of conidia, which appeared reduced in size and detached from the cell wall. In the untreated control, conidia germinated and produced elongated hyphae (Figure 8).

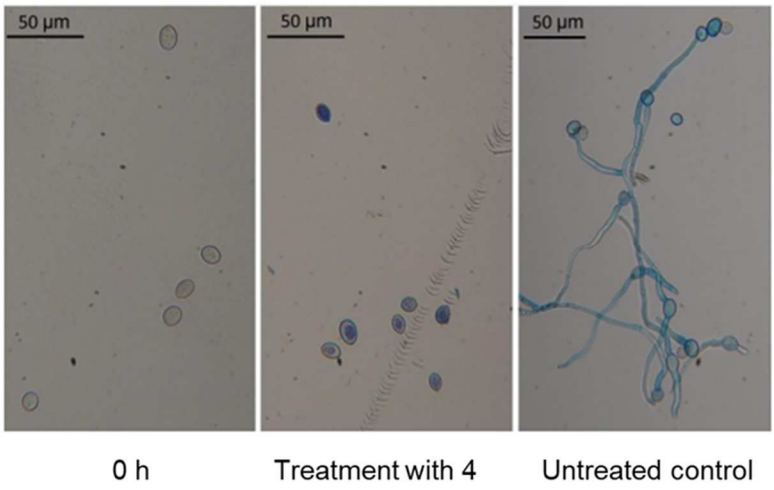

Figure 8: Evan's blue staining of *B. cinerea*. Conidia were stained at 0 h and after 24 h from the treatment with peptide 4 at 15 µM. Untreated conidia (control) were stained after 24 h.

Conidial suspensions treated with peptide 4 at 15 µM were rinsed with distilled water and plated on potato dextrose agar (PDA). No fungal growth was observed, revealing the fungicidal activity of the peptide at that concentration.

Further assays allowed to obtain the minimal inhibitory concentration (MIC) values for the most active peptides (4 and 5) against *B. cinerea*. Peptides 4 and 5 displayed MIC values between 1 and 5 µM (Figure 9).

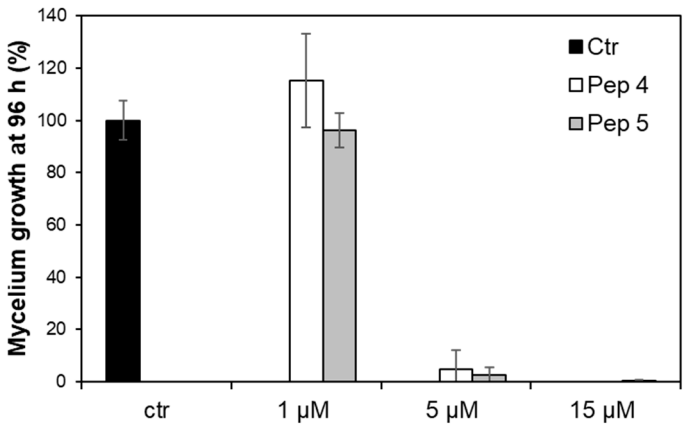

Figure 9: *In vitro* antimicrobial activity of 4 and 5 against the fungal pathogen *B. cinerea*. Peptides were supplied at 1, 5 or 15 µM concentration. After 96 h of incubation, the fungal growth was estimated by reading the absorbance at 450 nm, and it was expressed as a percentage of the maximum values measured in control samples containing the fungus not treated with the peptides. Each data point is the mean of 3 independent replicates ± standard error (SE).

In vivo experiments

Peptides 4 and 5 were identified as the most effective against *B. cinerea in vitro* and peptide **4** was used to treat grapevine leaves and berries to study efficacy against *B. cinerea* infection. At 50 µM, peptide 4 protected grapevine leaves and berries from *B. cinerea* infection. In particular, compared to the untreated control, peptide 4 reduced the infected leaf area by about 70% (Figure 10) and disease on berries by about 45% (Table 4; Figure 11). Interestingly, the peptide treatment resulted in a three fold increase in the number of asymptomatic berries (Table 4). No phytotoxic effects were noticed on either leaves or berries after treatment with the peptides (data not shown).

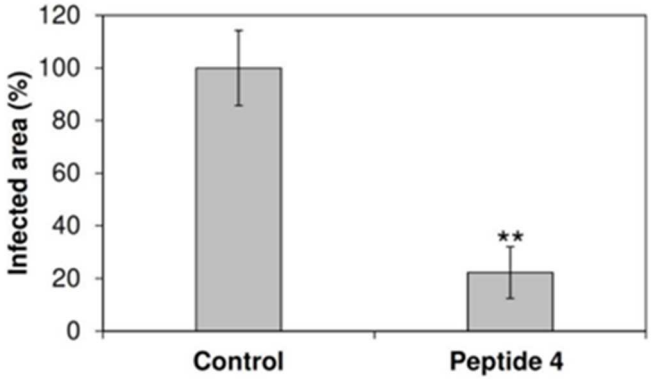

Figure 10: Symptomatic area of grapevine leaves cv. Glera treated with peptide 4 at 50 µM and inoculated with *B. cinerea* conidia. The sympthomatic leaf area was measured 7 dpi and is expressed as percentage compared to the control (treated with water). Bars indicate standard error. Three independent infection experiments were performed. Data were statistically analyzed by the Student's t-test. Each treatment was compared to the respective control. ** indicates significant differences at $p < 0.01$.

Table 4: Protection of grape berries from *B. cinerea* infection by treatment with peptide 4 at 50 µM.

	DI ± SE[a]	**Asymptomatic berries (%) ± SD[a]**
Control	1.93 ± 0.14	17.5 ± 8.7
Treated	1.03* ± 0.24	51.25* ± 18.0

[a] Symptoms are expressed as Disease Index (DI) ± standard error (SE). The mean percentage of asymptomatic grape berries ± standard deviation (SD) was also calculated. Four independent infection experiments were performed for each treatment. Data were statistically analyzed by the t-test. * indicate significant differences at $p > 0.05$.

Figure 11: Representative symptoms produced by *Botrytis cinerea* on grape berries (cv. Glera) untreated or treated with the peptide 4 at 50 µM at 4 dpi. Ripe grape berries were surface sterilized, rinsed with sterile water, separately arranged on a plastic tray, treated with peptide 4 (or water for control) and sprayed with *B. cinerea* conidia at a concentration of 1.85×10^6 ml^{-1}.

3.5. Discussion

Trichogin is an 11-residue lipophilic peptaibol with antimicrobial activity naturally produced by *T. longibrachiatum*. The presence of 3 Aib confers an helix structure able to interact with microbial membranes and a remarkable resistance toward proteolytic degradation (Yamaguchi *et al.*, 2003). The environmental stability of peptides is a suitable characteristic for the use of these molecules as fungicides in open field (Pertot *et al.*, 2017). However, trichogin has poor solubility in water and this feature is likely responsible for the inactivity recorded in this work against *P. viticola* and *B. cinerea*. The Lys->Gly substitution(s) carried out in the trichogin chain provides cationic properties, and, consequently, solubility in water, and a variable fungicidal activity against the two pathogens.

Although the experimental systems carried out to screen the activity of the peptides were different – i.e. the activity against *B. cinerea* was initially assayed *in vitro* while the activity against *P. viticola* was inevitably assayed on grapevine leaves – some of the 11-residues long peptides behaved similarly against both pathogens. In particular, peptide 4 was active, while peptides 2, 3 and 7 were inactive or poorly active. Other peptides, on the other hand, were differently active depending on the pathogen. This is the case of peptides 1 and 6, which are active against *P. viticola* and inactive against *B. cinerea*, while peptide 5, vice versa, was inactive against *P. viticola* and active against *B. cinerea* (Table 5). The mechanisms responsible for these differences are not easily identifiable. The variability in the effectiveness of these peptides could be related to the number, position and combinations of Lys-to-Gly substitutions on the trichogin chain. Summarizing, among the single Lys substitutions, the Lys^6 substitution (peptide 5) ensures fungicidal activity only against *B. cinerea*, while the Lys^5 substitution is active against *P. viticola* only providing an additional Aib in position 7 (peptide 6) (Table 5). Among the double Lys substitutions, only the Lys^5 + Lys^6 modification (peptide 4) provides significant activity against both pathogens, while the Lys^2 + Lys^5 combination (peptide 1) is active only against *P. viticola* (Table 5).

The 3D structure adopted by peptides (Figure 1) allows drawing a tentative 3D-structure-activity relationship. The two Lys of the peptide 4, which is active against both pathogens, are close. However, the two Lys point toward different directions, thus the electrostatic repulsion by the positively charged Lys can be avoided, resulting in satisfactory efficacy in pathogen control. In contrast, in peptide 7 the Lys^2 and Lys^6 are one helix-turn away, but they are spatially close and this Lys proximity could cause electrostatic repulsions that might influence peptide performance against the pathogens. A similar effect could be hypothesized for peptide 1, where Lys^5 is also close to Lys^2. However, in this case peptide 1 is oddly effective against *P.*

viticola. However, it is worth noting that these antimicrobial peptides target the plasma membrane, and the membrane of oomycetes is different from that of fungi. Indeed, sterols are absent in the membrane of oomycetes, while ergosterol, the main sterol found in fungal membranes, affects membrane fluidity and resistance to stress (Block, 1983; Wise *et al.*, 2014)
The position of Lys in the peptaibol chain can also influence the self-aggregation capacity of the peptides and the capacity to open pores in the membrane. It has been demonstrated that peptides with longer chains assemble and form channels in phospholipidic membranes (Milov *et al.*, 2016). Trichogin GA IV was demonstrated to self-assemble in trans-membrane dimers, which may induce membrane disruption, and, as a monomer, it aggregates the fatty acids disturbing the lipid homeostasis of bacterial membranes (Afanasyeva *et al.*, 2019).
In conclusion, the contemporary presence of two Lys in the sequence can increase but also decrease the peptide performances according to their relative position. The proximity of two Lys seems crucial in maintaining the activity against both pathogens. This feature is present also in the shorter analogs and in the 4r peptide. However, this is not true with the 4c1 peptide that, besides maintaining the proximity of the two Lys, is profoundly modified at the N-terminal region.

Table 5: Comparison of the performances of the Lys-containing peptide analogs against *P. viticola* and *B. cinerea*.

Peptide	Lys Position(s)	Antifungal Activity[a] *P. viticola*[a]	Antifungal activity[a] *B. cinerea*[a]
1	2 and 5	++++	+
2	2	+	++
3	5	+	-
4	5 and 6	++++	++++
5	6	+	++++
6	5 (and Aib^6)	++++	+
7	2 and 6	+	++

[a] +, level of antifungal activity; -, no activity.

From microscopic observations, it appears that spores of *P. viticola* and *B. cinerea* have different cytological responses after treatment with the peptide 4. Plasma membranes of *P. viticola* sporangia and zoospores are damaged by the treatment and cytoplasmic material is released, while in *B. cinerea* a shrinkage of protoplast occurs. Whether and how the membrane different composition of *P. viticola* and *B. cinerea* may be involved in providing a different sensitivity to the peptide analogs is unknown. Taken together, the findings obtained indicate peptide 4 as a candidate molecule for exploitation as a new biofungicide. The killing effect of peptide 4 on *P. viticola* zoospores occurs only after 30 minutes from the administration of 50

μM peptide (corresponding to 65 mg l^{-1}). To obtain a similar effect against *P. viticola* sporangia, the two common fungicides Cymoxanil and Mancozeb were used at 11 and 100 mg l^{-1}, respectively (Andrieu *et al.*, 2001). In comparison with copper, peptide 4 at 30 μM provided the same fungicidal effect of copper at 730 μM (a tenth of field dosage). Thus, the peptide 4 has a higher or similar fungicidal activity as compared to the active ingredients of commercial fungicides used to control downy mildew.

The activity demonstrated by peptide 4 against *B. cinerea in vitro* was confirmed in experiments *in planta*; at 50 μM (65 mg l^{-1}) the peptide 4 protected grapevine leaves and berries from gray mold. For a comparison, fenhexamid, a common botriticidal fungicide, is applied at 1.65 mM (0.5 g l^{-1}) (EFSA, 2014).

Besides the promising data obtained in the laboratory, open field trials are necessary to verify the effectiveness of peptide 4 in field conditions and to evaluate other important properties such as stability and persistence.

Peptide 4 analogs (4r, 4c, and 4c2) may be other interesting molecules to experiment with in field conditions. In particular, these peptides offer an interesting perspective for reducing the synthesis costs.

3.6. References

- Afanasyeva E F, Syryamina VN, De Zotti M, Formaggio F, Toniolo C, Dzuba S A. Peptide antibiotic trichogin in model membranes: Self-association and capture of fatty acids. Biochimica and Biophysica Acta-Biomembranes 1861: 524–531 (2019).
- Andrieu N, Jaworska G, Genet J-L, Bompeix G. Biological mode of action of Famoxadone on *Plasmopara viticola* and *Phytophtora infestans*. Crop Protection 20: 253-260 (2001).
- Auvin-Guette C, Rebuffat S, Prigent Y, Bodo B. Trichogin A IV, an 11-residue lipopeptaibol from *Trichoderma longibrachiatum*. Journal of the American Chemical Society 114: 2170–2174 (1992).
- Bloch K. Sterol structure and membrane function. Crititical Review in Biochemistry 14: 47-92. Block (1983).
- Cabanne C C, Donéche B. Polygalacturonase isozymes produced during infection of the grape berry by *Botrytis cinerea*. Vitis 41: 129–132 (2002).
- De la Fuente-Núñez C, Whitmore L, Wallace B A. Peptaibols. Chapter 22 in: Handbook of Biologically Active Peptides, Section II: Bacterial/Antibiotic Peptides, 150-156. Edited by Abba J. Kastin (2013).
- De Zotti M, Biondi B, Formaggio F, Toniolo C, Stella L, Park Y, Hahm K-S. Trichogin GA IV: An antibacterial and protease-resistant peptide. Journal of Peptide Science 15: 615–619 (2009).
- De Zotti M, Sella L, Bolzonello A, Gabbatore L, Peggion C, Bortolotto A, Elmaghraby I, Tundo S, Favaron F. Targeted amino acid substitutions in a *Trichoderma* peptaibol confer activity against fungal plant pathogens and protect host tissue from *Botrytis cinerea* infection. International Journal of Molecular Science 21:7521: 1-18 (2020).
- Degenkolb T, Fog Nielsen K, Dieckmann R, Branco-Rocha F, Chaverri P, Samuels G, Thrane U, von Döhren H, Vilcinskas A, Brückner H. Peptaibol, secondary-metabolites, and hydrophobin pattern of commercial biocontrol agents formulated with species of the *Trichoderma harzianum* complex. Chemistry & Biodiversity 12: 662-684 (2015).
- European Food Safety Authority. Conclusion on the peer review of the pesticide risk assessment of the active substance fenhexamid. EFSA Journal 12: 3744 (2014).
- Favaron F, Lucchetta M, Odorizzi S, Pais da Cunha A T, Sella L. The role of grape polyphenols on trans-resveratrol activity against *Botrytis cinerea* and of fungal laccase on the solubility of putative grape PR proteins. Journal of Plant Pathology 91: 579–588 (2009).
- Fraceto L F, Maruyama C R, Guilger M, Mishra S, Keswani C, Singh H B, de Lima R. Trichoderma harzianum-based novel formulations: Potential applications for management of Next-Gen agricultural challenges. Journal of Chemical Technology & Biotechnology 93:2056-2063 (2018).
- Gessler C, Pertot I, Perazzolli M. *Plasmopara viticola*: a review of knowledge on downly mildew of grapevine and effective disease management. Phytopathologia Mediterranea 50: 3-44 (2011).
- Granade T C, Hehmann M F, Artis W M. Monitoring of filamentous fungal growth by in situ microspectrophotometry, fragmented mycelium absorbance density, and 14C incorporation: Alternatives to mycelial dry weight. Applied and Environmental Microbiology Journal 49: 101–108 (1985).
- Islam M T, Laatsch H, von Tiedemann A. Inhibitory effects of Macrotetrolides from *Streptomyces* spp. on zoosporogenesis and motility of peronosporomycete zoospores are likely

linked with enhanced ATPase activity in mitochondria. Frontiers in Microbiology 7: 1824: 1-15 (2016).

- Kanellis A K, Roubelakis-Angelakis K A. Grape, in: G. Seymour, S.J. Taylor, G. Tucker (Eds.), Biochemistry of Fruit Ripening, Chapman & Hall Ltd., London, pp. 189–234 (1993).
- Kortekamp A. Expression analysis of defence-related genes in grapevine leaves after inoculation with a host and a non-host pathogen. Plant Physiology and Biochemistry 44: 58-67 (2006).
- Marik T, Tyagi C, Balázas D, Urbán P, Szepesi Á, Bakacsy L, Endr G, Rakk D, Szekeres A, Anderson M A, , Salonen H, Druzhinina I S, Vágvölgyi C, Kredics L. Structural diversity and bioactivities of peptaibols compounds from the *Longibrachiatum* clade of the filamentous fungal genus *Trichoderma*. Frontiers in Microbiology 10: 1437 (2019).
- Marra R, Lombardi N, d'Enrico G, Troisi J, Scala G, Vinale F, Woo S L, Bonanomi G, Lorito M. Application of *Trichoderma* strains and metabolites enhances soybean productivity and nutrient content. Journal of Agricultural and Food Chemistry 67:1814-1822 (2019).
- Martínez G, Regente M, Jacobi S, Del Rio M, Pinedo M, de la Canal L. Chlorogenic acid is a fungicide active against phytopathogenic fungi. Pesticide Biochemistry and Physiology 140: 30–35 (2017).
- Merz P R, Moser T, Höll J, Kortekamp A, Buchholz G, Zyprian E, Bogs J. The transcription factor VvWRKY33is involved in the regulation of grapevine (*Vitis vinifera*) defense against the oomycete pathogen Plasmopara viticola. Physiologia Plantarum 153: 365-380 (2015).
- Milov A D, Tsvetkov Y D, Raap J, De Zotti M, Formaggio F, Toniolo C. Conformation, self-aggregation, and membrane interaction of peptaibols as studied by pulsed electron double resonance spectroscopy. Journal of Ppetide Science 106: 6–24. (2016).
- Muthmann R. In Pierre N (ed) The use of Plant Protection Products in the European Union. Eurostat, Luxembourg (2007).
- Pertot I, Caffi T, Rossi V, Mugnai L, Hoffmann C, Grando M S, Gary C, Lafond D, Duso C, Thiery D, Mazzoni V, Anfora G. A critical review of plant protection tools for reducing pesticide use on grapevine and new perspectives for the implemention of IPM in viticulture. Crop protection 97:70-84 (2017).
- Sella L, Gazzetti K, Castiglioni C, Schäfer W, Favaron F. *Fusarium graminearum* possesses virulence factors common to Fusarium head blight of wheat and seedling rot of soybean but differing in their impact on disease severity. Phytopathology 104: 1201–1207 (2014).
- Semighini C P, Hornby J M, Dumitru R, Nickerson K W, Harris S D. Farnesol-induced apoptosis in *Aspergillus nidulans* reveals a possible mechanism for antagonistic interactions between fungi. Molecular Microbiology 59: 753–764 (2006).
- Speckbacher V and Zeilinger S. Secondary Metabolites—Sources and Applications; Vijayakumar, R., Raja, S.S.S., Eds.; IntechOpen: London, UK, pp. 37–55 (2018).
- Stoppacher N, Neumann N K N, Burgstaller L, Zeilinger S, Degenkolb T, Brückner H, Schuhmacher R. The comprehensive peptaibiotics database. Chemistry & Biodiversity 10: 734-743 (2013).
- Tamandegani P R, Marik T, Zafari D, Balázs D, Vágvölgyi C, Szekeres A, Kredics L. Changes in peptaibol production of *Trichoderma* species during in vitro antagonistic interactions with fungal plant pathogens. Biomolecules 10: 730 (2020).
- Terral J-F, Tabard E, Bouby Laurent, Ivorra S, Pastrol T, Figueiral I, Picq S, Chevance J-B, Jung C, Fabre L, Tardy C, Compan M, Bacilieri R, Lacombe T, This P. Evolution and history of grapevine (*Vitis vinifera*) under domestication: new morphometric perspectives to

understand seed domestication syndrome and reveal origins of ancient European cultivars. Annals of Botany 105: 443-455 (2010).

- Toniolo C, Crisma M, Formaggio F, Peggion C. Control of peptide conformation by the Thorpe-Ingold effect (Cα-tetrasubstitution). Peptide science 60: 396-419 (2001).
- Toniolo C, Peggion C, Crisma M, Formaggio F, Shui X, Eggleston D S. Structure determination of racemic trichogin A IV using centrosymmetric crystals. Nature Structural & Molecular Biology 1: 908–914 (1994).
- Verma M, Brar S K, Tyagi R D, Surampalli R Y, Valéro J R. Antagonistic fungi, *Trichoderma* spp.: panoply of biological control. Biochemical Engineering Journal 37: 1-20 (2007).
- Vinale F, Ghisalberti E L, Sivasithamparam K, Marra R, Ritieni A, Ferracane R, Woo S, Lorito M. Factors affecting the production of Trichoderma harzianum secondary metabolites during the interaction with different plant pathogens. Letters in Applied Microbiology 48: 705–711 (2009).
- Wise C, Falardeau J, Hagberg I, Avis T J. Cellular lipid composition affects sensitivity of plant pthogens to fengycin, an antifungal compound produced by Bacillus subtilis strain CU12. Biochemistry and Cell Biology 104: 10: 1036-1041 (2014).
- Yamaguchi H, Kodama H, Osada S, Kato F, Jelokhani -Niaraki M, Kondo M. Effect of α,α-dialkyl amino acids on the protease resistance of peptides. Bioscience, Biotechnology, and Biochemistry 67:2269-2272 (2003).
- Zeilinger S, Gruber S, Bansal R, Mukherjee P K. Secondary metabolism in Trichoderma-Chemistry meets genomics. Fungal Biology Reviews 30: 74-90 (2016).
- Zhao P, Ren A, Dong P, Sheng Y, Li D. Antimicrobial peptaibols, trichokonins, inhibit mycelial growth and sporulation and induce cell apoptosis in the pathogenic fungus *Botrytis cinerea*. Applied Biochemistry and Microbiology 54: 396–403 (2018).

MAIN CONCLUSIONS

This PhD thesis aimed to give a contribution to the global effort of finding new effective alternatives for protection of grapevine, whose production is strongly affected by two important pathogens, *B. cinerea* and *P. viticola*.

In a first contribution, the investigation of *B. cinerea* protease and grapevine class IV chitinase interaction provided evidences of another strategy adopted by this pathogen to facilitate its infection process by escaping plant defense mechanisms. The grapevine class IV chitinase is a PR- protein abundantly present in grape berries that can inhibit conidial germination and hyphal development. However, the early activity of fungal proteases cleaves the CBD of the chitinase halving its enzymatic activity and allowing a normal fungal development. Metalloprotease activity is involved in this two-steps cleavage process, which needs to be further investigated to identify possible new targets to be exploited for *B. cinerea* control.

In the second study, the effectiveness of several water-soluble analogs of a natural occurring peptaibol naturally produced by *Trichoderma longibrachiatum* aimed to explore the possibility to use modified natural metabolites of beneficial fungi to counteract *B. cinerea* and *P. viticola* infection. Several peptides have been identified as effective against one of the two pathogens, with one of them (peptide 4) being active against both. A promising result in perspective was obtained from the comparison of the effectiveness of peptide 4 with commercial active substances such as Fenhexamid, used against *B. cinerea*, and copper, Cymoxanil and Mancozeb, used against *P. viticola*. However, if the efficacy in open field conditions will be confirmed, the challenge will concern the synthesis cost. To this purpose, the shorter analogs of peptide 4 offer an interesting opportunity.

RINGRAZIAMENTI

Vorrei ringraziare il prof. Francesco Favaron per gli insegnamenti e gli stimoli indispensabili per l'attività di ricerca, il prof. Luca Sella per l'aiuto scientifico, il dott. Silvio Tundo per il supporto tecnico e scientifico. Vorrei ringraziare tutte le persone con cui ho lavorato in questi anni presso il Laboratorio di Patologia Vegetale del dipartimento TESAF. In particolare Carla Castiglioni e Silvana Odorizzi, per l'assistenza tecnica e morale, Alessandra Quarantin, Rocco Caracciolo, Rakshita Govind, Isabella Busato, Arianna Panichi, Ilhem Meriane and Riccardo Scrinzi per la condivisione di questa esperienza, la collaborazione ed il supporto. Un ringraziamento anche ad Ilirjan Allgjata che ha contribuito all'ottenimento di alcuni risultati del secondo capitolo.

Un ringraziamento va agli altri gruppi con cui ho avuto modo di interagire. In particolare alla prof.ssa Marta De Zotti per aver ideato e sintetizzato i peptidi testati nel terzo capitolo, alla prof.ssa Nathalie Pousserau per aver fornito i ceppi di *B. cinerea*, al prof. Simone Vincenzi per aver fornito del materiale necessario per alcuni saggi del secondo capitolo, al dott. Matteo Marangon per aver messo a disposizione esperienza e attrezzatura per le analisi all'HPLC e Edward Jhon Brearley-Smith per il supporto tecnico in tali analisi, al prof. Antonio Masi e Ilaria Battisti per l'identificazione delle proteine tramite MaldiTOF.

In fine, un ringraziamento a chi al di fuori del lavoro mi ha incoraggiato in questi anni, ognuno a modo suo, ognuno per la sua parte.

Angela Bolzonello