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Overcome of grape chemical barriers

by the fungal pathogen *Botrytis cinerea*

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DATA CONSEGNA TESI

31 Gennaio 2011

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Dedication

I dedicate my thesis to all the Angolan people who have died in the shadow of the illiteracy, not for they lack of willingness but for being denied access to schooling.

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LIST OF ABBREVIATIONS

4CL	4-coumarate: CoA-ligase
5CSA	5-Chlorosalicylic acid
%	Per cent
°C	Degree Celsius
µg	Microgram
ABC	ATP Binding Cassette
AcOH	Acetic acid
AgNO3	Silver nitrate
AOS	Active oxigen species
avr	Avirulent
BA	Benzoic acid
BcAP	Botrytis cinerea aspartic protease
Bclcc	Botrytis cinerea laccase
Bcpg	Botrytis cinerea polygalacturonase
BSA	Bovine Serum Albumine
BVX	Botrytis virus X
Ca^{2+}	Calcium
CA	Controlled atmosphere
CAD	Cinnamyl-alcohol dehydrogenase
CHS	Chalcone synthase
CH_2Cl_2	Dichloromethane
Cl	Chloride
cm	Centimetre
CO2	Carbon dioxide
$CuSO_4$	Cupper Sulphate
cv.	Cultivar
CWDEs	Cell wall degrading enzymes
Cz	Czapek
DAPG	Diacetylphloroglucinol
DNA	Deoxyribonucleic acid
e.g.	For example

et al.	et alii (and others)
ET	Ethylene
EtOH	Ethanol
$FeSO_4$	Ion sulphate
g	Gram
GMD	Glass-microfibre disc
GST	Gluthatione-S-transferase
h	Hour
H^+	Hydrogen
HPLC	High Performance liquid chromatography
HRGP	Hydroxiproline-rich glicoprotein
in vitro	Outside a living organism
in vivo	Inside a living organism
JA	Jasmonic acid
K^+	Potassium ion
K_2HPO_4	Dipotassium hydrogen phosphate
KCl	Potassium Chloride
kDa	Kilo Dalton
Kg	Kilogram
l	Liter
LTPs	Lipid transfer protein
M	Molarity
m	Metre
$MgSO_4$	Magnesium sulphate
min	Minute
ml	Millilitre
mm	Millimetre
nm	Nanometer
mM	Millimolar
MeJA	Methyl Jasmonate
MeOH	Methanol
MgCl	Magnesium chloride
mg	Milligram

MFS	Major Facilitator
MW	Molecular weight
NaCl	Sodium chloride
$NaNO_3$	Sodium nitrate
n.d.	Not defined
OA	Oxalic acid
O.D.	Optical density
OG	Oligogalacturonides
PDA	Potato Dextrose Agar
PCD	Programmed Cell Death
PCR	Polymerase chain reaction
PAGE	Polyacrylamide Gel Electrophoresis
RH	Relative humidity
RIPs	Ribose activating proteins
RNA	Ribonucleic acid
RT	Reverse transcription
SA	Salicylic acid
SOD	Super Oxide Dismutase
SDS	Sodium Dodecyl Sulfate
TLP	Thaumatin like protein
TPP II	tripeptidyl peptidase II
USA	United States of America
UV	Ultraviolet
v/v	Volume by volume
VvTL	*Vitis vinifera* Thaumatine like protein
w/w	Weight by weight
w/v	Weight by volume
$ZnSO_4$	Zinc sulphate

Abstract

Botrytis cinerea in contact with mature grape berries encounters an environment particularly rich in polyphenols and PR proteins, where the stilbenic phytoalexin *trans*-resveratrol may accumulate. To mimic conditions similar to those found in grape berries, *B. cinerea* was grown *in vitro* with grape PR proteins and polyphenols extracted from mature grapes and with *trans*-resveratrol. Results showed that in the presence of highly toxic amounts of *trans*-resveratrol, grape polyphenols allowed total recovery of fungal growth, and proteins allowed partial recovery. These resveratrol-polyphenol or resveratrol-protein combinations also induced a strong release into the medium of laccase activity, which is likely to be involved in *trans*-resveratrol detoxification. The grape protein pattern changed during fungal growth; most grape proteins quickly disappeared from the culture when polyphenols and *trans*-resveratrol were present together. Similar protein patterns were obtained *in vitro* by incubating grape proteins with grape polyphenols and/or *trans*-resveratrol with a purified *B. cinerea* laccase. Under these conditions, most proteins became insoluble. The grape protein pattern obtained from grape berries infected by *B. cinerea* strongly resembled that obtained *in vitro* by incubating grape proteins and polyphenols with fungal laccase. It seems that *B. cinerea*, through laccase secretion and activity and by exploiting the berry polyphenols, easily neutralizes the toxicity of grape stilbenic phytoalexins and makes the grape pathogenesis-related proteins insoluble. The effect of laccase, resveratrol and polyphenols on fungal spore germination was also studied. Results showed that resveratrol alone initially does not inhibit the spore germination. But the inhibition was completely relieved by the presence of grape polyphenols. Instead, the pre-incubation of resveratrol with laccase completely inhibited the spore germination. In addition, we investigate the involvement of *B. cinerea* proteases in the degradation of grape PR proteins. An aspartyl and a tripeptidyl protease were purified from *B. cinerea* in vitro culture. The purified proteases activities partially degraded PR proteins. The expression analysis of tripeptidyl and aspartic protease gene families revealed that several members of these families are expressed in the presence of grape PR proteins.

In conclusion, our results support that in a grape berry environment characterized by an abundance of polyphenols, *B. cinerea* laccase not only detoxify the trans-resveratrol but

also modifies the solubility of grape proteins and this environment may facilitate the fungal protease to degrade grape PR proteins.

Riassunto

Durante l'infezione dell'uva il fungo fitopatogeno *Botrytis cinerea* incontra tessuti particolarmente ricchi di polifenoli e proteine PR e dove si accumula la fitoalessina *trans*-resveratrolo. Per simulare condizioni simili a quelli trovati negli acini d'uva, *B. cinerea* è stato coltivato *in vitro* con proteine e polifenoli estratti da uve mature, e con trans-resveratrolo. I risultati hanno dimostrato che in presenza di livelli tossici di *trans*-resveratrolo, i polifenoli dell'uva favoriscono una normale crescita del patogeno mentre le proteine consentono un parziale recupero della crescita.

Le combinazioni polifenoli-resveratrolo o resveratrolo-proteina inducevano il rilascio di una forte attività laccasica nel mezzo, che sembra essere coinvolta nella disintossicazione del trans-resveratrolo. I risultati hanno di mostrato anche che il pattern delle proteine dell'uva era alterato durante la crescita del fungo. Infatti, le proteine dell'uva scomparivano rapidamente dalla coltura nella quale polifenoli e trans-resveratrolo erano presenti simultaneamente. Profili proteici simili sono stati ottenuti *in vitro*, incubando proteine con polifenoli dell'uva e /o trans-resveratrolo con le laccasi purificata di *B. cinerea*. In queste condizioni, la maggior parte delle proteine diventava insolubile. Questo pattern era molto simile a quello osservato negli acini infettati da *B. cinerea*. Pertanto, *B. cinerea*, attraverso la secrezione di attività laccasica e sfruttando i polifenoli, neutralizza facilmente la tossicità delle fitoalessine stilbeniche e rende le proteine PR insolubili. L'effetto di laccasi è stato studiato anche sulla germinazione delle spore di *B. cinerea*. Il resveratrolo da solo inizialmente non inibiva la germinazione delle spore, invece, la sua pre-incubazione con le laccasi, inducendo la formazione di trans-ε-viniferina, ne inibiva la germinazione. Invece se nel mezzo erano presenti anche i polifenoli non si osservava alcuna inibizione della germinazione. Successivamente è stato indagato il coinvolgimento delle proteasi di *B. cinerea* nella degradazione delle proteine d'uva. Del mezzo coltura è stata purificata una aspartyl e una tripeptidyl proteasi. Queste proteasi sono state in grado di degradare parzialmente le proteine d'uva. Un'analisi di espressione dei geni delle famiglie di tripeptidyl e aspartyl proteasi ha dimostrato anche altri membri di queste famiglie erano espresse in presenza di proteine PR dell'uva. I risultati permettono di concludere che nell'acino d'uva, caratterizzato d'una grande varietà di polifenoli, la laccasi non solo anulla

la tossicità del *trans*-resveratrolo, ma modifica anche la solubilità delle proteine dell'uva. Questo effetto potrebbe facilitare l'azione proteasica del fungo verso le proteine PR d'uva.

Objectives

During infection of the grape berry by *B. cinerea*, it is likely that the fungus encounters an environment particularly rich in polyphenols and PR-proteins, where the stilbene phytoalexin *trans*-resveratrol (here after referred as resveratrol) may accumulate. The effects on fungal growth of the interaction among these three factors remain to be investigated. Besides, little is known about the role involving *B. cinerea* proteases during infection of grape berries.

These topics have been addressed by:

1. studying the effect of grape proteins and polyphenols on resveratrol activity against *B. cinerea* and on grape PR proteins. To this aim the following experiments have been performed:

    1.1. *B. cinerea* was grown with grape proteins, polyphenols extracted from grape berries and resveratrol. Laccase activity and alteration in the grape protein pattern in the fungal culture was investigated;

    1.2. *B. cinerea* fungal spore germination was performed in presence of resveratrol and grape polyphenols with or without addition of fungal laccase;

    1.3. grape proteins were mixed together with polyphenols, resveratrol and fungal laccase. Alterations of PR protein pattern was determined.

2. Establishing the possible involvement of *B. cinerea* protease in the degradation of grape PR proteins. To this aim the following experiments were done:

2.1. protease was purified from *B. cinerea* grown with casein as a sole nitrogen source. The purified proteases were identified by Mass spectrometry. The activity of the proteases on grape PR proteins was performed in an *in vitro* assay;

2.2. the activity of these proteases was assayed on grape PR protein in presence of polyphenols or *B. cinerea* laccase;

2.3. The expression of the family members which include the purified proteases was established during the presence of grape PR proteins.

General introduction

Botrytis cinerea is one of the most important fungal pathogen of grapevine world-wide. In grapevine the fungus cause serious grey mold disease drastically reducing yield and wine quality (Ribéreau-Gayon *et al*., 1980; Ribérau-Gayon *et al*., 1998). Sometimes, *B. cinerea* infection can be also desirable when gives "noble rot" on late harvest grape varieties for the production of sweet wines in some areas of France and Germany are produced (Ribérau-Gayon *et al*., 1998).

During wet and warm conditions *B. cinerea* infect early bloom inflorescences, shoots, leaves and fruits, but infection of ripening berries is quite unlikely (Rosslenbroich and Stuebler, 2000). Besides the grapevine, this fungus infects more than 200 economically important plant species, including *monocot* and *Dicot* plants (Williamson *et al.,* 2007) during pre- and post harvest (Agrios, 2005). This fungus kills host cells through the production of cell wall degrading enzymes, phytotoxic metabolites and reactive oxygen species accumulating after the induction of a plant-produced oxidative burst. Thanks to an arsenal of degrading enzymes, *B. cinerea* is able for decomposition and consumption of different plant tissues (Van Kan, 2006).

Owing to its diversity of weapons and hosts and its survival strategy as *mycelia* and/or conidia or for extended periods as sclerotia in crop debris (Williamson *et al*., 2007), the control of the gray mould disease could be difficult. Fungicides treatments are effective for gray mould disease control in short run time but do not guarantee long term control. *B. cinerea* is a resilient pathogen that cope with multiple fungitoxic compounds from different origin. An example, is the tolerance to the fungicide fenpiclonil and the grapevine phytoalexin (Schoonbeek *et al*., 2002). The risk of resistant strains development to fungicides (Locke and Fletcher, 1988; Rosslenbroich and Stuebler, 2000; Yourman and Jeffers, 1999), such as dicarboximide and benzimidazoles is high (La mondia and Douglas, 1997; Sadfi-Zouaoui *et al*, 2008).

Resistance against broad spectrum antibiotics such as 2,4-DAPG (2,4-Diacetylphloroglucinol) has been demonstrated by Schouten *et al*., (2008). *B. cinerea* employs both non-degradative and degradative mechanisms to resist 2,4 - DAPG. The non-degradative mechanism involves efflux by the ABC transporter BcAtrB, whereas

degradation of 2,4-DAPG is mediated indirectly by the laccase 2 (BcLCC2). Laccases is a copper containing polyphenol oxidases well known for its ability to oxidize phenolic compounds (Baldrian, 2006), including resveratrol (stilbene phytoalexin) and tannins released by the plant hosts (Schouten *et al*., 2002).

Biological control using natural antagonistic microorganisms has been extensively studied, and some fungi and bacteria have been demonstrated to be effective against grey mold disease (Nicot *et al.,* 1993; Sutton and Peng 1993; Dick and Elad, 1999; Utkhede and Mathur 2002; Lee *et al*., 2006).

Plants have a different immune system comparing to animals. Plants have evolved a variety of potent defence mechanisms, including the synthesis of low-molecular-weight compounds and proteins and peptides that have antifungal activity (Caruso *et al*., 1996; Kitajima and Sato, 1999; Selitrennikoff, 2001). According to Selitrennikoff, (2001), chitinases, (1-3)β-glucanase, chitin-binding proteins, thaumatin-like (TL) proteins, defensins, cyclophilin-like protein, glycine/histidine-rich proteins, ribosome-inactivating proteins (RIPs), lipid-transfer protein (LTPs), killer proteins (killer toxins), and protease inhibitors are the most important PR-proteins. In grape, research has been focused on plant defensive mechanism such as stilbene phytoalexins and pathogenesis related proteins (PR-proteins).

For an efficient control of this fungus, it is necessary to understand the complexity of biochemical and molecular mechanisms involved in *B. cinerea* pathogenicity.

My research was aimed to investigate some aspects of the interaction between *B. cinerea* and grape PR-proteins (Chitinases and Thaumatin like protein), grape polyphenols and resveratrol. The first objective was to evaluate the effect of above mentioned compounds on spore germination and mycelium growth. The second one was the measurement of mycelium biomass and laccase activity during interaction with these compounds. The third one, was the study of how proteins, polyphenols and resveratrol, alone or combined together can affect the protein solubility. The third objective concerned the evaluation of grape PR protein degradation by *B. cinerea* proteases. Finally, the expression analysis of protease gene expressed during fungal growth in presence of grape PR proteins.

1. Literature review

1.1. Botrytis cinerea as pathogen of grapevine

1.1.1. Botrytis grey mould disease or bunch rot

Botrytis cinerea is the etiological agent of grey mould disease or bunch rot. This pathogen inflicts serious damages in the grape production by rotting of berries either in the field or in post-harvest. This fungus is responsible also for important damages in the winery. The quality of the wine is affected due to the conversion of sugar into glycerol and gluconic acid and by the oxidation of phenolic compounds inclucing the stilbene phytoalexins (Bavaresco, *et al*., 1997). *B. cinerea* also changes the pattern of the protein contained in the must and decreases the foamability of sparkling wines, such as champagne (Marchal *et al*., 2006).

Gray mould is the state in which infected organs become covered by a gray film of prolific conidiophores and conidias of the fungal pathogen. This pathogen produces abundant gray mycelium and long branched conidiophores that have rounded apical cells bearing clusters of colorless or gray, one-celled, egg-like conidia formed on branched conidiophores over the surface. This arrangement of the spores gives the genus its name, from the Greek *botrys*, meaning a cluster of grapes (Horst, 2008). Conidia are released readily in humid weather and are carried by air currents. The fungus frequently produces irregular sclerotia (small, flat, dark, hard resting structures).

1.1.2. Taxonomy and classification

Table 1. Taxonomic classification of *Botrytis cinerea* and *Botryotinia fuckeliana* (*ten Have, 2000*).

	Asexual	Sexual
Kingdom	Fungi	Fungi
Division	Deuteromycota	Ascomycota
Class	*Hyphomycete*	Discomycete
Order	*Moniliales*	Helotiales
Family	Moniliaceae	Sclerotiniaceae
Gen	Botrytis	Botryotinia
Species	*Botrytis cinerea*	*Botryotinia fuckeliana*

According to Williamson *et al*., (2007) *B. cinerea* is classified in the following taxonomy: Kingdom: Fungi, *Phylum*: Ascomycota, *Subphylum*: Pezizomycotina; Class: Leotiomycetes; Order: Helotiales; family; *Sclerotiniaceae*; Genus *Botryotinia*; Species *Botryotinia fuckeliana* and Type species *Botrytis cinerea* (Pers. Fr) (Table 1).

Botryotinia fuckeliana (de Bary) Whetzel is the apothecial stage of *B. cinerea.* However, the teleomorph stage is rarely observed in the field (Faretra *et al.*, 1988).

1.1.3. Symptoms

B. cinerea is responsible for a number of symptoms that cannot easily be generalized across plant organs and tissues (Williamson *et al*., 2007). Symptoms of toxic damage to a plant may be readily observed by the naked eye in many instances but the primary lesion is usually at the biochemical level (Strange, 2003). There are two major sorts of symptoms caused by *B*. *cinerea*: localized infection and spreading necrosis (Elad, 1997). The localized lesions are associated with the occurrence of factors such as low inoculum level, absence of free water on the host surface and high vapor pressure deficit, absence of highly susceptible or senescing tissue, lack of exogenous nutrients or presence of host-derived inhibiting compounds. If one or more of these factors does not limit the infection, then the lesion spreads and typical grey mould symptoms of the disease become evident (Elad, 1997). Soft

rots, accompanied by collapse and water soaking of parenchyma tissues, followed by a rapid appearance of grey masses of conidia are perhaps the most typical symptoms on leaves and soft fruits (Williamson *et al*., 2007).

In grapevine, ripe berries are the preferred organs attacked by *B. cinerea*. Infected berries first appear soft and watery. The berries of white cultivars become brown and shriveled, and those of purple cultivars develop a reddish colour. At this stage of infection, rubbing causes the skin to slip over the inner pulp, a condition known as 'slip skin' (Pearson and Goheen, 1988; Nicholas *et al.*, 1994).

Under high relative humidity and moisture conditions, infected berries usually become covered with a gray growth of fungus mycelium. Rotted berries generally shrivel with time and drop to the ground as mummies. The fungus also can cause a blossom blight that can result in significant crop loss early in the season. Although uncommon, leaf infections also occur, but appear to be of no economic importance. Leaf infection begins as dull, green spots, commonly surrounded by a vein. Those spots rapidly become necrotic lesions. Typically, leaf infection causes reduction on chlorophyll content and photosynthetic rate (Zhang *et al*., 1996) which result in the visible disease symptoms.

Figure 1 - Grapevine berries showing typical gray mould disease and bunch rot

(Foto: O. R.Sônego *in* http://www.sistemasdeproduçao.cnptia.embrapa.com.br)

1.1.4. Disease cycle of *B. cinerea* in grapevine

The *B. cinerea* is present in vineyards as part of the environmental microflora (Keller *et al*., 2003). The fungus overwinters as sclerotia or mycelium in grape mummies and/or in dead canes, in grape tissues or other organic debris in the vineyard and around it (Figure 1). In the spring, when the environment conditions become suitable, Sclerotia germinate and *mycelia* develop from or within dying tissues. Then the fungus produces conidiophores and multinucleate spores (conidia). This will be the primary source of inoculum for pre-bloom infection of leaves and clusters. These spores are produced throughout the growing season (Pearson and Goheen, 1988) and can be released either singly or in clusters (Coertze *et al.*, 2001) and it can contribute up to 70% of disease levels on the vine at flowering and about 28% at harvest (Nair *et al.*, 1995). As long as the wet condition persists, as greater the probability of infection occurs. Relative humidity (RH) is a crucial environmental factor for *B. cinerea*, but RH is extremely difficult to regulate experimentally (Harrison *et al*., 1994; Williamson *et al*., 2007). Therefore, persistence of high RH during blossom periods leads to successive cycles of infection and sporulation (Williamson *et al*., 2007). As infected blooms die, the spores germinate and colonize dead flower parts and then the fungus invades living tissues such as young and succulent shoots, especially if injured. Normally, after blossom infection occur a period of latency or quiescence inside the clusters berries without causing disease symptoms, generally until grape berries begin to ripen (McClellan *et al*., 1973; Nair *et al*., 1995; Pezet and Pont 1986). After latency the fungus starts to infect mature berries and senescent plant tissues. This phase is believed to be an important in the epidemiology of *B. cinerea* in grapevine (Nair *et al*., 1995).

In summer pedicels or rachides can also be infected causing withering of bunches below the infected areas (Pearson & Goheen, 1988). Immature berry can be infected by soft brown rot in early summer (Nicholas *et al.*, 1994). However, infection over this period is unusual because immature berries are resistant to *B. cinerea* infection due to the high skin strength (Nicholas *et al.*, 1994), low concentrations of soluble solids such as sugars (Huang *et al.*, 2001), high concentrations of phenolic compounds (tannins), organic acids (Pearson and Goheen, 1988; Coertze *et al.*, 2001) and phytoalexins (Bavaresco *et al.*, 1997; Schouten *et al.*, 2002). During ripening grape berries become more susceptible. Berry skins become soft, the sugar content increases and many antimicrobial compounds such phytoalexin

drastically decrease. Consequently, the fungus can infect the berries and quickly spread from berry to berry within ripening bunches especially if berries are wounded or split. Sometimes, the damage is due to the cracking of the cuticle because the pressure increasing within the berries and to the physical damage by insects.

In grapes there are several insects associated with dispersion of viable conidia (Fermaud and Le Menn, 1989; Fermaud and Gaunt, 1995; Louis *et al*., 1996; Engelbrecht, 2002; Mandy *et al*., (1998) showed the existence of a mutualistic relationship between larvae of the grape berry moth (*Lobesia botrana*) and *B. cinerea*. Hail and wind also predispose clusters to berry infection, together with wet conditions lead to disease expression of "bunch rot".

Regarding to the sexual cycle, it involves the spermatization of sclerotia, leading to the production of apothecia and asci with eight binucleate ascospores (Williamson *et al*., 2007). However the apothecia (teleomorph stage) are rarely recorded in most crops attacked by *B. cinerea* in the field (Faretra *et al.*, 1988; Beever and Weeds, 2004).

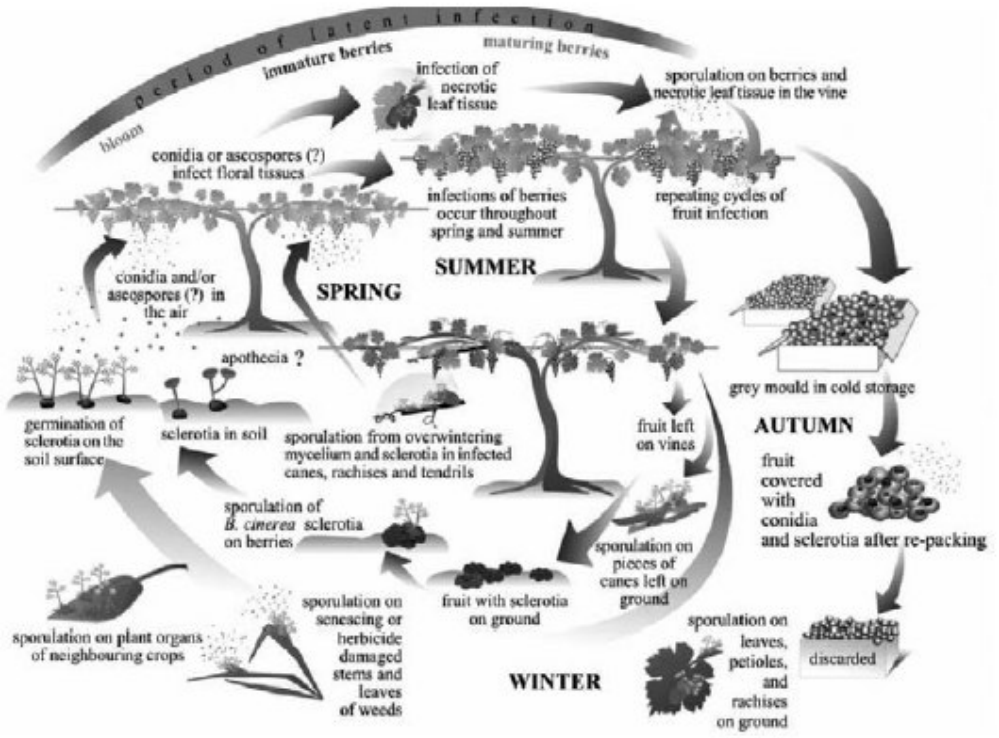


Figure 2. Proposed life cycle of Botrytis cinerea and disease cycle of grey mould in wine and table grape vineyards (Adopted from Elmer and Michailides in Elad *et al.*, 2007)

1.1.5. *Botrytis cinerea* pathogenicity

The term pathogenicity refers to the capacity of an organism to induce disease in its hosts. During infection Botrytis activate a battery of weapons to invade and/or kill the host.

1.1.5.1. Cell wall degrading enzymes

Botrytis spp. can enter the host via stomata and other natural openings (Clark and Lorbeer, 1976; Fourie and Holz, 1995; Hsieh *et al*., 2001) or alternatively by tissues by active penetration, directly through the cuticle following the attachment and germination of conidia under favourable conditions (Cole *et al*., 1996; Williamson *et al*., 1995). Usually this mechanism is not assisted by physical damage or abrupt mechanical penetration of the cuticle by *B. cinerea* (Williamson *et al*., 1995; Cole *et al*., 1996) suggesting that enzymatic activity is playing a crucial role in penetrating intact host surfaces (Salinas and Verhoeff, 1995; Elad and Evensen, 1995).

B. cinerea produces cutinase and lipase, both of which break down cutin. Lipase possesses cutinolytic activity and specifically hydrolyses long chain fatty acid esters (Commenil *et al.,* 1995) such as those present in waxes and cutin (Commenil *et al.,* 1997). In addition, lipases play a central role in the modification of the waxes and cuticle to favor the adhesion of conidia to the plant surface (Comménil *et al*., 1998). Experiments conducted by Salinas (1992) using specific monoclonal antibody against cutinase prior the inoculation of the gerbera flowers, showed reduction on the number of lesions formed, suggesting that cutinase was important for the penetration (Elad *et al*., 2007). In contrast, molecular genetic studies did not confirm the previous studies of Salinas (1992) since mutants lacking the 18-kDa cutinases were also virulents as the wild types isolates either on gerbera or in tomato fruits, and the fungus remained able to penetrate intact cuticle surfaces (Van Kan *et al*., 1997). Although, the role of cutinase in the penetration was not proved (Elad *et al*., 2007) electron microscopic examinations suggests that enzymatic hydrolysis of the cuticle is involved during penetration of the infection structure into the plant cell (Rijkenberg *et al.,* 1980).

Pectinases are also thought to play a major role in pathogenicity (Clark and Lorbeer, 1976; Collmer and keen, 1986; Cole *et al*., 1998) since they are involved in the penetration of the epidermal cell wall, because the carbohydrates released are a carbon source for the fungal

growth (ten Have *et al*., 2002). *B. cinerea* produces (Cabanne and Donèche, 2002), at least six endopolygalaturonase genes (Wubben *et al*., 1999) which are differentially expressed during pathogenesis on different hosts, and may contribute to the broad host range of this species (Wubben *et al*., 2000; ten Have *et al*., 2001). In earlier experiments, endopolygalacturonase activity was detected in ungerminated *B. cinerea* conidia (Verhoeff and Warren, 1972) and two polygalacturonase isozymes were associated with the penetration stage of the infection process (van den Heuvel and Waterreus, 1985). It was suggested that the early, constitutive production of polygalacturonases enables fast penetration of the host tissue (Kapat *et al*., 1998). It was later demonstrated the straight relation between *B. cinerea* invasion and growth *in planta* and degradation of pectin compounds and expression of endoPG genes (*Bcpg*) during invasion of different plant tissues, indicating the importance of endoPGs for virulence of *B. cinerea* (ten Have *et al*., 1998; Kars *et al*., 2005). The deletion of *bcpg1* genes encoding polygalacturonases 1 (ten Have *et al*., 1998) or of a gene encoding a pectin methylesterase gene (Valette-Collet *et al*., 2003) reduced the virulence of the pathogen.

1.1.5.2. Laccase (Benzenediol oxygen oxidoredutases)

Laccases produced by *B. cinerea* have been studied over two decades by Mayer and co-workers (1990, 1995). Laccases are polyphenol oxidases that require molecular oxygen to oxidize phenols, polyphenols, aromatic amines and different non phenolic substrates by one-electron transfer, resulting in the formation of reactive radicals (Schouten *et al*., 2002). Detoxification of phytoalexins pterostilbene and resveratrol by *B. cinerea* was studied over 3 decades by Mansfield (1980). Oxidation of resveratrol catalyzed by a laccase was reported by Adrian *et al*., (1998). Laccases are part of a more general 'attack' machinery designed to detoxify phenolic defences from many host plants (Staples and Mayer, 1995), including resveratrol and tannins, released by plants (Schouten *et al*., 2002; Schouten *et al*., 2008). Laccase activity displayed by the fungus is assumed to detoxify resveratrol and to facilitate colonization of grape. However, its production by *B. cinerea* increases during late stages of infection (Roudet *et al*., 1992; Manteau *et al*., 2003) when resveratrol concentration in the host is decreasing (Adrian *et al*., 2000; Monteiro *et al*., 2003).

Laccase can be stimulated *in vitro* by the presence of plant compounds such as polyphenols, pectins, pH and copper compounds (Viterbo *et al*., 1993a; Manteau *et al*., 2003)). Proanthocyanidins act as competitive laccase inhibitor (Pezet *et al*., 1992). Some other secondary plant metabolites inhibit laccases of *B. cinerea*. For example, the secondary metabolite cucurbitacin protect cucumber fruit and cabbage leaves from infection by *B. cinerea* (Bar-Nun and Mayer, 1990). Cucurbitacin suppressed the production of laccases in a *B. cinerea* culture but the activity of other fungal enzymes were not affected (Viterbo *et al*., 1993a). Some cucurbitacin forms were more effective than others in reducing secreted laccase activity (Viterbo *et al*., 1993b), leading to the hypothesis that laccase plays an important role in pathogenesis (Viterbo *et al*., 1993a; Staples and Mayer, 1995).

The role of laccase in *B. cinerea* have been studied by gene disruption. Deletion of either *Bclcc1* or *Bclcc2* gene did not result in detectable reduction of virulence on a range of host species tested (Schouten *et al*., 2002). It led to conclude that at least the laccases *Bclcc1* and *Bclcc2* are not important virulence factors. Deletion of the *Bclcc3* gene did not show any important role in early steps of pathogenesis (Schouten *et al*., 2002).

Some fungal laccases are capable of degrading lignin (Evans, 1991). In addition to its role, laccases are considered important enzymes because of their diverse applications in industry, such as for delignification of lignocellulosics and crosslinking of polysaccharides, for bioremediation applications such as waste detoxification and textile dye transformation, (Mayer and Staples, 2002).

1.1.5.3. Proteolytic enzymes

The involvement of fungal enzymes in plant pathogenesis was suggested by de Bary (1886). However the role of proteases in *B. cinerea* pathogenicity remains unclear. Proteolytic enzymes are potentially able to degrade proteins of the host cell wall and plasma membranes (Poussereau *et al*., 2001). Proteases may also counter host defense-related proteins such as antifungal enzymes (Poussereau *et al*., 2001). Two decades ago Movahedi and Heale (1990a,b) found an aspartic protease secreted by *B. cinerea* both in culture and in infected carrots. Extracellular aspartic protease activities were detected in

both ungerminated conidia and during germination, prior to the appearance of pectinases activity (Movahedi and Hale, 1990a). A decade later, Prins *et al*., (2000), studied the role of the aspartic protease *BcAP1* in the infection of detached tomato leaf tissues by gene disruption. They didn't observe any remarkable loss of symptoms for the *BcAP1*-deficient mutant, indicating that this protease is not essential for virulence. But these authors did not exclude the involvement of other aspartic protease in *B. cinerea* pathogenicity. Interestingly it was found that when spores of *B. cinerea* were treated with pepstatin, a specific inhibitor of aspartic protease, there was a marked reduction in disease symptoms, in a number of hosts (Strange, 2003). These results suggest that aspartic protease may play a role in the pathogenicity of *B. cinerea*.

Studies conducted by Gronover *et al*., (2004) via Suppression Subtractive Hybridization (SSH) analysis revealed that during host infection, *B. cinerea* expresses numerous protease encoding genes including acid protease, aorsin, metalloproteinase, penicillolysin precursor, polyporopepsin and tripeptidyl peptidase I precursor. These proteases are thought to be involved in virulence by degrading PR proteins (Gronover *et al*., 2004). In contrast, Marchal *et al*., (2006) reported that degradation of grape proteins by *B. cinerea* is difficult. However, Gronover *et al*., (2004) suggested that the six protease like genes he studied, may have a role in degrading the cell wall and PR proteins of the host plant, and also in fungal nutrition during the following invasive growth.

B. cinerea protease has been also studied in winery and evidences suggested that *B. cinerea* proteases are able to degrade grape proteins that cause haze in wines. Marchal *et al.* (1998) analyzed musts from healthy grapes and grapes infected with *B. cinerea*. Protein pattern analysis revealed that *B. cinerea* infected musts had less protein than the healthy musts. Equally, Girbau *et al*., (2004) analysed juice from healthy and *B. cinerea* infected Chardonnay and Semillon grapes. When compared with uninfected grape juice, *B. cinerea* infected grape juice had lower levels of proteins. Significantly, the proteins responsible for haze in white wine were absent from some of the *B. cinerea* infected juices.

1.1.5.4. Killing the host

B. cinerea is an important necrotrophic plant pathogen that kills the host and decomposes the plant tissues. Colonization of necrotic tissues provides nutrients for growth and infection of healthy plant tissues. As a necrotrophic pathogen *B. cinerea* induces host cell collapse possibly by secretion of toxic metabolites causing necrotic lesions in plant tissues (Van Kan, 2006).

The induction of cell program death seems to be one of the most important strategies for Botrytis to kill the host cells probably through the releases hydrogen peroxide or superoxide, which is converted rapidly by superoxide dismutase into hydrogen peroxide (Agrios, 2005). A secreted superioxide dismutase (BcSOD1) is active and displays an important role during cuticle penetration by the appressorium, (Rolke *et al*., 2004; Williamson *et al* 2007). *B. cinerea* is also able to exploit the host production of Active Oxygen Species (AOS) to induce host cell death (Von Tiedemann, 1997; Govrin and Levine, 2000). This activates and stimulates the plant host resistance (HR) pathway (Agrios, 2005). Thus, the fungus, by usurping the HR signaling and programmed cell death, subsequently invades the dying tissue and colonizes the plant by mimicking the HR signals (Agrios, 2005). Oxalic acid (OA) which is a phytotoxin produced by many plant pathogenic fungi, including *B. cinerea* contributes to the pathogenic success of the fungus. Effects of oxalic acid in fungal pathogenicity have been reported more than a century ago (de Bary 1886). It is believed that due to the acidification of infected tissues, OA led to destruction of host cell walls, by enhancing activities of polygalacturonases and other hydrolytic enzymes that have an acidic pH optimum (Bateman and Beer 1965; Lumsden 1979). Can also inhibit defence responses of host plants (Ferrar and Walker 1993; Cessna *et al.* 2000) and induce a programmed cell death (PCD) (Kim Su *et al*., 2008).

Also the production of phytotoxins by *B. cinerea* has been proposed to promote cell death in the host (Rebordinos *et al*., 1996).

1.1.6. Detoxification of plant chemicals by *Botrytis cinerea*

Plant produces a large number of fungitoxic compounds which constitute a chemical defense against plant pathogens. Among them, plant secondary metabolites with

antimicrobial activity (Rice, 1987; Osbourn, 1996, 1999; Morrissey and Osbourn, 1999) play a relevant role. To be a successful pathogen on multiple host species *B. cinerea* must cope with plant defense compounds (Williamson *et al*., 2007). The ability of *B. cinerea* to detoxify plant chemical defense mechanism have been associated as a potential pathogenicity determinant (Verhoeff and Liem, 1975; Pezet *et al*., 1991; Sandrock and VanEtten, 1998; Sbaghi *et al*., 1996; Quidde *et al*. 1999; Prins *et al*., 2000). As suggested by Prins *et al*., (2000) probably *B. cinerea* possesses a large set of enzymes capable to detoxify the phytoanticipins. Quidde *et al*. (1999) demonstrated that *B. cinerea* can deglycosylate the saponins digitonin, avenacin, and avenacosides. At least three different saponinases; two glycosidases (avenacosidase) and a xylosidase (tomatinase and digitoninase) are likely involved in this process.

B. cinerea is able to withstand toxic effects of other polyphenols such as isoflavonoids and coumarins, and sesquiterpenos (Schoonbeek *et al*., 2001). ATP-binding cassette (ABC) transporters might provide protection against plant defense compounds and fungicides by a ATP-driven efflux mechanisms. Botrytis also possesses Major Facilitator Superfamily Transporter which together with ABC transporter excretes a spectrum of fungitoxic compounds (de Waard, 1997; Schoonbeek *et al*., 2001; Waard *et al*., 2006; Williamson *et al*., 2007; Stefanato *et al*., 2009).

As reported before *B. cinerea* may detoxify the stilbenic phytoalexin resveratrol by a laccase stilbene oxidase activities (Adrian *et al*., 1998).

Some researcher have demonstrated that *B. cinerea* has the ability to detoxify the phytoalexins camalexin (Kliebenstein *et al*., 2005; Williamson *et al*, 2007) and *tomatine* (Quidde *et al*., 1999; Agrios, 2005). Exposure of *B. cinerea* to camalexin induces expression of *B. cinerea* ABC transporter (BcatrB), and *B. cinerea* strains lacking functional BcatrB is more sensitive to camalexin *in vitro* and less virulent on *A. thaliana* wild-type plants (Stefanato, 2009). The author concluded that ABC transporter is a virulence factor that increases tolerance of the pathogen towards a phytoalexin (Stefanato, 2009).

Some ABC-transporters are located in the vacuolar membranes and are involved in pumping complexes of glutathione and toxic compounds generated by glutathione-S-

transferase (GST) into the vacuoles (Ishikawa *et al*., 1997; Prins *et al*., 2000). In addition, the removal of potentially toxic external AOS by *B. cinerea* is likely to be mediated by extracellular enzymes such as superoxide dismutase (SOD) and catalase (Prins *et al*., 2000).

1.1.7. Control of gray mold disease

Control of Botrytis gray mould disease is performed in several ways according to the principles of integrated pest management.

Cultural practices: this practice start with reduction of the overwintering inoculum in the vineyard by eliminating the crop debris in the ground and the pruning material. During growth the excess of vegetation should be removed, especially the leaves near the grape cluster (Pscheidt, 2007) in order to promote a rapid leaf drying (Williamson *et al*., 2007). According to Williamson *et al*., (2007) an high relative humidity (RH) promotes conidial generation and allows germination and penetration of the host. Therefore conditions for proper aeration by ventilation inside the storage, rooms and between the clusters should be provided (Agrios, 2005).

During cultivation, adequate level of calcium ions enhances the plant resistance to *B. cinerea*. It is well known the effect of calcium on the composition of cell walls and their resistance to penetration by pathogens. Calcium reduces the severity of several diseases caused by root and stem pathogens (Agrios, 2005).

Chemical approach: timing of fungicide application is critical for *B. cinerea* control. Sprays with a number of broad-spectrum or systemic fungicides give excellent control of Botrytis on a wide variety of crops. However, it is well known that Botrytis is able to acquire resistance to several systemic and even to some broad-spectrum fungicides. Therefore, the use of different fungicides and fungicide combinations is recommended to reduce the appearance and establishment of resistant strains (Agrios, 2005). This can be achieved by using mixtures of specific systemic and wide-spectrum protectant fungicides and alternating sprays with systemic and protectant fungicides, (Agrios, 2005).

Research conducted on blackcurrant (*Ribes nigrum*) demonstrated that strobilurin fungicides (azoxystrobin, kresoxim-methyl and trifloxystrobin), fenhexamid, dichlofluanid and tolyfluanid applications during flowering can decrease the number of *B. cinerea* infection sites per shoot thereby increasing yield (Duben and Rosslenbroich 2002; Walter *et al*., 2007). Fungicides are currently applied during the high-risk periods associated with particular vine growth stages and when weather conditions are conducive for infection and sporulation (e.g. more than 48 h of >95% RH and 15-25ºC). Under high disease pressure fungicides are commonly applied when shoots are 10-15 cm long twice over flowering, at pre-bunch closure, and then as required after *veraison* according to the weather conditions (Nicholas *et al.*, 1994).

Biological approach: Spraying of the fungal antagonist *Trichoderma viride* spores on strawberry blossoms and young fruits, reduced severely the disease during pre and postharvest (Agrios, 2005). Sprays with *Trichoderma* in the field also reduced Botrytis rot either of strawberries or grapes at the time of harvest and in storage. Li *et al*., (2002) demonstrated that conidia and germ tubes of *B. cinerea* are highly susceptible to infection by the mycoparasite *Gliocladium roseum*, when both organisms were grown as a mixed culture.

Several antagonistic yeasts has been also reported to protect grapes and tomatoes from *B. cinerea* infection (Agrios, 2005). Yeast *Candida guilliermondii* strains 101 and US 7 significantly reduced disease incidence, when applied simultaneously with the pathogen (Saligkarias *et al*., 2002). Other yeast species such as *Aureobasidium pullulans*, *Metschnikowia pulcherrima* and *Pichia guilliermondii* showed, on average, higher biocontrol activity (Raspor *et al*., 2010).

Mycovirus have been reported as potential biocontrol of gray mould disease (Chen *et al*., 1994; Howitt *et a1*., 1995; Leslie and Yamashiro, 1997; Zhou and Boland, 1998; Nuss, 2005; Pearson, *et al*., 2009). Some mycoviruses reduce the ability of their fungal hosts to cause disease in plants in a mechanism known as hypovirulence (Nuss, 2005). *B. cinerea* cells infected with this virus showed an important degree of cellular degeneration (Castro *et al*., 1999). Also Howitt *et al*., (2006) characterized a flexuous rod-shaped mycovirus,

Botrytis virus X (BVX), infecting *B. cinerea* suggesting that modified viruses could provide a new approach for the biocontrol of grey mould (Howitt, 1998).

1.1.8. Grapevine defense mechanisms against *B. cinerea*

Resistance of plants to infection by pathogens is the result of multiple defense reactions comprising both constitutive or preformed and inducible barriers (Jeandet, P., *et al*., 2002) or biochemical factors (Hammerschmidt, 1999). The typical preformed, constitutive defenses are the structural barriers components like waxes, cutin, suberin, lignin, phenolics, cellulose, callose, and cell-wall proteins (Jeandet *et al*., 2002). Besides, active defense mechanisms mainly involve the oxidative burst, rapid and localized cell death (hypersensitive response), accumulation of phytoalexins, and synthesis of pathogenesis-related (PR) proteins.

Defense response mechanisms are not only activated upon infection by pathogenic microorganisms, but can also be induced by abiotic stresses such as induction with UV-light, or by chemicals (respiratory inhibitors, surfactants, antibiotics, plant regulators, or the salts of heavy metals), as well as elicitors released by the pathogens or products resulting from the activity of fungal degrading-enzymes on host cell-walls (Harborne 1999; Jeandet, *et al*., 2002).

Structural and biochemical mechanisms are both involved in passive and active defence in grapevine.

In grapevine, the synthesis and accumulation of phytoalexins and PR-proteins are the most frequently observed and best characterized defense mechanisms (Derckel, *et al*., 1999; Jeandet *et al*. 2002).

Grape berry phenolics, for example catechin, may play a role in resistance of young berries to *B. cinerea* (Goetz *et al*, 1999), since these compounds were shown as inhibitors of stilbene oxidase an enzyme implicated in *B. cinerea* pathogenesis (Sbaghi *et al*., 1996). Unlike grape berries, leaves tend to become more resistant to *B. cinerea* as they mature, a possible consequence of cell wall-bound phenolics being released during attempted infection (Weber *et al*., 1995; Elad *et al*., 2007).

1.1.8.1. Constitutive or pre- infectional defenses

Biochemical and physical barriers may form constitutive defense mechanisms that may account for the high relative resistance of berries to *B. cinerea* (Nair and Hill, 1992; Barnavon *et al*., 2001; Keller *et al*., 2003).

1.1.8.1.1. Physical or morphological barriers

Preformed morphological features act as a physical barrier, and more likely to play an important role in the mature berries, when they are more susceptible to Botrytis infection (Nair and Hill, 1992).

In grapevine, the number and thickness of epidermal and hypodermal cell layer have been positively correlated with resistance to *B. cinerea* (Mlikota-Gabler *et al*., 2003). Physical resistance to infection also depends on the cuticle as well as the cell wall structure and composition in the berry skin (Commeli *et al*., 1997; Mlikota-Gabler *et al*., 2003; Vorwerk *et al*., 2004)

a. Cuticle

Cuticle is the first barrier between the host plant and the pathogen (Rosenquist and Morrison, 1989; Vidhyasekaran, 2004). The cuticle regulates the water loss during transpiration and contributes to the control of gaseous exchanges (Rosenquist and Morrison, 1989) irradiation and xenobiotics and is involved in the delimitation of organs during development (Kolattukudy, 1985; Nawrath, 2002).

B. cinerea is able to enter through undamaged plant cuticle (McKeen, 1974; Rijkenberg *et al,* 1980; Pie and de Leeuw, 1991; Salinas, 1992) and cuticular breakdown products have been shown to be potent inducers of cutinase processes in fungal pathogens (Chassot *et al*., 2007).

The susceptibility of grapes to bunch rot disease have been particularly related with arrangement of the pellicular cells (Bernard, 1976), the cuticular thickness (Galet, 1977; Prudet *et al*., 1992) and the density of the cuticle layers (Percival *et al.,* 1993). The susceptibility of grape berries to *B. cinerea* infections greatly increase at the onset of

ripening of grapes (Bulit and Lafon, 1977; Hill *et al.,* 1981; Jeandet and Bessis, 1989), when cuticle thickness (Prudet *et al*., 1992) and chemical composition change as berries ripen, often at a time coinciding with increased susceptibility to *B. cinerea* (Rosenquist and Morrison, 1989; Nair and Hill, 1992; Commenil *et al*., 1997).

b. Cell wall

The plant cell wall consists of pectic substances, hemicelluloses, cellulose, and hydroxyproline rich-glycoproteins (Vidhyasekaran, 2004). The amount of cellulose and polysaccharides in the cell wall and their stage of breakdown will determine the strength and rigidity of the cell wall and this may affect the firmness of the grape berry (Plessis, 2008).

The susceptibility of the plant cell wall to degradation by cell wall-hydrolyzing enzymes can affect the severity of disease caused by *B. cinerea*. Fungal polygalacturonases (PGs) hydrolyze the homogalacturonan of plant cell-wall pectin and are important virulence factors for some necrotrophic fungi, whereas the plant polygalacturonase-inhibiting proteins (PGIPs) contribute to resistance by counteracting fungal PGs (Powell *et al*., 2000; Ferrari *et al*., 2003b; Prusky and Gullino, 2010).

Polygalacturonase-inhibiting proteins (PGIPs) are leucine rich repeat-containing proteins that may inhibit endopolygalacturonases (endoPGs) of plant pathogenic or non-pathogenic fungi (Juge, 2006; Williamson *et al*., 2007). PGIPs is associated with plant cell walls apart from being efficient inhibitor might also have additional functions linked to cell wall strengthening (Williamson *et al*., 2007).

A PGIP purified from grapevine was shown to inhibit crude polygalacturonase extracts from *B. cinerea* (Joubert *et al*., 2006). A transgenic plant overexpressing the PGIP in tobacco plants showed a reduced susceptibility to infection by this pathogen (Joubert *et al*., 2006).

1.1.8.1.2. Biochemical or physiological defenses

Phytoanticipins are low-molecular-weight antimicrobial compounds present in plants before pathogen infection. Phenolics, terpenoids, glucosinolates, alkaloids, dienes, saponins, and cyanogenic glucosides compounds belong to the group of phytoanticipins (VanEtten *et al*., 1994). Phytoanticipins such as phenolic compounds, tannins and some fatty acid-like compounds dienes are present in high concentrations in cells of young fruits, leaves, or seeds and have been proposed as responsible for the resistance of young tissues to pathogenic microorganisms such as *B. cinerea* (Agrios, 2005).

1.1.8.2. Post-infectionally activated defenses

In grapevine, several studies have reported the induction of genes encoding PR proteins and enzymes of the phenylpropanoid pathway after inoculation with various pathogens (Kortekamp, 2006; Robert *et al*., 2002; Van Loon *et al*., 2006; Wielgoss and Kortekamp, 2006). Genes encoding enzymes of the phenylpropanoid pathway involved in the synthesis of antimicrobial phenolics are also induced upon inoculation of *V. vinifera* cv. Riesling with *Pseudoperonospora cubensis*, a non-host pathogen (Kortekamp, 2006). Moreover, a positive correlation has been found between the resveratrol level in grapevine cultivars and their resistance to *B. cinerea* (Langcake and McCarthy, 1979).

1.1.8.2.1. Pathogenesis related proteins (PR-proteins)

Pathogenesis related proteins are low molecular weight proteins with antimicrobial activity against a vast group of plant pathogens. Due to this role in plant defense, PR proteins have been intensively studied in many plants in both agriculture and in biotechnological studies. To date are classified in 17 different families (Van loon *et al*., 2006) as they represent a large array of proteins coded by the host plant that are coordinately and specifically expressed under environmental stresses either infection or related situation (Elad, *et al*., 2007; Stermer, 1995; Sticher *et al*., 1997). PR proteins are also involved in systemic acquired resistance (SAR) proteins (Ward *et al*., 1991; Kessmann *et al*., 1994; Sticher *et al*., 1997).

PR proteins accumulate either in extracellular space or in the vacuole (Stermer, 1995; Sticher *et al*., 1997). However, accumulations of PR proteins in organs are not homogeneous, fruit tissues accumulate high concentrations of PR proteins compared to other organs (Derckel *et al*., 1998). But this accumulation is most during fruit ripening (Derckel *et al*., 1998).

a. PR-proteins in grape

Grapes mainly accumulate one thaumatin-like proteins and chitinases which make up 80% of the total soluble protein content of the fruit at harvest (Derckel *et al*., 1998; Salzman *et al*., 1998; Waters *et al.*, 1998).

Isoelectric focusing, SDS-PAGE electrophoresis and chromatography studies indicated that there are up to 13 different forms of chitinase in grapevine, with most of the activity resulting from acidic forms of the enzyme (Giannakis *et al*, 1998).

Among the PR proteins, grape chitinase has one of the highest botryticidal activities. It inhibits germination of conidia with an EC50 value of 7.5 µg/ml (Derckel *et al*., 1998) and it restricts the elongation of hyphae (Salzman *et al*., 1998).

Most the chitinase activity in *V. vinifera* berries is in the pulp (Derckel *et al.*1998) and grapevine chitinase is present at levels up to 26 $\mu g.g^{-1}$ in fruit of resistant cultivars (Derckel *et al*., 1998; Salzman *et al*., 1998).

Purified grape chitinases have shown antifungal activity when tested *in vitro* against the grape pathogens *Guignardia bidwellii, Botrytis cinerea,* and *Uncinula necator* (Derckel *et al.,* 1998, Giannakis *et al*. 1998, Salzman *et al*. 1998; Derckel *et al*., 1999). Besides transgenic grapevines plants overexpressing chitinase have shown higher resistance to *Botrytis cinerea* than untransformed vines (Derckel *et al*., 1999; Kikkert *et al*. 2000).

Thaumatin like proteins (PR-5) are antimicrobial proteins shown to be induced in some plant tissues only after pathogen infection (Ruiz-Medrano *et al*., 1992). Many PR-5 proteins have been shown possessing antifungal activity (Fagoaga *et al*., 2001) altering the permeability of fungal membranes (Vidhyasekaran, 2004) by causing leakage of cytoplasmic material and hyphal rupture. The majority of PR-5 proteins have molecular

masses of 22 kDa and are stabilized by eight disulfide bonds. This highly stabilized structure allows PR-5 proteins to be very resistant to protease degradation (Roberts and Selitrennikoff, 1990).

Thaumatin exhibits strong *in vitro* antifungal activity by blocking the growth of *Phomopsis viticola* and *Botrytis cinerea* mycelia (Monteiro *et al*., 2003).

Two thaumatin like proteins (VvTL-1 and VvTL-2) were described from grapevine (Tattersall *et al*. 1997; Jacobs *et al*. 1999). VvTL-1 was identified as a developmentally-regulated, fruit-specific protein (Tattersall *et al*. 1997). VvTL-2, expressed in leaves and fruits, previously was named grapevine osmotin (Loulakakis 1997; Salzman *et al*. 1998), but was renamed when it was determined to be acidic. VvTL-2 shows considerable homology to VvTL-1, but is more responsive to biotic than abiotic stresses (Jacobs *et al*., 1999). Antifungal properties of VvTL-1 have not been determined, whereas purified VvTL-2 inhibited fungal growth in the presence of sugars (Salzman *et al*., 1998).

1.1.8.2.2. Phytoalexins

Phytoalexins are low molecular weight antimicrobial compounds synthesized and accumulated *de novo* after the plant tissues being exposed to microorganisms (Muller and Borger, 1940; Paxton, 1981) or after injury or stress (Ebel, 1986; Kuc, 1995). Accumulation is a result of specific elicitors released either by the fungal cell walls or by the plant cell walls (Ebel, 1986).

Stilbene phytoalexin and their oxidation products play important roles in the defense system of the grapevine, by showing the antifungal activity against a wide range of pathogens (Pezet *et al*., 2004). The major molecules involved are the 3,5,4' - trihydroxystilbene resveratrol and its derivatives: pterostilbene, a 3,5-dimethylated resveratrol, ε and δ viniferin, dehydrodimers of resveratrol, piceid, a glucoside of resveratrol. Their fungitoxicity differs from one to another and numerous biotic and abiotic factors can induce their synthesis (Elad *et al*., 2007).

Phytoalexins can affect fungal growth by inhibiting germ tube elongation and colony growth (Elad, 1997). Direct contact of phytoalexins with fungal cell walls resulted in fungal plasma membrane disruption and loss of the ultrastructural integrity (Elad, 1997).

However, the concentration of stilbene phytoalexin decreases from ripening to maturity and these ripening berries became susceptible to *B. cinerea* infection (Bais *et al*., 2000). This may be due to a detoxification of phytoalexin and/or a laccase-like stilbene oxidase activity and a competition between chalchone synthase and stilbene synthase (Bavaresco *et al*., 1997). Mohr and Cahill (2001) provided evidence that phytoalexins may be more important than phenolic and lignin deposition in disease resistance.

a. Resveratrol

Resveratrol (*trans*-3,5,4'-trihydroxystilbene) is the major stilbenic phytoalexin in grapevine which protects the host from fungal infection (Breuil *et al*., 1999) including *B. cinerea* (Langcake, 1981). In phenylpropanoid pathways resveratrol is derived from the acetate-malonate (Langcake and Pryce 1977b). Stilbene synthase (STS) is the key enzyme that catalyses the formation of resveratrol via the phenolic substrates, malonyl-CoA and 4-coumaroyl-CoA (Fritzemeier and Kindl 1981; Melchior and Kindl 1991).

Resveratrol is one of the most soluble and least toxic stilbenes, as it can be stored safely at high concentrations in vacuoles and cell walls (Morales *et al*., 2000). The translocation can only be activated upon contact with a microorganism (Elad, 2007). In grape berry resveratrol was found in the skin in response to fungal attack mainly *B. cinerea* (Ebel, 1986).

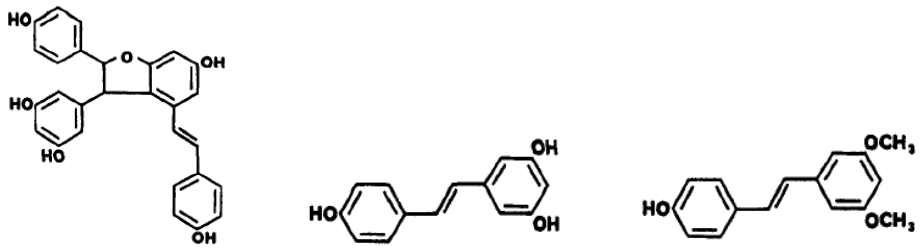
Resveratrol has been demonstrated to inhibit radial growth of fungal mycelia (Hoos and Blaich, 1990) however, resveratrol do not display immediate toxicity towards *B. cinerea* (Pezet and Pont, 1995). During the interaction of grapevine and *B. cinerea*, the host plant synthesizes resveratrol as a pathogenesis response in order to arrest the fungal invasion. When resveratrol comes in contact with Botrytis conidia, it can induce a series of morphological anomalies in the fungus. Including effects on the fungal plasmatic membrane integrity (Adrian *et al*., 1997), cytoplasm anomalies in the conidia, germ tube enlargement or retraction in the growth, protoplasmatic retraction in the hyphae and

formation of tertiary germ tube in survived conidia have been reported (Adrian, 1998; Jeandet *et al*., 2002). Pezet and Pont (1990) demonstrated that application of Pteroestilbene in conidia of *B. cinerea* induces strong modifications in the endocellular membrane and the fast destruction of the reticule endoplasmatic, nuclear and mitochondrial membranes. In addition, complete retention in the cellular respiration process and cytoplasm coagulation has been reported also. Adrian *et al*., (1997) reported that the presence of phytoalexins in the infection area does not always result in the death of fungal invaders. Also, in the experiments where conidia were incubated with resveratrol or pterostilbene, the percentage of non-germinated conidia was generally greater than dead ones (Adrian *et al*., 1997).

Resveratrol may be converted in *trans*-ε-viniferin, a resveratrol dehydrodimer (Langcake and Pryce, 1977b; Langcake, 1981). This conversion is due to the oxidative dimerisation of resveratrol by *B. cinerea* laccase (Pezet, 1998; Schouten *et al*., 2002) or probably resveratrol is oxidazed in planta by the plant enzyme peroxidase (Langcake and Pryce, 1977a,b).

Pterostilbene is also a derivative compound of resveratrol synthesized by grapevine. It has the highest antifungal activity, but its concentration is low (less than 5 μg g^{-1}) in leaves (Douillet-Breuil *et al*., 1999) and in fruits (Adrian *et al*., 2000). The potency of pterostilbene increases in the presence of glycolic acid, an organic acid that accumulates to high concentrations in immature grape berries.

Besides resveratrol, numerous other stilbenes have been characterized in grapevine. These include a 3-O-β-glucoside of resveratrol called piceid that is formed by the action of a glycosyl transferase on resveratrol (Waterhouse and Lamuela Raventos, 1994; Romero-Perez *et al*., 1999). Piceids are a water-soluble form of resveratrol that can be reconverted into resveratrol by plant glycosidases (Ayran *et al.,* 1987).



ε-viniferin resveratrol pterostilbene

Figure 3 - Chemical structure of trans-resveratrol, trans-pterostilbene and ε-viniferin (Adrian *et al*., 1997)

1.1.8.3. Induced Programmed Cell Death

Programmed cell death (PCD) is a pathological process of cell necrosis as a result of normal tissue homeostasis and stress responses (Wertz and Hanley 1996). This hypersensitive response is the culmination of the immediate plant defense responses initiated by the recognition by the plant of specific pathogen-produced signal molecules, known as elicitors (Agrios, 2005). As described before, elicitors will induce several local responses surrounding the infection site cells including in the accumulation of citotoxic compounds: deposition of callose and lignins in the cell wall, and the synthesis of stilbene phytoalexins and PR proteins (Ouchi, 1983; Calderón, 1993) and the rapid localized cell death forming the necrotic lesion (Dixon and Harrison, 1990).

During incompatible interactions, e. g., the infected leaf does not sustain pathogen growth so that lesions do not spread and no evident symptoms of host cell killing. However, only with compatible interactions does the pathogen infect and colonize the host. In addition, directly damage to cell membranes by the pathogen or by induced free radical, also triggers the production of several membrane-bound kinases that are components of signalling pathways that ultimately lead to programmed cell death. This host response is important for defence against biotrophs, but would presumably increase its vulnerability to necrotrophs such as *B. cinerea (*Van Baarlen *et al*., 2004*).*

The regulation of PCD involves many potential signal-transducing molecules including reactive oxygen species, ion fluxes, G proteins, jasmonic and salicylic acids, protein

phosphorylation cascades, activation of transcription factors and protein recycling by the polyubiquitin system (Dangl *et al.,* 1996; Hammond-Kosack and Jones, 1996).

Grapevine leaves undergo a PCD response associated with localized cell death of the host plant to inhibit the growth of the pathogen. This hypersensitive response is often accompanied by a rapid production of phytoalexins (Darvill and Albersheim, 1984), oxidative response burst in the driven by rapid production of H_2O_2 (Calderon, 1993). Grapevine cells also respond to the *T. viride* elicitor through a plasmolytic response such response may be mediated by the synthesis of osmotin, which may form a part of signal transduction during plant defence responses (Linthorst, 1991). But the role of the plasmolytic response in plant defence still unclear (Linthorst, 1991; Ye *et al.,* 1992).

Derckel *et al*., (1999) suggested that grapevine leaves undergo a hypersensitive-like response when challenged with the less virulent strain of *B. cinerea*, T4. Several defense compounds, secondary metabolites, chitinases, and β-1,3-glucanases, were produced around a very localized group of dead cells. A necrotrophic pathogen like *B. cinerea*, the HR could facilitate the plant infection (Govrin and Levine, 2000) instead of confining the pathogen (Aziz, *et al*., 2006).

2. Material and Methods

2.1. Biological material, reagents and culture media

2.1.1. Plant material

White ripe grape (*Vitis vinifera*, cv. IM 6.0.13) was harvested in the first week of September 2008 from a typical vineyard near Conegliano (north east Italy) and stored for 3 - 6 days at 4°C before protein and polyphenol extraction and *B. cinerea* inoculation.

2.1.2. Culture media

2.1.2.1. Potato Dextrose Agar

The potato dextrose agar (PDA) was prepared by diluting 39 grams of PDA (Difco, USA) in 1 liter of distilled water and sterilized in autoclave at 121 °C for 20 min.

2.1.2.2. Czapek-Dox medium

Liquid cultures were produced in a modified Czapek-Dox medium (2 g l^{-1} $NaNO_3$, 0.5 gl-1 KCl, 0.5 gl^{-1}.$MgSO_4.7H_2O$, 1 g l^{-1} K_2HPO_4, 0.01 g l^{-1} $FeSO_4$, 1 ml of a solution of 1% ZnSO4 plus 0.5% $CuSO_4$, and 20 g l^{-1} glucose), adjusted to pH 3.5 with tartaric acid (about 10 mM). The medium was prepared at twice working concentration.

2.1.3. Fungal strain

Botrytis cinerea strain PM-10 isolated from grape was kindly provided by Professor Giuseppe Firrao, University of Udine (Italy).

The fungus was grown on Petri dishes on PDA at 24°C. For spore production, completely colonized plates were incubated under near UV light for 16 h as reported by Schouten *et al.* (2002). After 15 days, conidia were collected into 5 ml of sterile water by gently scraping the plates with a glass rod. Conidia were filtered through sterilized gauze to remove mycelial fragments and counted using a hemacytometer. The obtained spore suspensions were then kept at -80 °C with 30 % glycerol.

2.2. Grape protein extraction and analysis

Proteins were extracted from about 1 liter of grape juice obtained by hand crushing about 1.6 kg of berries at 4°C in a beaker. The grape juice was immediately filtered through a nylon gauze, and cysteine was added to give a final concentration of 4 mM to prevent polyphenol oxidation. All successive operations were also performed at 4°C. The juice was centrifuged at 30,000 . *g* for 30 min, and the supernatant was filtered in succession through glass-microfibre discs (GMD) and cellulose acetate filters (0.8 µm) (Sartorius, Germany) and dialyzed overnight (membrane cut off =10 kDa) against 10 mM potassium tartrate buffer, pH 3.5. The dialyzed material was adjusted to 20% saturation (106 g l-1) with (NH4)2SO4 and stirred for 1 h. After centrifugation at 30,000 *g* for 30 min, the supernatant was filtered in succession through 0.8, 0.45, and 0.2 µm membranes, concentrated 8 times with a VivaFlow 5000 apparatus (Sartorius, Germany), and loaded on a Sephadex G-25 column (PD-10, GE Healthcare, UK). The protein was eluted with water and further concentrated with a VivaFlow 5000 apparatus and then assayed for protein (Bradford, 1976) and phenol (Folin-Ciocalteau assay) amounts using BSA and gallic acid as the standards, respectively. The protein recovered, about 20 µg g-1 of berry fresh weight, was approximately one-third that measured initially in the grape juice. The estimated weight ratio between protein and phenols in the final protein preparation was about 5:1. This protein was used for *B. cinerea* cultures and *in vitro* assays. The protein profile was analyzed after precipitation of the protein with four volumes of cold ethanol at -20°C for 2 h. The pellet was washed once with cold 70% ethanol. After centrifugation at 12,000 *g* for 15 min, the precipitated protein was air dried and resuspended in the sample buffer (Laemmli, 1970), and separated by sodium dodecyl sulphate gel electrophoresis on 16% (w/v) polyacrylamide gels (SDS-PAGE). The gel was stained with the colloidal Coomassie G-250 blue silver method (Candiano *et al.*, 2004).

To compare the protein patterns of healthy and infected berries, 10 healthy and 10 infected berries were crushed, and the juice was centrifuged, filtered, and passed through a PD-10 column as described above. A volume of extract of healthy berries containing 5 µg of protein and an identical volume from the infected berries were precipitated and analyzed by SDS-PAGE, as described above.

2.3. Grape polyphenols extraction and analysis

Once crushed and divested of seeds, the solid grape residue was mixed with potassium metabisulphite (0.5 g kg-1 of fresh grape) and stored at -20°C. Polyphenols were extracted by the method of Kammerer *et al.* (2004) with some modifications. A total of 50 g of the stored material was stirred with four volumes of methanol/0.1% HCl (v/v) for 2 h under nitrogen at room temperature. The extract was filtered through filter paper and vacuum dried using a Rotavapor at 30°C. The residue was dissolved in 100 ml of acidified methanol, centrifuged at 8,600.*g* for 20 min, and dried again. The residue was dissolved in 40 ml of deionized water brought to pH 3.5 with HCl. The aqueous suspension was centrifuged at 8,600.*g* for 15 min.

The supernatant was filtered through 0.45 μm membranes, and 10 ml aliquots were loaded onto SPE DSC- 18/6 ml tubes (Supelco, USA) equilibrated with deionized water. After washing with 5% methanol in water, the phenols were eluted with absolute methanol and concentrated using the Rotavapor, as described above. The residue was dissolved in 2 ml of deionized water, and the phenol concentration was determined by the Folin-Ciocalteau assay, using gallic acid as a standard.

About 500 μg of phenols per gram of grape fresh weight were obtained, and stored at -20 °C until required.

2.4. Production of *Botrytis cinerea* enzymes

2.4.1. *B. cinerea* laccase production, purification, and assay.

B. cinerea laccase was extracted as described by Slomczynski *et al.* (1995). After culturing for 7 days, the 50-ml contents of several 250 ml Erlenmeyer flasks, inoculated with *B. cinerea* mycelia disks, were pooled and filtered through GMD and then 0.8 and 0.45 μm cellulose acetate membranes (Sartorius, Germany).

The filtrates were dialyzed against deionized water, concentrated to 40 ml using a VivaFlow 5000 apparatus, adjusted to pH 6.0 with 10 mM potassium tartrate, and loaded onto a Q-sepharose column (16.120 mm, GE Healthcare, UK). Bound protein was eluted

with a 60 min linear gradient of 0-0.5 M NaCl dissolved in 10 mM Potassium tartrate buffer.

Laccase activity was assayed in the eluted fractions (2 ml), and the fraction with the highest activity was used in the following assays. This fraction showed a 96 kDa band when analyzed by SDS-PAGE (Fig. 4A, lane 1), similar in size to the protein purified by Slomczynski *et al.* (1995).

Laccase activity was determined spectrophotometrically as described by Wolfenden and Willson (1982) in a volume containing 0.7 ml of 0.1 M acetate buffer, 0.1 ml of 2 mM 2,2'-azinobis-(3-ethylbenzthiazoline-6-sulphonate) (ABTS) substrate, and variable volumes of samples (1-30 µl). The assay was performed at 30°C by monitoring the A420. One laccase unit (U) was defined as the amount of enzyme that oxidizes 1 µmol ABTS per min.

2.4.2. Production of proteases, purification and assays

B. cinerea extracellular proteases were induced in liquid mineral cultures in a modified Czapek-Dox medium (as described before) containing casein (5.0 g/l) as sole nitrogen source and sterilized by autoclaving. All cultures were maintained at 24 °C on a rotary shaker (120 rpm) for 7 days. Protease activity was surveyed from day 3 to 7. After culturing for 7 days, the 50-ml contents of several 250 ml Erlenmeyer flasks were pooled and filtered through GMD and then 0.8 and 0.45 µm cellulose acetate membranes (Sartorius, Germany). The filtrates were dialyzed (12-kDa cut-off) against deionized water overnight at 4 °C. After that protease broth was 10 times concentrated to 30 ml using in VivaFlow 10000 apparatus (Sartorius, Germany) and loaded on a sephadex G-25 column (PD-10, GE Healthcare, UK). The protease was eluted with sterile water and again concentrated with a VivaFlow 10'000 apparatus. Concentrated protease was then adjusted to pH with 10 mM potassium tartrate and connected to an AKTA purifier system (GE, Helthcare, UK) loaded onto a resource Q column (16.120 mm, GE Healthcare, UK). Bound protein was eluted with a 60 min linear gradient of 0.5 M NaCl dissolved in 10 mM Potassium tartrate buffer.

Proteolytic activity was assayed either in crude supernatant and in the eluted protease fractions (1 ml). Protease was mixed with 450 µl of Haemoglobin (1%) and incubated for 1 hour at 37 °C, next reaction was stopped by addition of same volume of TCA (20 %) and

centrifuged at 10 000 rpm. Supernatant was mixed with an equal volume of NaOH 500 mM. Protease activity was then determined spectrometrically (Spectronic Genesys 10 Bio, USA) at 280 nm. The increase of Absorbance was determined by subtracting the blank from the sample values. Each assay was performed in triplicate and values represent the mean of three replicates. The fraction with the highest activity was used in the following assays.

For the analysis of protease profile, aliquost of protease containing 0.02 and 0.04 U/ml activity were mixed with the samples buffer buffer (Laemli, 1970) and analyzed by SDS-PAGE.

2.5. Inoculation of *B. cinerea* grape berries

For inoculation, the berries were detached from the bunch and surface-sterilized with 99 % ethanol for 1 min, then rinsed with sterile water. A 3.2 mm piece of PDA, cut from the margin of an actively growing fungal colony, was placed on a small wound created on the berry surface with a razor blade. The berries were placed on a moist filter paper and closed in a plastic bag at about 22 °C. After 7 days, the inoculated berries appeared extensively brownish, and were stored at -20 °C until extracted. Berries inoculated with non-colonized PDA were incubated in a similar way and used as the control. Healthy berries and naturally infected ones with grey mould symptoms were harvested from the vineyard at the beginning of November 2008.

2.6. Interaction of grape proteins, grape polyphenols, resveratrol in *B. cinerea* culture.

Two milliliters of aliquots of a modified Czapek-Dox medium (as described above) were plated onto Petri dishes (3 cm diameter) and diluted 1:2 with filter-sterilized stock solutions of grape proteins, grape polyphenols, resveratrol (Sigma-Aldrich, USA. Purity >99%), and/or water, and with the concentrated spore suspension to obtain a final concentration of 10^4 conidia ml^{-1}. Absence and presence of each factor (proteins, polyphenols, and resveratrol) were compared in a factorial design. Proteins, polyphenols, and resveratrol

were supplied at 100 µg ml^{-1}, 200 µg ml^{-1}, and 200 µg ml^{-1}, respectively. The resveratrol stock solution (25 mg ml^{-1}) was in 95% ethanol, and the same amount of ethanol (0.8%, v/v) was added to the cultures not treated with resveratrol. Crystals formed when resveratrol was added at the beginning of the experiment, but the compound dissolved during the course of culturing. A higher concentration of ethanol (4%, v/v) has been recommended to increase the solubility of resveratrol (Adrian *et al.*, 1998), but was not used because this concentration reduced growth of the fungus. Each treatment was replicated three times. The cultures were maintained in the dark at 24°C for 4 days. Aliquots of 100 µl were harvested daily from each culture to assess laccase activity and the protein pattern. At the end of the experiment, the content of each culture medium was transferred into 5-ml pre-weighed tubes and centrifuged at 12'000 *g* for 30 min. In addition, each plate was rinsed with 3 ml of water, which was then added to each tube, and the mycelium mat was briefly vortexed and centrifuged again. The supernatant was discarded, and the tubes were oven dried at 80 °C for 3 days and then weighed.

2.7. *In vitro* interaction of grape proteins, grape polyphenols and *B. cinerea* laccase

Grape proteins, grape polyphenols, and resveratrol were mixed in the same weight ratio of 1:2:2 as used for the *B. cinerea* cultures. Grape proteins (50 µg ml^{-1}), resveratrol (100 µg ml^{-1}), and polyphenols (100 µg ml^{-1}) were dissolved in 0.1 M potassium tartrate buffer pH 3.5 in the presence or absence of purified laccase (0.02 U ml^{-1}). After 24 h of incubation at 24°C, 100 µl aliquots of the mixtures were centrifuged at 16'000 *g* for 20 min, and the pellet containing the precipitated protein was washed once with cold 70% ethanol. The protein in the supernatant (soluble protein) was precipitated with 4 volumes of cold ethanol at -20°C for 2 h and then washed once with cold 70% ethanol. Both soluble and insoluble proteins were recovered by centrifugation and air dried. Proteins were run on SDS-PAGE gels and stained as described above.

Statistical analysis. Data for laccase activity and mycelium dry matter were subjected to analysis of variance according to a multifactorial design with three replicates.

2.8. Effect of resveratrol, polyphenols and grape proteins and/or Laccase in *B. cinerea* spore germination.

B. cinerea spores germination was assayed in eppendorf tubes in a volume of 400 µl with $1x10^4$ spore concentration. Grape polyphenols (100 µg ml^{-1}) and resveratrol (100 µg.ml^{-1}) were transferred to the eppendorf tubes contained 10 mM potassium tartrate buffer at pH 3.5 and grape polyphenols 100 mg.$µl^{-1}$ or/and resveratrol (100 µg ml^{-1}) treatment were also performed by pre-incubating the mixtures with *B. cinerea* laccase (0.02 AU ml^{-1}) before the addition of B. cinerea spores. Conidial germination was monitored by microscope at 1 h interval until 16 h. Conidia were considered to have germinated when germ tube length was equal to or greater than conidial diameter. Each treatment was done in triplicate.

2.9. Digestion of grape proteins with *B. cinerea* proteases

Grape protein (50.0 µg ml^{-1}) was firstly diluted in sodium succinate buffer (20 mM, pH 3.5) and sterile water. Later on Botrytis protease (0,02 U ml^{-1}) was added into respective tubes (see table), mixed well and incubated at 24°C for 72 hours. Aliquots of 100 µl were harvested at 24, 48 and 72 hours respectively from each treatment to assess the protein pattern.

2.10. Evaluation of effect of *B. cinerea* protease in the insolubility of the grape proteins.

Grape proteins (50 µg ml^{-1}), resveratrol (100 µg ml^{-1}), polyphenols (100 µg ml^{-1}) were dissolved in 10 mM potassium tartrate buffer pH 3.5 in the presence or absence of purified laccase (0.02 U ml^{-1}) and/or protease (0.01 U ml^{-1}) (See table). After 24 h of incubation at 24°C, 100 µl aliquots of each treatment were centrifuged at 16,000 *g* for 20 min, and the pellet containing the precipitated protein was washed once with cold 70% ethanol. The protein in the supernatant (soluble protein) was precipitated with 4 volumes of cold ethanol at -20°C for 2 h and then washed once with cold 70% ethanol. Both soluble and insoluble

proteins were recovered by centrifugation and air dried. Proteins were run on SDS-PAGE gels and stained as described above.

Table 2 - Evaluation of the effect of *B. cinerea* protease in the solubility of the grape proteins

		Grape protein **mg ml^{-1}**	**Grape Polyphenols** **mg ml^{-1}**	**Laccase** **U ml^{-1}**	**Protease** **U ml^{-1}**	**Potassium Tartrate** **mM**	**Sodium Succinate** **mM**
Stock		**300**	**32**	**0.5**	**0.7**	**20**	**20**
Treatments	(1)	-----	-----	-----	-----	10	10
	(2)	-----	-----	-----	0.02	10	10
	(3)	50.0	100	-----	-----	10	10
	(4)	50.0	100		0.02	10	10
	(5)	50.0	100	0.02	0.02	-----	10
	(6)	50.0	100	0.02	-----	-----	10

2.11. Expression analysis of *B. cinerea* tripeptidyl peptidase and aspartyl proteinase members.

2.11.1. RNA Extraction

RNA was extracted from *B. cinerea* mycelium grown for 72 h in the modified Czapek-Dox medium (described above) strain PM10 were inoculated on 25 ml of modified Czapek-Dox medium (described before) supplemented with grape proteins (10 µg ml^{-1}) or casein (5 mg ml^{-1}). Cultures were performed with $1x10^4$ spore ml^{-1} in an orbital shaker (120 g). Mycelia were harvested by centrifugation (10'000 g, 10 min, 4° C) and the resulting pellet was washed twice with sterile water, centrifuged again and immediately frozen with liquid nitrogen and stored at -80 °C.

Total RNA was extracted according to Foissac *et al*. (2001). *B. cinerea* mycelia were grounded in liquid nitrogen to a fine powder and 1 ml extraction buffer [6 M Guanidine thiocyanate, 0.2 M Sodium acetate (pH 5.2), 1 M Potassium acetate, 25 mM EDTA, 2.5% PVP-40 and 1% Sodium metabisulphite] was added. After centrifugation at 16 000 g for 10 min, 300 µl of the supernatant were transferred to a new tube with addition of 150 µl phenol and 150 µl chloroform and again centrifuged as before. The obtained supernatant was then added to 300 µl NaI (6 M Sodium iodide and 0.15 M sodium sulphite), 150 µl ethanol and 50 µl silica particles suspension (1 g ml^{-1}, pH 2.0). After centrifugation at 6000 rpm for 1 min, the supernatant was discarded and the pellet was washed with washing buffer [10 mM Tris-HCl (pH 7.5) , 0.05 mM EDTA, 50 mM NaCl and 50% ethanol]. The pellet was then resuspended with 150 µl of RNase free water and incubated for 4 min at 70°C and then centrifuged at 13 000 g for 3 min. RNA extracted was treated with 1 U of RQ1 DNase (Promega) and incubated at 37 °C for 20 min. Finally RNA was stored at -80°C.

The amount and purity of the purified RNA was checked by using the spectrophotometer and then by loading an amount of RNA on an agarose gel.

Table 3 – Total RNA extracted and used for the cDNA production

RNA Samples	RNA yield (µg/ml)	OD260 / OD280
Control	1286,7	2,61
Protein (10 µg/ml)	1000,0	3,95
Casein (5,0 µg/ml)	0713,4	3,96

The quality as well as the size of the RNA was checked also by agarose gel electrophoresis in 1 X TAE (Tris-Acetate-EDTA). One µg of each RNA sample was mixed with sterile loading buffer (50% glycerol, 1mM EDTA and 0.4% bromophenol blue). Before loading RNA samples were denatured by heating at 65 °C for 5 min. Electrophoresis was performed at 90 V. RNA was visualized by adding 100 ng of ethidium bromide to each sample.

2.11.2. Reverse transcription

RNA previously treated with DNase was used for first strand cDNA synthesis by using the Improm-II Reverse Transcriptase (Promega) according to the manufacturer's guide. one microliter of RNA was used as template. Five hundred nanogram of oligo dT were added to the RNA template and the mixtures were kept at 70 °C for 5 min. After 5 min of incubation on ice, 5 µof this mixture were added to 15 µl of the RT mixture containing reaction buffer, $MgCl_2$, dNTP, and RT as reported below (Table 9).

Table 4 - Reverse transcription mix

Reagents	Final concentration	Volume
ImProm-II™ 5X Reaction Buffer	1 X	4,0 µl
$MgCl_2$, 25 Mm	3 mM	2,4 µl
dNTP mix 10 mM	0,5 mM	1,0 µl
Improm-II™ Reverse Transcriptase	1 U	1,0 µl
Nuclease free water		1,6 µl
Final volume RT Mix per 20 µl reaction		20,0 µl

First strand cDNA synthesis was obtained by using the following conditions: Annealing at 25 °C for 5 min, Extension at 42 °C for 60 min and heat-inactivation at 70 °C for 15 min.

2.11.3. Oligonucleotide primers design

Seven genes of the tripeptidyl peptidase family and fine genes belonging to the aspartyl proteinases family were selected from the *B. cinerea* genome database (http://www.broadinstitute.org).

These genes encoding proteases were selected according to their expression in the extracellular matrix location, using the TargetP1.1 server (http://www.cbs.dtu.dk) and Psort program (http://www.psort.org).

Gene sequences were aligned using the CLUSTAL W program (Thompson *et al*., 1994; http:// www.pasteur.fr/) and conserved regions were identified. Specific primers were then designed on the coding sequence excluding conserved gene regions among genes. The primer sequences are reported in the tables below:

Table 5 - Gene-specific primers for tripeptidyl peptidase encoding genes.

Gene code	Sense	Primer Sequence 5' – 3'	Tm	T ann	Product (bp)
BC1G_00978	FOR	TACTCCACCGTCAAACACCA	60.00	57.00	630
	REV	CATGTGCCATTGTCTCCAAC	60.00	57.00	
BC1G_01073	FOR	AGGTCTCACGCAACAGAACC	60.31	61.00	419
	REV	CCTGCTTCACCTGACTCCTC	59.99	61.00	
BC1G_01803	FOR	GCTTCCAACAGATGCTCCTT	59.97	55.00	546
	REV	ACGGTGACACCTTGAAGACC	60.01	55.00	
BC1G_02944	FOR	GGGTTTGTACGAGGGTGCTA	59.99	57.00	637
	REV	CAAGGGAACGGTGTCTTCAT	59.97	57.00	
BC1G_12776	FOR	TCCAGAATGGTCTTTGCCTC	60.20	59.00	525
	REV	CCTCCGGTTGTGTAAGCAAT	59.99	59.00	
BC1G_14820	FOR	AAGAGGTTGCCACAACCAAC	60.01	57.00	543
	REV	AGGATGGGATGGGGAAATAG	59.97	57.00	

Table 6 - Gene-specific primers for aspartyl proteinase encoding genes.

Gene code	Sense	Primer Sequence 5' – 3'	Tm	T ann	Product (bp)
BCAP5	FOR	TGTCGCTTTGGTCACTTCTG	60.00	57.00	433
	REV	AGCATTGGCCATCTTGGTAG	60.00	57.00	
BCAP7	FOR	CTCAGGCTTCAAGCGTATCC	60.00	57.00	714
	REV	CATGGCACAGAGTAGGCAAA	60.00	57.00	
BCAP9	FOR	CAGGGTGCTACTTGGTCCAT	60.00	57.00	630
	REV	ATCAGAACCACGGACGGTAG	60.00	57.00	
BCAP6	FOR	CAGGCTCTTTTGCTTCCAAC	60.00	57.00	426
	REV	GCCCATTGTTGTTTTCGAGT	60.00	57.00	

2.11.4. RT-PCR and PCR conditions

After reverse transcription the cDNA obtained was used for the PCR of the genes of interest. The following PCR mixtures and conditions were used.

Table 7 - PCR reaction mixture

cDNA	Master-mix (2x)	REV (1 µM)	FOR (1µM)	H_2O
3,0 µl	12,5 µl	1,6 µl	1,6 µl	6,3 µl

Table 8 - Amplification conditions

T (°C)	95	94	See table 5-6	72	72
Time (minute)	3'	30"	30"	60"	10'
		32 cycles			

The amplified products obtained by PCR were separated on a 1% agarose gel containing ethidium bromide (0.6 µl ml^{-1}). DNA bands were visualized by U.V. light.

3. RESULTS

3.1. Protein pattern in grapes infected by *B. cinerea*.

Mature grape was inoculated with *B. cinerea* mycelium and changes in the protein pattern were analysed after 7 days, infection with the fungus.

Grape berries showing grey mould disease symptoms in the vineyard were also analyzed. Compared with healthy grapes which displays three many protein bands of 25, 31 and 66 kDa respectively (Fig. 4), both artificially or naturally infected berries did not show the 31 kDa band and the 25 kDa band showed strongly reduced intensity (Fig. 4A, lane 2 and Fig. 4B, lane 3). A new band at about 45 kDa was observed in the healthy control berries harvested from the field (Fig. 4B, lane 2). This band was not visible in the control grapes used for artificial inoculation, and is possibly linked to the different harvesting dates of the two control samples.

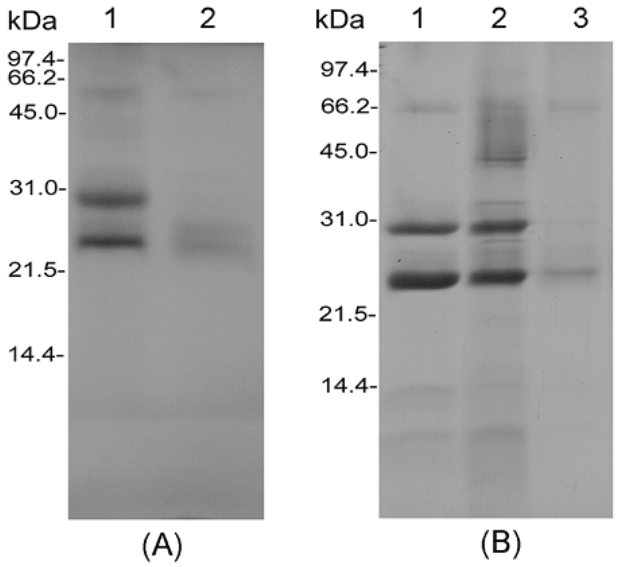


Figure 4 - SDS-PAGE of protein extracted from grape berries either healthy or infected with *B. cinerea*. A volume of extract of healthy berries containing 5 µg of protein and an identical volume from the infected berries were precipitated with four volumes of cold ethanol and analyzed. (A) Protein extracted from healthy (lane 1) and artificially infected berries (lane 2) at 7 days from inoculation. (B) Protein extracted from healthy (lane 2) and infected berries (lane 3) harvested from a vineyard; a sample (approximately 5 µg) of the partially purified grape protein used in the experiment reported in Fig. 9 was also loaded (lane 1).

3.2. Effects of resveratrol, grape proteins, and polyphenols on *B. cinerea* laccase activity and growth.

B. cinerea was grown on a Czapek-Dox mineral medium supplemented with two concentrations each of resveratrol (0 and 200 μg ml^{-1}), grape polyphenols (0 and 200 μg ml^{-1}), and grape proteins (0 and 100 μg ml^{-1}) in a factorial design. Two experiments were performed successively using different preparations of polyphenols, proteins, and spores. Laccase activity was monitored daily from 1 to 4 days of culture. Some results for laccase activity were similar between the two experiments: levels were negligible in all treatments after one day of culture with the highest activity detected on subsequent days when resveratrol was combined with polyphenols or with both polyphenols and proteins (Fig. 5). Finally, activity was lower when polyphenols and proteins were supplied without resveratrol, and activity was very low in cultures supplied with resveratrol only (Fig. 5). Comparing the two experiments, the major difference was that activity in the first experiment increased earlier and was much higher at day 4 in the three-factor treatment (polyphenols, protein, and resveratrol together). In addition, in the second experiment, higher laccase activity was detected at day 4 in the cultures treated with proteins or polyphenols and in untreated controls (Fig. 5). The main effects and the first- and second-order interactions of the three factors were significant at $P<0.01$ for all data points except in the first experiment; in that experiment, the interaction of proteins x polyphenols and the second-order interaction were not significant at day 3 of culture. During the course of the experiments, the cultures containing polyphenols alone or in combination with proteins and resveratrol became brownish.

Mycelia were collected and dried at day 4 of culture. In both experiments, the dry weights were higher than in untreated controls (zero level of each factor) when proteins were supplied alone, in combination with polyphenols, or with both polyphenols and resveratrol (Fig. 6). A few sparse mycelium flakes were observed in the culture treated with resveratrol alone (7B), and the mycelium dry weight was almost negligible (fig. 6). Mycelium growth was low when resveratrol was supplied with proteins, and the detrimental effect of resveratrol on mycelium growth was completely abolished when polyphenols were present (Fig. 6). The main effects of the three factors and first-order interactions of protein x resveratrol and polyphenols x resveratrol were significant at $P<0.01$. Overall, the growth

data indicated that grape polyphenols abolish the toxicity of resveratrol and that grape proteins promote fungal growth but mostly in the absence of resveratrol.

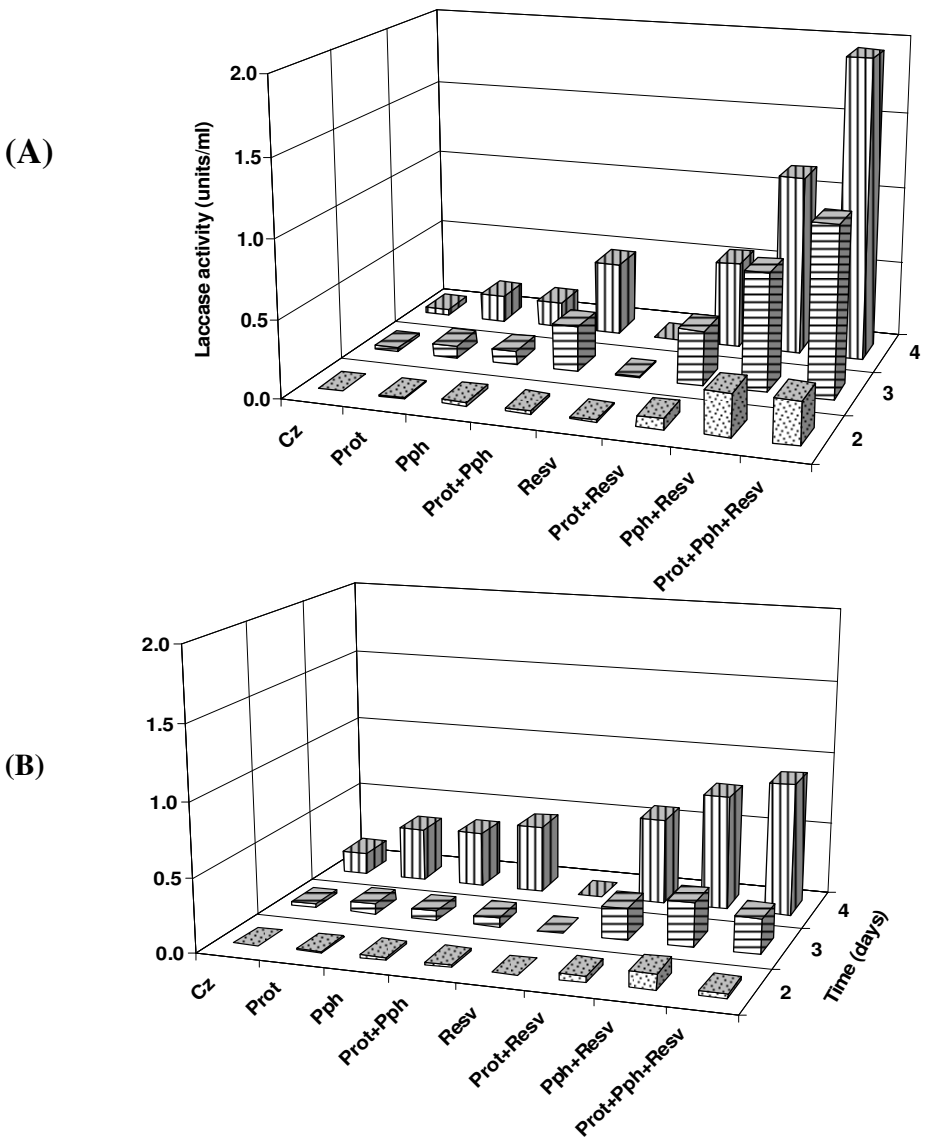
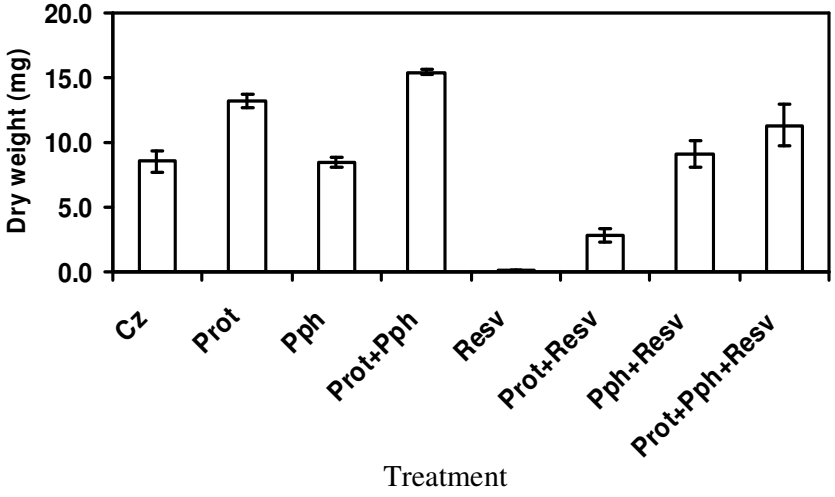


Figure 5 - Laccase activity detected in *B. cinerea* cultures at days 2, 3, and 4 after spore inoculation (104 spores ml-1). In a factorial design, the 4-ml cultures were incubated in Czapek-Dox medium at pH 3.5 and supplied without (Cz) or with different combinations of grape proteins (Prot), grape polyphenols (Pph), and resveratrol (Resv) at 100 µg ml-1, 200 µg ml-1, and 200 µg ml-1, respectively. At each data point, 1–30 µl aliquots of each culture were assayed for laccase activity. Data from two experiments performed with different batches of proteins, polyphenols, and spore preparations are presented (A and B). Each data point was obtained from three replicated cultures. The main effects and the first- and second-order interactions of the three factors were significant at $P<0.01$ at all data points except in experiment (A), in which the interaction of proteins x polyphenols and the second order interaction were not significant at day 3 of the culture.

(A)



(B)

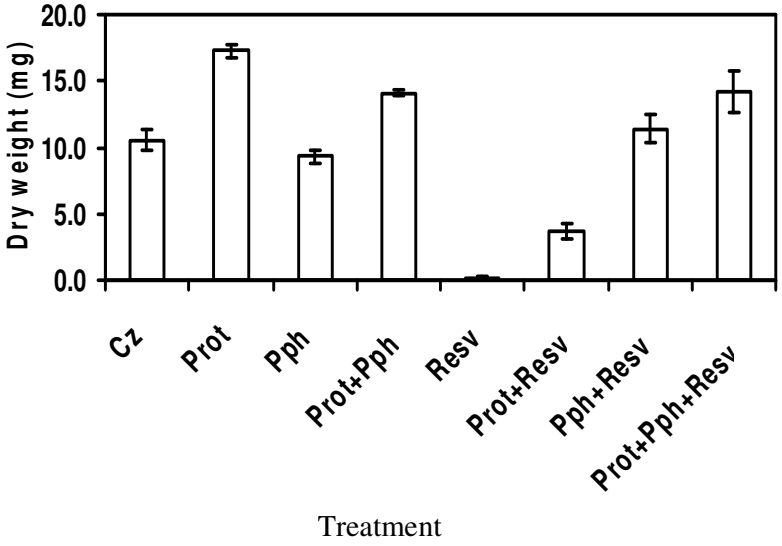
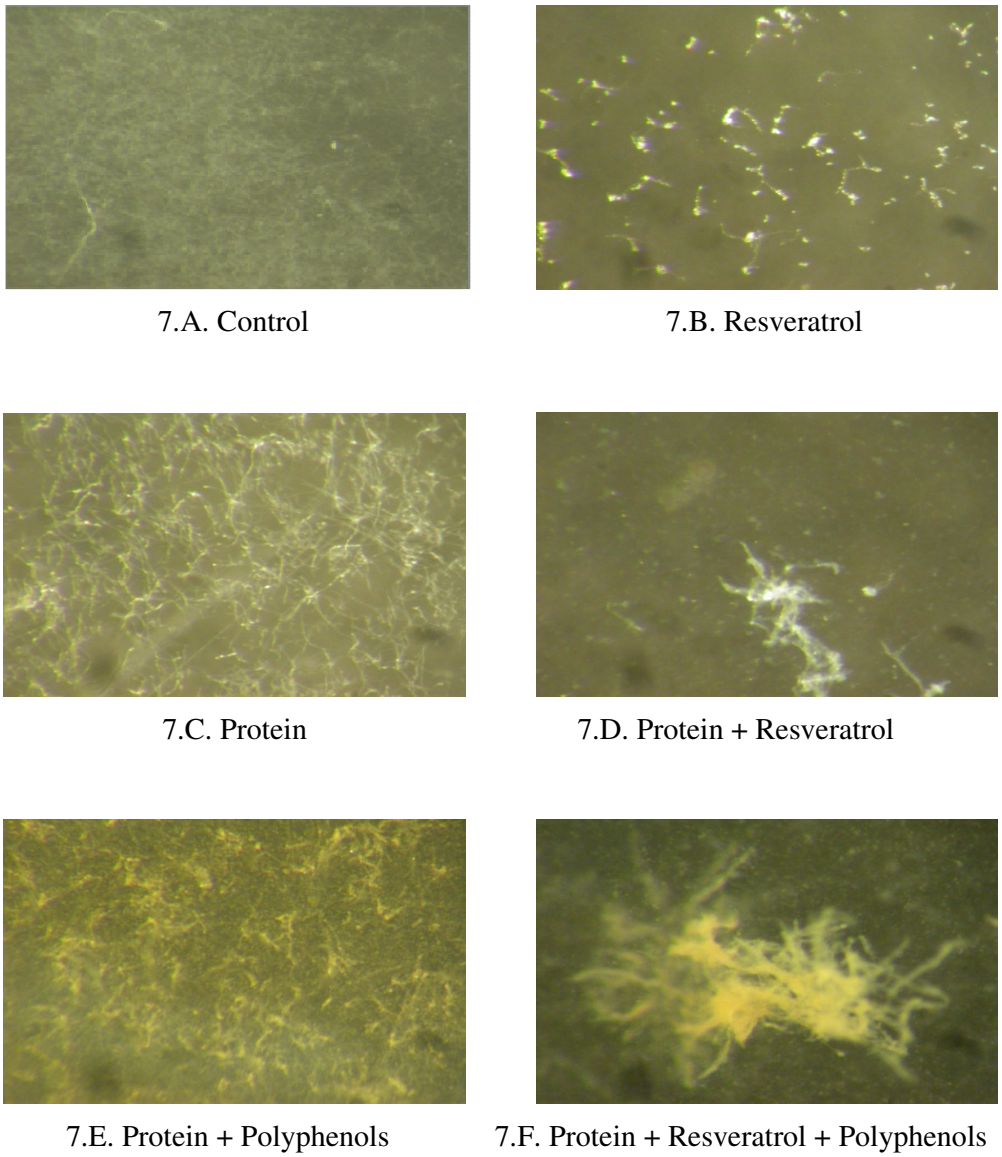


Figure 6 - *B. cinerea* dry weight harvested at day 4 after spore inoculation (10^4 spores ml^{-1}) from the two experiments (A and B) described in Fig. 2. In a factorial design, the cultures were incubated in 4 ml Czapek-Dox medium at pH 3.5 supplied without (Cz) or with different combinations of grape proteins (Prot), grape polyphenols (Pph), and resveratrol (Resv) at 100 µg ml^{-1}, 200 µg ml^{-1}, and 200 µg ml^{-1}, respectively. Each data point is the mean ±SD of three replicated cultures.

Following the treatments some changes in the morphology of colonies were observed. In particular, in comparison to the untreated control, proteins induced aggregation of hyphae (Fig. 7). The simultaneous presence of protein and resveratrol induced the formation of large mass of mycelium (Fig. 7).



7.A. Control 7.B. Resveratrol

7.C. Protein 7.D. Protein + Resveratrol

7.E. Protein + Polyphenols 7.F. Protein + Resveratrol + Polyphenols

Figure 7 – Morphology of *B. cinerea* mycelia after 4 days of colture in 4 ml Czapek-Dox medium at pH 3.5 supplied with different combinations of grape proteins, grape polyphenols, and resveratrol at 100 μg ml^{-1}, 200 μg ml^{-1}, and 200 μg ml^{-1}, respectively.

3.3. Alteration of grape protein in *B. cinerea* cultures.

Estimation of the total grape protein present in the cultures during fungal growth was impaired because polyphenols and resveratrol interfere with standard protein assays. Therefore, aliquots of the *B. cinerea* cultures containing the grape proteins were analyzed by SDS-PAGE. Control samples incubated without the fungus showed the typical grape protein pattern of three main broad bands at about 25, 31, and 66 kDa and a few bands of minor intensity (Fig. 8, controls). The pattern changed with the type of treatment in the *B. cinerea* cultures. In the culture treated with protein only, the intensity of the band at 31 kDa gradually attenuated and a new band of lower size was formed. This band is probably the degradation product of the 31 kDa protein, which finally, after 4 days, localized at about 27 kDa (Fig. 8A). A similar pattern was obtained in the culture with resveratrol, except that the 25 kDa band was also less intense at the end of the culture, and the 66 kDa band disappeared (Fig. 8B). In the culture supplied with grape polyphenols, the 31 kDa band gradually disappeared and the 25 kDa band faded, becoming poorly visible by the end of the culture period (Fig. 8C). Protein bands almost completely disappeared in the culture containing both polyphenols and resveratrol (Fig. 8D).

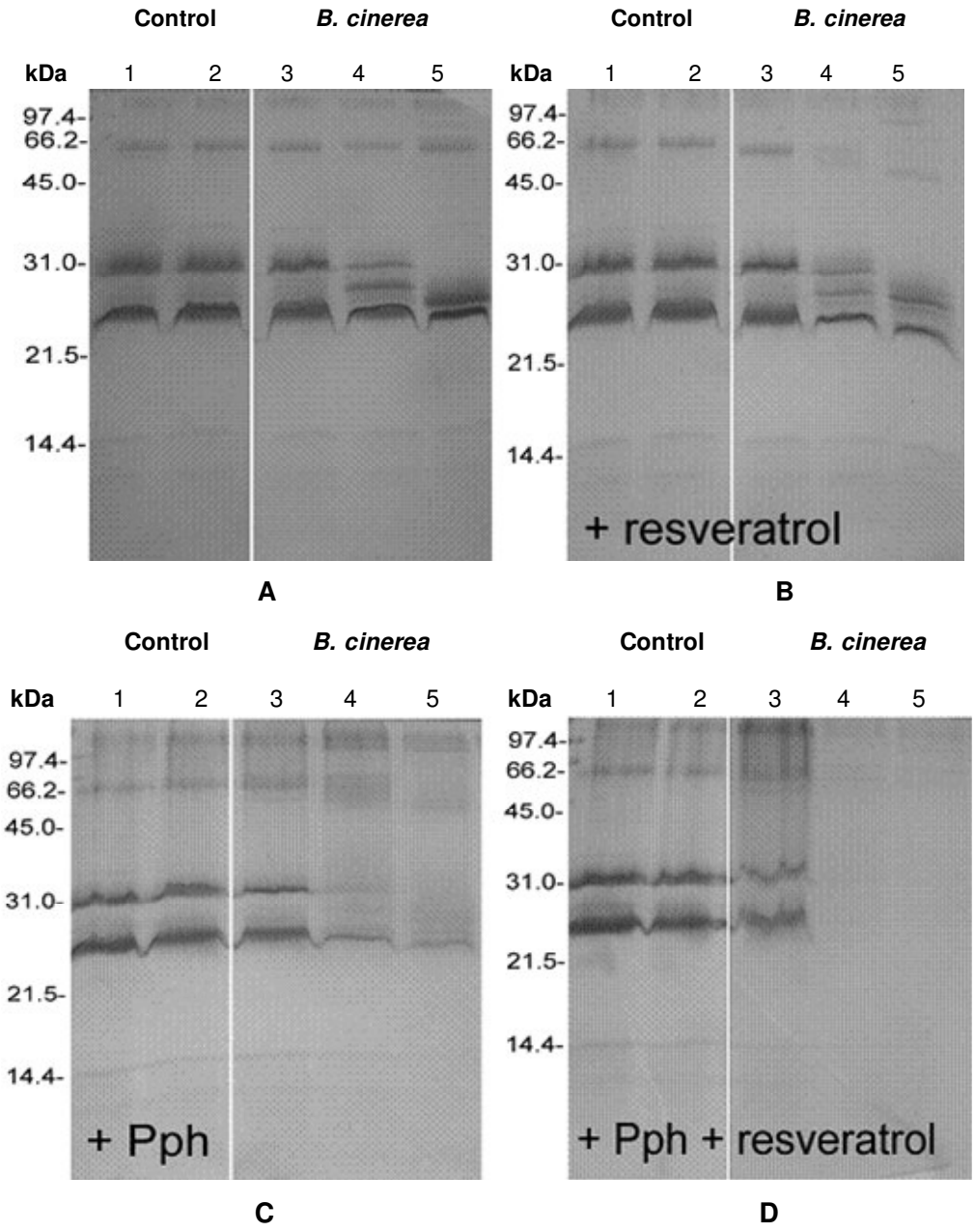
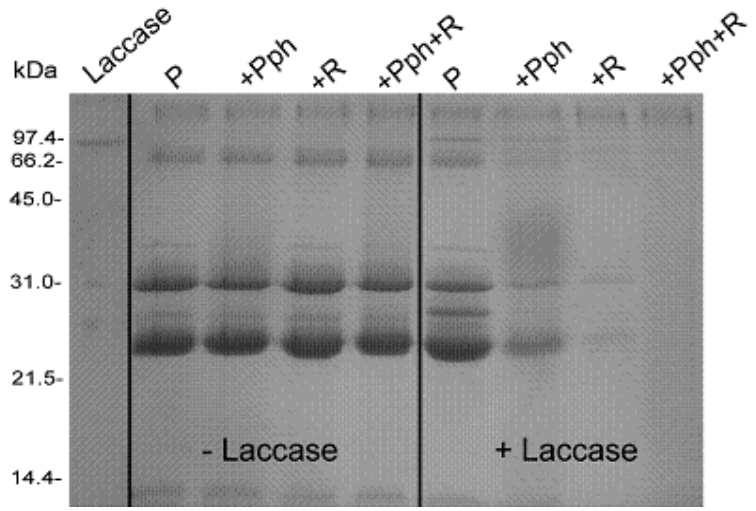


Figure 8 - SDS-PAGE patterns of proteins collected from the *B. cinerea* cultures described in Fig. 6A. Only the cultures supplied with the grape proteins (100 µg ml^{-1}) alone (A) or in combination with resveratrol (B) or grape polyphenols (Pph) (C), or both resveratrol and Pph (D) were analyzed. Samples of 40 µl of inoculated cultures collected at 1, 2, and 4 days (lanes 3, 4 and 5, respectively), and of culture media, incubated without the fungus, collected at 1 and 4 days (lanes 1 and 2, respectively), were centrifuged, precipitated and analyzed as reported in Materials and Methods. The gels were stained with the Coomassie G-250. The low resolution of several bands was probably the result of salts in the medium contaminating the proteins during ethanol precipitation.

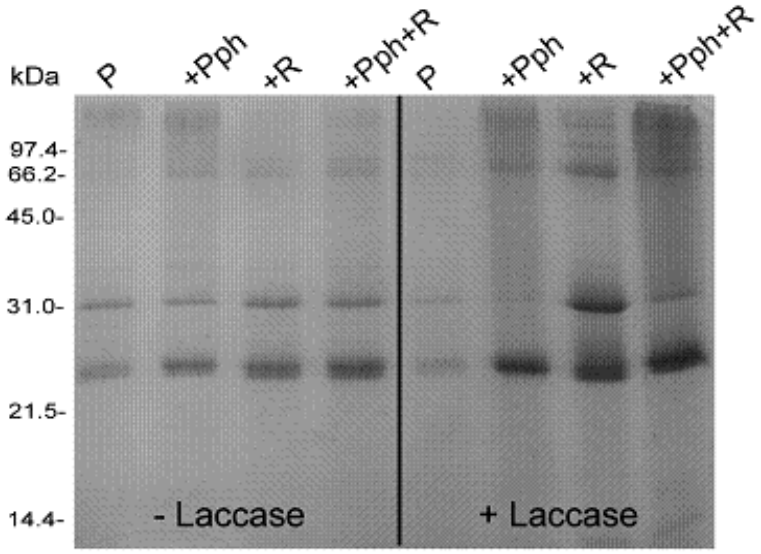
3.4. Alteration of grape protein pattern by *B. cinerea* laccase.

From the above results, it seems that polyphenols and resveratrol alter the grape protein pattern, mostly when both compounds are added concurrently to the culture. Strong laccase activity was also induced in these cultures. To establish whether *B. cinerea* laccase can mimic these protein alterations, incubations were performed by mixing together proteins with polyphenols and/or resveratrol and the fungal laccase. A low laccase concentration (0.02 U ml^{-1}) was used that was less than or comparable to the values measured at the second day of the *B. cinerea* cultures when proteins were mixed with polyphenols and/or resveratrol.

After 24 h incubation, these mixtures were centrifuged to separate soluble and insoluble components which were analyzed by SDS-PAGE (Fig. 9). In the supernatants of samples treated with laccase, the major proteins bands became much less intense in the mixture with polyphenols, became weakly visible in the mixtures containing resveratrol, and were undetectable in the sample with resveratrol and polyphenols (Fig. 9A). These proteins were at least partially recovered from the precipitated materials (Fig. 9B). However, it is worth noting that in the mixture containing polyphenols, the 31 kDa band, which was weak in the soluble fraction (Fig. 9A), was almost undetectable in the insoluble fraction (Fig. 9B). In addition, in the mixture with polyphenols and resveratrol, the 31 kDa band, which had disappeared from the soluble fraction (Fig. 9A), was strongly reduced in the insoluble fraction (Fig. 9B); a smear of protein was clearly visible above 66.2 kDa, probably indicating the formation of high molecular weight aggregates (Fig. 9B). Controls without laccase or with protein plus laccase showed that most protein remained in solution (Fig. 9A). In the control with protein and laccase, a band at 28 kDa was more intense, possibly as a consequence of proteolytic activity in the laccase preparation (Fig. 9A).



(A)



(B)

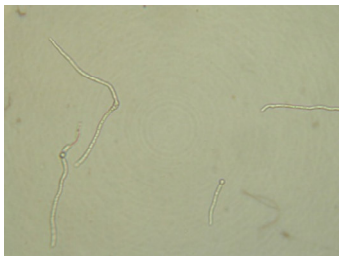
Figure 9 - SDS-PAGE of grape proteins (P) incubated with 0.02 U ml^{-1} of *B. cinerea* laccase activity and with grape polyphenols (Pph), resveratrol (R), or polyphenols plus resveratrol (Pph+R). Grape proteins, grape polyphenols, and resveratrol were supplied at 50, 100, and 100 µg ml-1, respectively (1:2:2 ratio). After a 24-h incubation, 100 µl of each mixture was centrifuged to separate the soluble proteins in the supernatant (A) from the insoluble ones in the precipitate (B). The soluble protein was recovered from the supernatant after precipitation with 4 volumes of cold ethanol. The laccase preparation used in the experiment was also loaded onto the gel (A, first lane from the left). The gels were stained with the Coomassie G-250 as reported in "Materials and Methods".

3.5. Effect of laccase, resveratrol and polyphenols on *B. cinerea* spore germination.

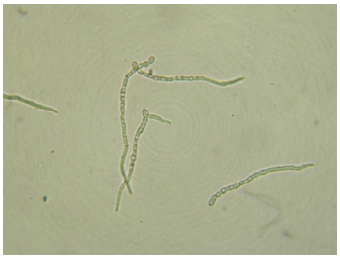
As shown above polyphenols abolish the toxic effect of resveratrol on *B. cinerea* mycelium with a possible involvement of the fungal laccase. The effect of resveratrol and polyphenols was also analyzed on *B. cinerea* spore germination. Germination was also analyzed after pre-treatment of resveratrol and polyphenols with the *B. cinerea* laccase.

Results showed that resveratrol alone does not inhibit the spore germination (Fig. 10.A). Instead, the pre-incubation of resveratrol with laccase completely inhibited the spore germination (Fig. 10.D). This inhibition effect was relieved when resveratrol was pre-incubated with grape polyphenols and laccase before the addition of the fungal spores (Fig. 10.C and 10E). Thus resveratrol does not prevent the spore germination and the toxic effect occurs later during the mycelium development (7B).

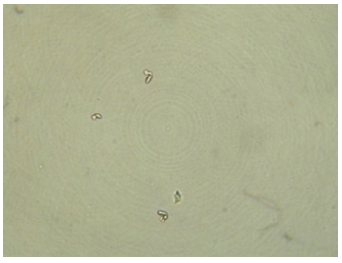
10A. Resveratrol



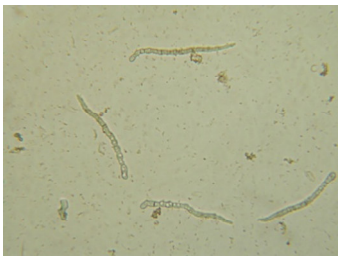
10B.Untreated(Control)



10C. Polyphenols + Resveratrol



10D. Resveratrol + Laccase



10E. Polyphenol+ Resveratrol+Laccase

Figure 10 - Effect of resveratrol and polyphenols on *B. cinerea* spore germination with or without pre-incubation with laccase. In the sample containing laccase pre-incubation lasted 5 h before the addition of *B. cinerea* spores. Spore at concentration of 10^4 spores ml^{-1}. Germination was observed after 16 h of incubation. Observations were made with magnification (100x) using a light microscope.

3.6. Purification of *B. cinerea* protease activities

Above results suggested that *B. cinerea* protease are possibly involved in the degradation of grape proteins. To ascertain the ability of *B. cinerea* proteases to digest the grape protein we first purified the protease activity from the culture. To this aim *B. cinerea* was grown in a modified Czapek-Dox medium supplemented with casein (5.0 g l^{-1}). The obtained protease activity was purified by FPLC using a resource S column. Most protease activity was collected in correspondence of the highest protein peak (fractions 29, 30, 31), (fig. 11 and 12).

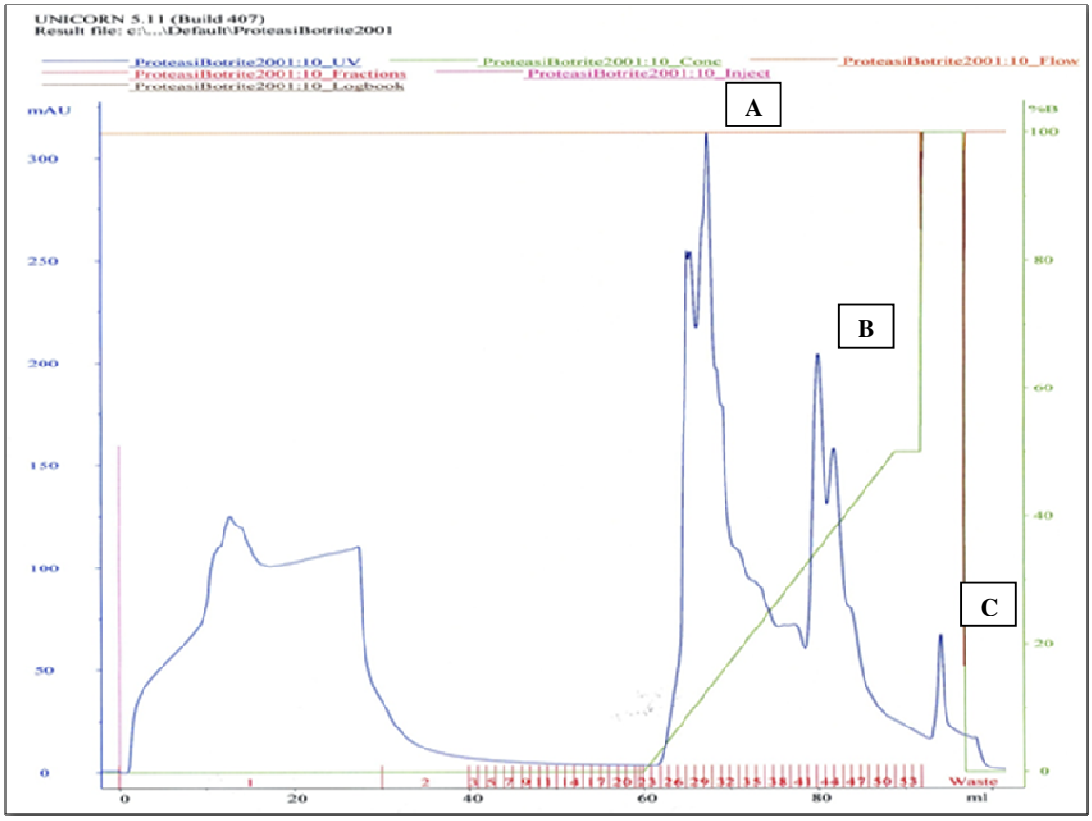


Figure 11 – Mon S Chromatographic separation of the culture filtrate of *B. cinerea* grown on Czapeck-Dox medium supplemented with casein as sole nitrogen source. Only fractions 29, 30 and 31 corresponding to peak A presented proteinase activity.

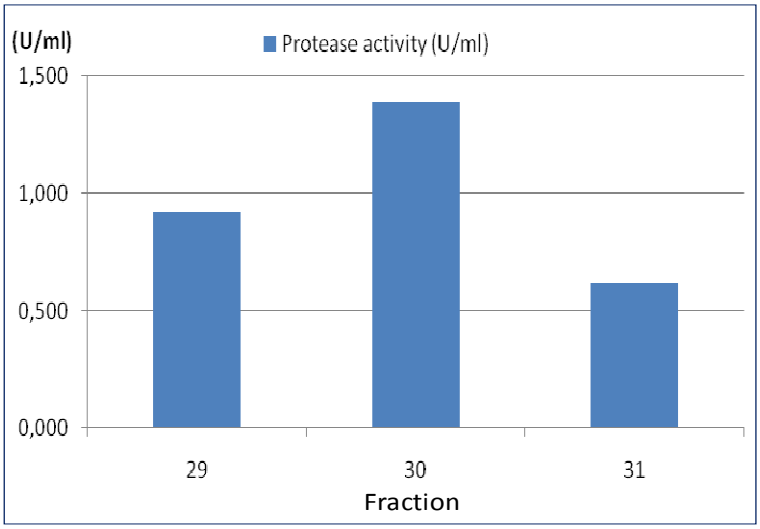


Figure 12 - Protease activity of the factions eluted from the Mono S column. Activity was determined on 1% haemoglobin. FPLC showing protease activity when incubated with haemoglobin. Subsequent elutions from 32 (0.233 U ml^{-1}) and the two other peaks didn't contain enough protease activity in comparison to fractions 29, 30, 31 which presented 0.933, 1.40 and 0.62 U ml^{-1} respectively.

Aliquots of the most active fraction (n° 30) was analyzed by SDS-PAGE. This fraction showed two bands with a mass of 45 and 33 kDa (13).

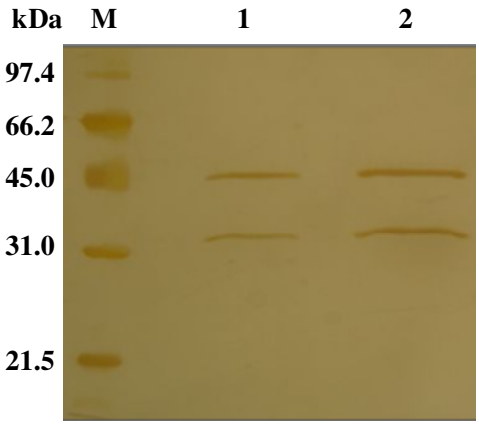


Figure 13 - SDS-PAGE of purified *B. cinerea* protease induced in presence of casein. The gel was stained by silver nitrate (AgNO3) according to the protocol used in California Davis University (http://www.ucdavis.edu). Lane M, (standard protein makers); lanes 1 and 2 (fraction 30; 0.004 U and 0.008 U).

The two bands, after tryptic digestion were analysed by mass spectrometry using Maldi-TOF-MS instrument of the Centre of Mass Spectrometry of the University of Padova. The bands of 33 and 45kDa were identified as aspartyl BC1G_03070 and tripeptidyl peptidase BC1G_02944 respectively (Fig. 13), by the Mascot program and at the *B. cinerea* initiative database (http://www.broadinstitute.org) (table 9). The tripeptidyl protease has been labeled previously as BCAP8 by ten Have *et al*., (2009) and this labeling was maintained throughout the text.

Table 9 – Identification of the purified *B. cinerea* protease obtained after the analysis of LC-MALDI-TOF-MS.

***B. cinerea* proteases**	**Mass**	**Peptide sequence**	**Code and related gene**
Band of 33 kDa	39820 Da	K.YTGTLTYFPK.A K.EYTDTVSFGGLTVK.A K.FLEQYYSVFDTTNSR.I K.TSTGVASGGTVSVSYGSGSFSGK.E K.YYAWINDGGASGVNTIIGQK.F K.TDSSSGLAVFTTKPTSNFGIK.F K.FGSTTYTLTPAQYLVPTAQYSEFGLSSGK.Y	Aspartyl protease BC1G_03070 rhizopuspepsin-2 precursor or BCAP 8
Band of 45 kDa	62235 Da	K.TGLIGIAGFLK.Q K.AGLGFLNPWLYTSAK.S K.TDLTTFMNNYAYFANK.A	BC1G_02994 Tripeptidyl peptidase I –precursor

The lower masses determined by SDS-PAGE in comparison to those deduced from the aminocidic sequence of the proteins is due to the post-translational and post-transcriptional modifications that most secreted proteases undergo to become active.

3.7. Alteration of grape proteins pattern by *B. cinerea* proteases

To establish the ability of *B. cinerea* proteases to digest the grape proteins, the extracellular proteases (fraction No. 30) purified from culture of *B. cinerea* grown on the medium supplied with casein were mixed with grape proteins and aliquots of the digestion mixtures were analyzed by SDS-PAGE. Results showed that by increasing the time of incubation the intensity of all the grape protein bands progressively decreased (Fig. 14).

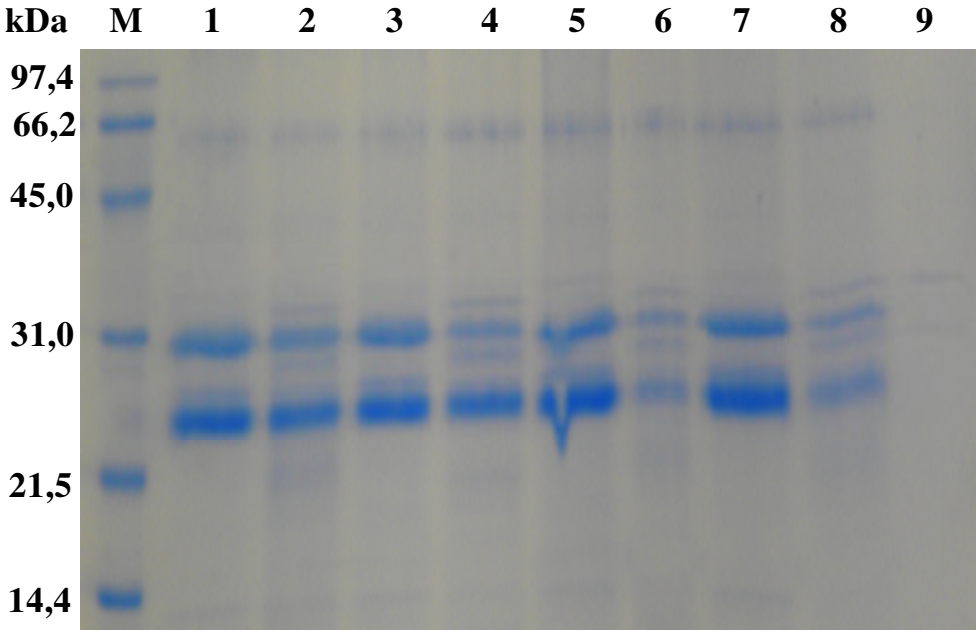


Figure 14 - SDS-PAGE of grape protein incubated for various times with or without *B. cinerea* protease fraction 30 (0.02 U ml^{-1}). Lanes: M (Protein Marker), 1 (Protein, 0 min), 2 (Protein + protease, 0 min); 3 (Protein, 24 h); 4 (Protein + Protease, 24 h); 5 (Protein, 48 h); 6 (Protein + Protease, 48 h); 7 (Protein, 72 h); 8 (Protein + Protease, 72 h).

3.8. Effect of *B. cinerea* protease on grape proteins in presence of grape polyphenols and laccase.

In order to verify whether the activities of *B. cinerea* protease is affected by grape polyphenols and laccase, an experiment was performed by mixing these components together. Also in this case soluble and insoluble proteins were separated by centrifugation before SDS-PAGE analysis.

The results showed that in comparison to the treatment with protease alone, a further decrease of band intensity of soluble protein occurs when polyphenols were added to the mixture (Fig. 15A). When laccase was added, the protein bands completely disappeared from the solution. A small amount of protein was recovered from the precipitated fraction but only when polyphenols were present in the mixture.

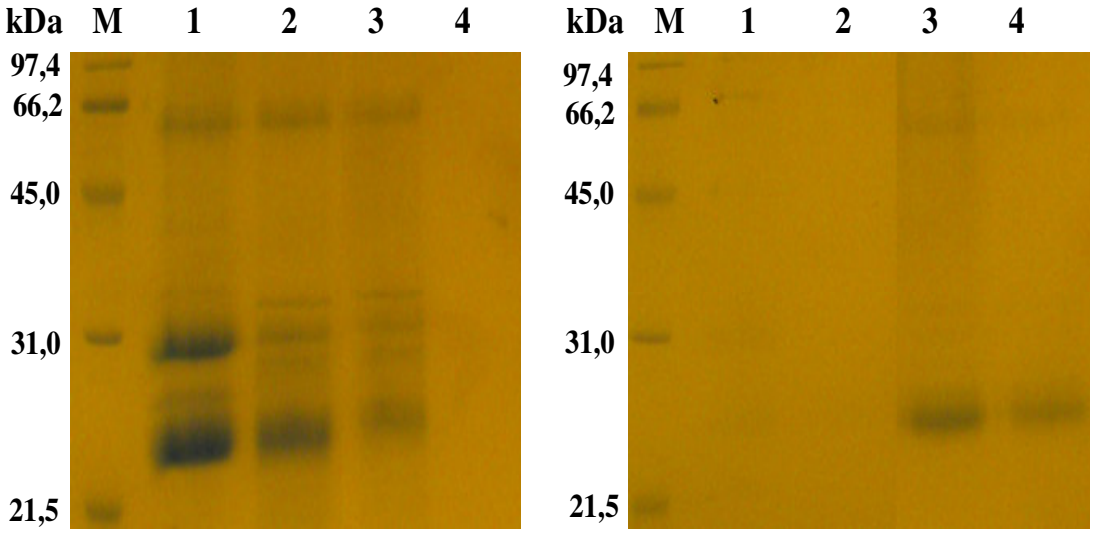


Fig. 15 - SDS-PAGE of grape protein incubated with 0.02 U $ml^{-1}$of *B. cinerea* protease activity and with grape polyphenols. Grape proteins, grape polyphenols and *B. cinerea* laccase were supplied at 50, 100 µg ml^{-1} and 0.02 U ml^{-1}, respectively. After a 24-h incubation, 100 µl of each mixture was centrifuged to separate the soluble proteins in the supernatant (A) from the insoluble ones in the precipitate (B). The soluble protein was recovered from the supernatant after precipitation with 4 volumes of cold ethanol. The gels were stained with the Coomassie G-250 as reported in "Materials and Methods". Lanes: M (Protein standard), P (protein), 2 (protein + protease), 3 (protein + polyphenols + protease) 4 (protein + polyphenols + laccase + protease).

3.9. Expression analysis of *B. cinerea* tripeptidyl peptidase and Aspartyl proteinase gene families.

As shown before, proteinases of *B. cinerea* capable to degrade the grape proteins were purified in a medium containing casein. The demonstration that these proteases are also secreted in a medium containing grape proteins is difficult because the complexity of the protein pattern (not shown). Therefore, an expression experiment was performed to establish if the aspartyl and tripeptidyl proteases genes identified were induced by grape protein. The expression of other members of the two families, selected as putatively secreted in the medium by the PSORT program, was also investigated. After 72 hours mycelia grown on mineral medium containing grape proteins, casein or inorganic nitrogen were collected and RNA was extracted. The purified RNA was quantified by agarose gel electrophoresis (Sambrook *et al*., 1989).

Fig. 16 - Total RNA (1 µg) purified from mycelia of *B. cinerea* grown 72 hours in different conditions. Lane 1(control), lane 2 (grape proteins), lane 3 (Casein). RNA stained with ethidium bromide was separated in 1% agarose gel.

One microgram of RNA was retro-transcribed to produce specific complementary DNA (cDNA) by using oligo dT primer as reverse primer. cDNA was then amplified by PCR with primers for each single protease gene.

All the aspartyl protease genes examined, including the one purified from casein (BCAP8) were expressed in the presence of grape proteins except the *Bcap9* gene. Casein seemed to induce a greater expression of the *Bcap7* and *Bcap8* genes. All the genes analyzed showed an appreciable expression in the control medium containing inorganic nitrogen (Fig. 17).

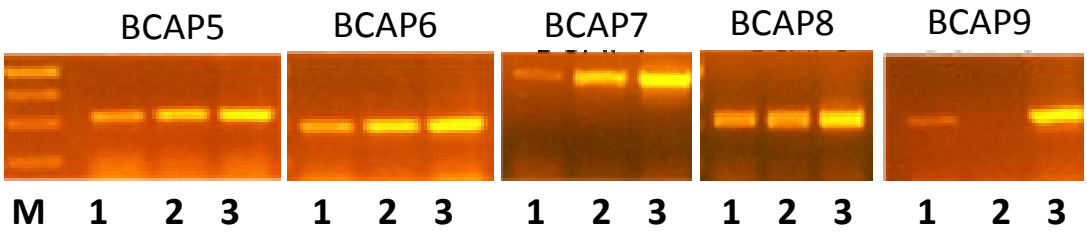


Fig. 17 – RT-PCR of BCAP genes expressed after 72 hours of *B. cinerea* growth in the presence of inorganic nitrogen (1), grape protein (2) or casein (3).

A similar behavior was observed with the tripeptidyl perptidase genes. All genes were expressed at a basal level in the presence of inorganic nitrogen. However, grape proteins and, most of all, casein increased the amount of the transcripts (Fig. 18).

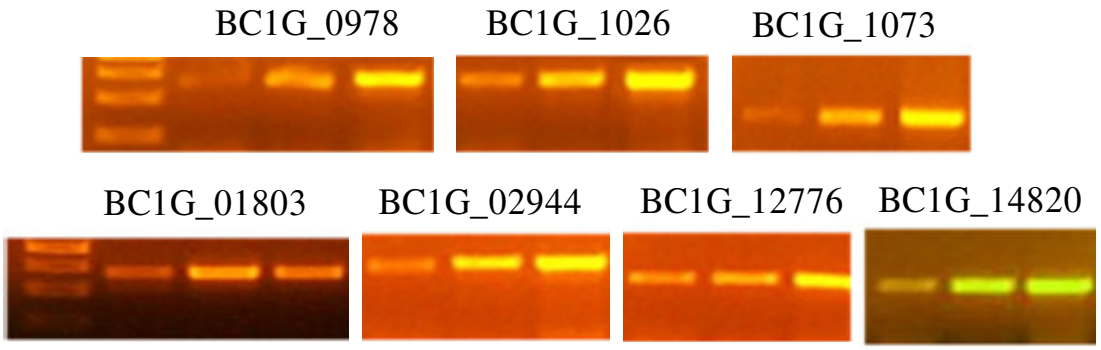


Fig. 18 – RT-PCR of tripeptidyl genes expressed after 72 h of *Botrytis cinerea* growth in the presence of inorganic nitrogen (1), grape proteins (2) or Casein (3).

4. Discussion and Conclusion

4. Discussion and conclusion

During grape infection, the necrotrophic fungus *B. cinerea* encounters an environment rich in phenols and proteins. Among the phenols, the stilbenic resveratrol is a phytoalexin precursor toxic to several fungal pathogens, including *B. cinerea* (Adrian *et al.*, 1997).

Other grape phenols, which here we generically designated as polyphenols, have no reported effect against pathogenic fungi. In addition, some grape berry proteins have been reported to be toxic against *B. cinerea* and belong structurally and functionally to the PR proteins (Derckel *et al.*, 1998; Monteiro *et al.*, 2003).

To mimic conditions similar to the natural infection of grape berries, the combined effect of resveratrol, grape proteins, and grape polyphenols on the growth of *B. cinerea* was studied. It is possible that the grape polyphenol preparations used (about 500 μg g^{-1} of grape fresh weight) could have contained minimal amounts of resveratrol. However, the concentration is estimated to be less than 2 μg g^{-1} of skin fresh weight in healthy *Vitis vinifera* grapes (Li *et al.*, 2006).

In our experiments, resveratrol (200 μg ml^{-1}) was added to cultures at concentration considered lethal for *B. cinerea* (Adrian *et al.*, 1997). An equal amount of polyphenols was also applied. In the literature, there is no clear determination of the resveratrol content in grape berries infected by B. cinerea, but it is likely to be exceeded by that of polyphenols.

The selected resveratrol concentration almost completely prevented fungal growth and laccase activity was negligible in these cultures. In contrast to these findings, Schouten *et al.* (2002) observed only a reduction in fungal growth and a strong induction of the fungal laccase that converts resveratrol into the more toxic dimer *trans*-ε-viniferin. The discrepancy between these results may be explained by the lower resveratrol concentration (50 μg ml-1) and the high titre of spore suspension (5.10^5 ml^{-1}) used by Schouten *et al*., (2002). Induction of laccase activity and partial fungal growth recovery was also observed after lowering the resveratrol concentration to 100 or 50 μg ml^{-1} and increasing the spore concentrations to 10^5 ml^{-1} (data not shown). Hoos and Blaich (1990) previously showed that the effect of resveratrol largely depends on the ratio between this molecule and the spore titre. Membrane-bound ATP-binding cassette (ABC) transporters are likely involved

in protecting *B. cinerea* germlings from the effects of sub-lethal concentrations of resveratrol (Schoonbeek *et al.*, 2001).

Resveratrol became completely ineffective in reducing fungal growth when grape polyphenols where added to the culture, and laccase activity released into the culture was greatly stimulated by this combination of compounds. Thus it can be inferred that under these conditions laccase activity is involved in neutralizing resveratrol toxicity. This role of laccase activity and polyphenols in abolishing the effect of resveratrol was confirmed in the experiment of spore germination. In this experiment the pre-treatment of polyphenols and resveratrol with laccase permitted a normal germination of spores while the absence of polyphenols inhibited the spore germination. This result can be explained by considering that oxidized resveratrol can for a dimer compound, the ε-viniferin which is very toxic to the fungus as demonstrated by Schouten *et al.*, (2002). Instead the presence of grape polyphenols and fungal laccase, which catalyzes a number of phenol coupling reactions (Baldrian, 2006; Riva, 2006), could lead to the formation of more complex compounds, hampering the formation of the toxic *trans*-ε-viniferin.

Recently, Schouten *et al.*, (2008) invoked a similar mechanism for the tannic acid-mediated degradation of the phenolic antibiotic 2,4-diacetylphloroglucinol and also suggested that, at the developing stage, the ABC efflux pump could give *B. cinerea* spores sufficient time to initiate the degradation process.

Grape proteins may also contribute towards making the grape environment inhospitable to fungal attack because they have been described mostly as PR or PR-like proteins. The most highly-expressed proteins in the skin of mature berries are chitinases and β-1,3-glucanase (Deytieux *et al.*, 2007), which together with thaumatin like proteins are also largely represented in the mesocarp of mature berries (Sarry *et al.*, 2004), and particularly enriched in grape juice (Tattersall *et al.*, 1997). The protein pattern obtained from grape juice in this study is similar to that obtained in previous studies and is characterized by three major bands at about 25, 31, and 66 kDa, tentatively identified as thaumatin-like proteins, chitinase, and invertase, respectively (Tattersall *et al.*, 1997; Davies and Robinson, 2000).

Grape thaumatin-like proteins and chitinases have significant antifungal activity against *B. cinerea* (Derckel *et al.*, 1998; Monteiro *et al.*, 2003). In contrast, this current study showed

an increase in mycelium growth upon supplying *B. cinerea* cultures with the grape proteins at a concentration comparable to that measured in grape juice. The discrepancy with the results of other studies may depend on differences in the bioassay used, including protein concentrations and purity, and the sensitivity of the fungal strain. Indeed, protein preparations used in this work were not pure, and phenol contamination may have played a role in diminishing the effect of grape proteins against the fungus.

The grape protein preparations used also allowed partial recovery of the fungal growth inhibited by the resveratrol, suggesting reduction of phytoalexin concentration in the culture, possibly because of interaction with the grape proteins. Indeed, the protein band intensities obtained in the presence of resveratrol seem quantitatively less than those obtained without resveratrol. However, the major change in the grape protein pattern in the *B. cinerea* culture was observed in the presence of polyphenols or when polyphenols and resveratrol were mixed together. In the latter case, almost all proteins were removed from the culture.

We suggest that this disappearance of proteins from liquid culture in the presence of polyphenols and resveratrol is the consequence of reduced solubility of grape proteins mediated by the fungal laccase. Covalent and non-covalent interactions between model and food proteins and selected phenolic compounds have been described (Kroll *et al.*, 2003; Rawel *et al.*, 2005; Prigent *et al.*, 2007). Covalent interactions are mediated by the formation of phenol radicals and quinones, which may occur spontaneously at alkaline pH and are produced by laccase activity at acidic pH (Kroll *et al.*, 2003; Baldrian, 2006). Quinones, which include the laccase oxidized products of *o*-diphenols and *p*-diphenols, may undergo attack by nucleophilic amino acids located on the protein surface (Kroll *et al.*, 2003). This interaction may lead to change in the physicochemical properties, solubility, degradability, and availability of proteins, as well as to alteration in the antioxidant properties of the plant polyphenols (Rawel *et al.*, 2007).

The *in vitro* experiments performed by mixing *B. cinerea* laccase, polyphenols, and/or resveratrol with grape proteins confirmed that laccase favours grape protein precipitation, probably through production of oxidized phenol intermediates. However, in the mixture containing polyphenols, the fate of the 31 kDa protein was unclear; its concentration diminished in the soluble fraction and was almost zero in the insoluble fraction.

The *in vitro* experiments strongly supported the conclusion that similar reactions catalyzed by laccase may occur in *B. cinerea* cultures, which would explain the protein alterations observed. The only difference is that in the *B. cinerea* cultures, supplied with resveratrol and proteins, some proteins remained soluble, while they were totally insoluble in the resveratrol-protein mixture used *in vitro* with laccase. A possible explanation is that in the presence of the fungus, a certain amount of resveratrol or oxidized derivatives is degraded by the fungus and is less available for interaction with grape proteins.

In general, grape proteins are considered resistant to fungal digestion (Waters *et al.*, 1992; Ferreira *et al.*, 2002). However, this contrasts with the observation that *B. cinerea*-infected grape berries showed a simplified protein pattern with bands of reduced intensity. The pattern observed in infected grape berries resembles that obtained with protein-polyphenol mixtures after addition of laccase. Considering that laccase activity is usually produced by *B. cinerea* in infected berries (Dewey *et al.*, 2008) we propose here that this interaction is responsible for the protein pattern observed in the grey mould-rotted grape. In conclusion, these results suggest that probably there is a combined effect of laccase, protease and polyphenols determining the insolubility and/or degradation of the proteins. However, the effect of the fungal laccase in the presence of grape polyphenols seems to prevail upon the activity of the fungal proteases. However, the change observed in grape protein from *B. cinerea* culture in absence of polyphenols and resveratrol also suggests the possible involvement of *B. cinerea* proteases.

The protease activity detected after purification was ascribed mainly to a mixture of aspartyl and tripeptidyl proteases. This preparation was capable to partially digest the grape proteins. However the pattern of degradation was not completely similar to that obtained by growing the fungus with the grape proteins. Expression analysis confirmed that both the enzymes produced on casein are also expressed in the presence of grape proteins; but, other members of these families were also expressed. Therefore the protein pattern observed with the fungus may result from the action of different members of this family or of other families of proteases.

The finding that several members of proteases are induced by casein but only two were purified from the culture filtrate open a question about the relationship between transcripts and protein levels.

It is well known that end point RT-PCR is not a reliable approach to establish quantitative measure of gene transcript expression, however, in this case it is also likely that some transcript product although present in the medium could not be detectable by enzyme assay.

In general fungal proteases are produced as pre-proteases which are often secreted from cells as inactive enzyme that may be activated in the external environment in dependence of factors like pH or temperature (Rolland *et al*., 2009).

The data presented here support the conclusion that in a grape environment characterized by an abundance of polyphenols, *B. cinerea* laccase not only detoxifies the *trans*-resveratrol phytoalexin but also modifies the solubility of grape proteins. In addition, this environment may facilitate the effect of on the degradability of the proteins by fungal proteases.

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