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CORSO DI DOTTORATO DI RICERCA IN SCIENZE DELLE PRODUZIONI VEGETALI

CICLO: 29°

Effects of Thaumatin-like proteins, chitinase and plant secondary metabolites on *Botrytis cinerea* development

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1/11/2016

Riccardo Marcato

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PREMISE

To attack plants pathogens have evolved pathogenicity factors and plants have evolved complex mechanisms of defense against pathogens. Many authors define this interaction as trench warfare or arms race. As defense mechanisms, plants produce fungitoxic compounds of high molecular weight such as pathogenesis-related proteins (PR-proteins) (van Loon et al., 2006) and several plant secondary metabolites (PSMs) of low molecular weight such as phytoalexins and phytoanticipins (Ahuja et al., 2012). On the other side, fungal pathogens release effectors to avoid the recognition by the plant surveillance system or to suppress or manipulate the host defense machinery, and have developed mechanisms to tolerate or detoxify PR-proteins and PSMs produced by their host plants (Pedras et al., 2005 and 2011; Milani et al., 2012; Hasegawa et al., 2014). All these mechanisms may be deployed simultaneously or in succession during the plant-fungus interaction, contributing together to a successful infection.

The goals of this work are to study the effect of two grape PR proteins (a class IV chitinase and a Thaumatin-like protein – TLP -) and sixteen PSMs on development of *Botrytis cinerea*, a necrotrophic fungal pathogen attacking several important crops worldwide, widely studied for its ability to cause rot on a number of fruits and vegetables in open field, greenhouse and during storage (Nicot et al., 2016).

TLPs and chitinases are important not only for their crucial role in the innate immunity of plants against fungal pathogens (van Loon et al., 2006) but also because grape TLP and chitinase are studied for their importance in oenological industry (Waters et al., 2005). In fact these proteins are undesirable in fruit juice processing and wine production because they are responsible for haze formation (Marangon et al., 2011). Therefore, the possibility to find out a mechanisms useful to eliminate the grape proteins from must or wine is an attracting topic for food technologists (van Sluyter et al., 2013). These two aspects, i.e. the relevance in plant defence and the practical exploitation in oenological industry, make these two proteins particularly interesting to study.

Similar concept can be expressed for PSMs: these molecules are important not only for their crucial role in the innate immunity of plants against fungal pathogens (Kim et al., 2014, Pierpoint, 2000; Pusztahelyi et al., 2015) but also because PSMs can be used in crop protection as alternative to normal fungicides (Romanazzi et al., 2016). The relevance in plant defence and the practical exploitation in plant protection make these compounds particularly interesting for studying the chemical characteristics related to their biological activity.

CHAPTER 1: *Botrytis cinerea* displays different mechanisms to counteract grape TLP and chitinase.

ABSTRACT

In this work the ability of the necrotrophic plant pathogen *Botrytis cinerea* to counteract two plants antimicrobial proteins – a chitinase and a grape thaumatin-like protein (TLP) from grape– was characterized. These two proteins extracted from grape berries (*cv*. IM 6.0.13) were added to the *B. cinerea* culture. The growth of the fungus was not affected by these proteins which, as determined by RP-HPLC, were partially removed from the medium. It was observed that aspartic and serine protease produced by *B. cinerea* cleave chitinase only, while the *B. cinerea* mycelium absorbs passively both proteins. In fact, both chitinase and TLP were released from the fungal cell wall when the mycelium was treated with an alkaline buffer and a laminarinase (β-1,3 glucanase) preparation. In particular, a stronger interaction between chitinase, TLP and a β-1,3-1,6-glucan polymer (scleroglucan) was observed.

Therefore, *B. cinerea* can entrap TLP and chitinase in its cell wall, and particularly by the layer of β-glucan that surrounds fungal hyphae. To investigate if TLP and chitinase can induce the synthesis of new fungal cell wall material, the expression of β glucan synthase, chitin synthase and chitin deacetylases was investigated. The expression of the β glucan synthase genes was not affected by both TLP and chitinase while the expression of genes encoding chitin synthase and chitin deacetylases, responsible for the synthesis of chitin and chitosan, respectively, was lowered following the treatment with the grape proteins.

Localization experiments of the cell wall components in the mycelium of *B. cinerea* showed that chitin is mainly present in the vegetative hyphae, chitosan predominate in infection cushions and β-glucan is evenly distributed. A model describing the mechanisms by which *B. cinerea* could escape the action of plant TLP and chitinase is proposed.

RIASSUNTO

In questo lavoro è stata studiata la capacità del fungo necrotrofo *Botrytis cinerea* di contrastare l'effetto di due proteine antimicrobiche – una chitinasi e una proteina thaumatin-like (TLP) di vite – presenti in elevata quantità negli acini di uva. Queste proteine estratte dall'uva (*Vitis vinifera cv*. IM 6.0.13) e somministrate alle colture di *B. cinerea* non hanno alterato la crescita del fungo neppure ad alte concentrazioni delle due proteine. Analisi mediante RP-HPLC hanno dimostrato che TLP e chitinasi vengono rimosse dal mezzo di coltura. Le attività aspartil- e serin-proteasica prodotte da *B. cinerea* in presenza di proteine dell'uva sono in grado di degradare la chitinasi ma non la TLP. Il micelio di *B. cinerea*, invece, si è dimostrato capace di adsorbire passivamente ambedue le proteine di vite. Infatti, dopo trattamento con un buffer alcalino e con β-1,3-glucanasi, il micelio rilascia significative quantità di TLP e chitinasi. In particolare una forte interazione è stata osservata tra le due proteine e il β-1,3-1,6-glucano (scleroglucano) di parete del fungo.

Queste evidenze suggeriscono un ruolo protettivo dei β-glucani che rivestono le ife del fungo dall'azione delle proteine della pianta. Per verificare se TLP e chitinasi inducono la sintesi di nuovo materiale di parete, è stata studiata l'espressione della β-glucan sintasi, della chitin sintasi e della chitin deacetilasi di *B. cinerea* in seguito al trattamento con TLP e chitinasi. Queste analisi hanno evidenziato che il livello di espressione della β-glucan sintasi di *B. cinerea* non è influenzato dal trattamento con TLP e chitinasi mentre l'espressione dei geni codificanti chitin sintasi e chitin deacetilasi – responsabili, rispettivamente, della sintesi di chitina e chitosano – diminuiscono in seguito al trattamento con le proteine dell'uva. Esperimenti di localizzazione nel micelio di *B. cinerea* dei polimeri di parete hanno evidenziato che la chitina è presente principalmente nelle ife vegetative, il chitosano prevale nei cuscinetti di infezione e i β-glucani sono presenti ubiquitariamente. I risultati ottenuti consentono di proporre un modello che descrive i meccanismi con cui *B. cinerea* può sfuggire all'azione della TLP e della chitinasi di pianta.

Introduction

The concept of PR proteins was introduced in 1980 to designate any protein of the host plant induced only in pathological situations (Antoniw et al., 1980). Today much information about these proteins is available. The biosynthesis of PR proteins is considered an important defense mechanism against fungal pathogens (Odjakova et al., 2001; Derckel et al., 1996; Monteiro et al., 2003). There are 17 families of PR proteins, which have different structure, function and cellular target, but are well conserved in plant kingdom (van Loon et al., 2006; Ferreira et al., 2007). Among them, the PR-3 (Chitinase class IV) and the PR-5 (Thaumatin-like protein – TLP) are the most studied families for their abundance and accumulation in plant tissues as antimicrobial compounds and, secondarily, for their allergenic property when present in foods (Hsieh at al., 1995). Genes of both classes are present in large families in plant genomes, with some members constitutively expressed in plant tissues and further induced after infection and others expressed only following infection (Robinson et al., 1997; Liu et al., 2010).

Chitinases are enzymatic PR proteins that hydrolyses chitin (β-(1→4) N-acetyl D – glucosamine), a structural polymer of fungal cell wall (Grover, 2012). Plant chitinases, cleaving chitin, block the growth of hyphae and are also involved in plant recognition of potential fungal pathogens by detaching chitin oligomers from the fungal cell wall activating defense responses in plant (Kaku et al., 2006).

TLPs are non-enzymatic antimicrobial proteins that permeabilize the cell membrane causing osmotic imbalance of fungal cells (Vigers et al., 1992). Several TLPs have a glucan binding site and conserve structural analogy with glucanases (PR-2 family) (Menu-Bouaouiche et al., 2003; Fierens et al., 2007). Recently, the X-ray structure of *Vitis vinifera* TLP (O04708_VITVI) has been published (PDB database). This TLP has a compact structure characterized by presence of β-sheets stabilized by seven disulphide bonds (Marangon et al., 2014).

Members of chitinase and TLP families act synergistically against the fungal hyphae, and genes encoding these proteins have been expressed together or over-expressed in transgenic plants obtaining an increased resistance against fungal pathogens (Maruthasalam et al., 2007; Tobias et al, 2007; Liu et al., 2012; Cletus et al., 2013; Shah et al., 2013).

The biological activity of both chitinases and TLPs is variable and depends on the proteins' structural features and on the particular fungal species (Saito et al., 2011; Wurms et al., 2011). For example the growth of *B. cinerea* is unaffected by 100 μg mL^{-1} of grape proteins, which are mostly TLP and chitinase (Favaron et al. 2009). Besides, spore germination is unaffected when the purified grape TLP and chitinase are administered separately or together at a concentration of 100 μg mL^{-1}

each, and only a delay in spore germination rate is observed (personal observation). Also the growth of the other ascomycetes fungi (*Sclerotinia sclerotiorum* and *Sclerotinia minor*) was not negatively affected by these two proteins and, moreover, both TLP and chitinase were completely removed from the medium by the basidiomycete *Sclerotium rolfsii* (Marcato et al., 2016). It is likely that the plant pathogen fungi have developed mechanisms to counteract the action of TLP and chitinase. For example, *Cladosporium fulvum,* interacting with tomato leaf tissue, secretes a chitinase-binding lectin that protects its cell wall by plant chitinase activity and contrasts the release of chitin oligomers (van den Burg et al., 2006).

Another mechanism proposed for detoxifying the PR-proteins is their enzymatic cleavage by fungal proteases (Poussereau et al., 2001a and 2001b; ten Have et al., 2004 and 2010). During host infection, pathogens express protease genes and secrete several proteases believed to contribute to virulence (Rao et al., 1998; Schulze Gronover et al., 2004). Typically, fungal proteases are considered as factors involved in the degradation of plant cell wall proteins and in nitrogen assimilation after killing of the plant tissue (Billon-Grand et al., 2012). However, few studies examined the role of fungal proteases in the cleavage of TLPs and chitinase (Olivieri et al., 2002; Naumann et al., 2011; Karimi Jashni et al., 2015; Price et al., 2015) because, in general, these proteins are considered refractory to protease degradation (Younes et al., 2013).

In order to characterize the capacity of *B. cinerea* to neutralize plant PR proteins, the mechanisms exploited by this fungus to remove the grape TLP and chitinase were investigated. Grape TLPs and chitinase have been previously used in experiments for characterizing the aspartic protease family of *B. cinerea* (ten Have et al., 2010) and to elucidate the mechanism of PR protein detoxification mediated by the laccase activity of this fungal pathogen (Favaron et al., 2009). As a first possible mechanism, we characterized the involvement of *B. cinerea* proteases, whose activity was hypothesized to contribute to plant infection (Billon-Grand *et al.*, 2002 and 2012; Meléndez *et al.*, 2009; ten Have *et al.*, 2010). The second investigated mechanism was the possible sequestering of TLP and chitinase by fungal cell wall polysaccharides that are localized at the mycelium surface. The binding between the PR proteins and the gluco-chitinic fungal matrix was also verified by a docking simulation; the localization of glucan, chitin and chitosan was established by specific labelings. Finally, possible changes induced by these PR proteins on the expression of fungal cell wall biosynthetic enzymes were also analyzed.

Material and methods

Extraction and characterization of grape proteins

Proteins were extracted from grape (*Vitis vinifera*, cv. IM 6.0.13) juice as previously reported (Favaron et al., 2009). Protein analysis was performed by reverse phase (RP) HPLC with an AKTA purifier (GE Healthcare, UK) equipped with Vydac 214 TP C4 protein/peptide column (cat. 214TP5415, Grace, Columbia, MD, USA) equilibrated with 0.1% trifluoroacetic acid in water (buffer A) and 5% of 0.1% trifluoroacetic acid in 95% acetonitrile (buffer B). After the loading of 100 µl of sample, the column was washed for 5 min with buffer A and then eluted with a gradient, from 5% to 75%, of buffer B in 42 minutes. The flow rate was 1 mL min^{-1} and the proteins in peaks corresponding to thaumatin-like protein (TLP) and chitinase were quantified by using external standards purified as reported by Vincenzi et al. (2011 and 2014). The protein preparation used was also characterized by the presence of chitinase activity using a protocol reported in Byrne et al. (2008).

Fungal cultures and growth

The fungal pathogen *B. cinerea* (strain B05.10) was grown on Petri dishes on potato dextrose agar (PDA, BD, Difco, USA) at 25 °C. For culture inoculation, PDA disks (0.4 cm of diameter) were taken from the marginal zone of actively growing colonies.

For examining fungal growth, a PDA disk of *B. cinerea* mycelium was inoculated in 2 mL of Czapek-Dox medium (6.7 mM KCl, 2 mM MgSO$_4$·7 H$_2$O, 7.3 mM K$_2$HPO$_4$, 0.03 mM FeSO$_4$, 10 mM citric acid, 111 mM glucose, pH 3.5) containing 150 µg mL^{-1} of grape proteins or 35 mM of NaNO$_3$ as nitrogen source. After 5 days at 25 °C in the dark, the growth of the colonies was examined by a stereomicroscope.

Removal of grape proteins by *B. cinerea*

In order to study the capacity of the fungus to remove chitinase and TLP from the culture, a PDA disk of mycelium of each fungus was inoculated in 2 mL of the Czapek-Dox medium above reported containing 150 µg mL^{-1} of grape proteins as nitrogen source. After 5 days of incubation at 25 °C, culture samples were centrifuged for 10 min at 14000 g, filtered by 0.2 µm membrane of cellulose acetate (Sartorius cat. 11107) and residual grape proteins remaining in the fungal cultures were determined by RP-HPLC as above reported. As a control, an aliquot of the same medium without the fungus was analyzed in RP-HPLC after 5 days of incubation. The experiments were performed in microtiter plates and replicated at least 3 times.

Induction and quantification of protease activity

To induce protease activity, *B. cinerea* was inoculated and grown as described in the previous subsection with 150 µg mL^{-1} of grape proteins or 5 mg mL^{-1} of enzymatically hydrolyzed casein (NZ-Amine-A, Sigma). After 5 days of growth, the protease activity was measured in triplicate by incubating at 37 °C 100 µl of fungal culture with 450 µl of hemoglobin 1% (w/v), adjusted to pH 3.5 with 1M HCl. The reaction was stopped after 90 minutes with 450 µl of 20% (w/v) trichloroacetic acid. Samples were centrifuged at 14000 g for 10 minutes and then 400 µl of supernatant were mixed with an equal volume of 0.5 M NaOH. Blanks were made by adding trichloroacetic acid before the addition of the sample. The absorbance values (in AU) were read at 280 nm. One enzyme unit was defined as the amount yielding 1 AU min^{-1}.

Characterization of *B. cinerea* protease activity by inhibition assays

Culture filtrates of *B. cinerea* grown for 5 days on Czapek-Dox medium with grape proteins (150 µg mL^{-1}) were used to characterize the type of protease secreted by protease inhibition assays. The experiments were carried out as described above in absence or in presence of the following protease inhibitors: 0.1 mM pepstatin A (aspartic and acidic protease inhibitor), 10 mM EDTA (a metalloprotease inhibitor), 5 mM PMSF (Phenylmethylsulfonyl Fluoride, a serine protease inhibitor), 0.1 mM leupeptin (cysteine and serine protease inhibitor) and 0.1 mM E-64 (cysteine protease inhibitor). As a positive control, the protease activity was also measured in the presence of water or ethanol (0.5%; v/v), the solvent of pepstatin A and PMSF.

Digestion of grape proteins with the fungal culture filtrate

An aliquot of 300 µl of the *B. cinerea* culture filtrate grown on Czapek-Dox medium with hydrolyzed casein was incubated at 25 °C with an equal volume of protein preparation containing 300 µg mL^{-1} of grape proteins. After 3 days, a 100 µl aliquot of the incubation mixture was analyzed in RP-HPLC as above reported to determine the residual amount of TLP and chitinase. As a control, the grape proteins were incubated for 3 days at 25 °C with the Czapek Dox medium. The digestion experiments were repeated 3 times. In a separate experiment, 400 µl of the purified grape chitinase (200 µg mL-1) and 400 µl of the *B. cinerea* culture filtrate induced with casein hydrolysate were incubated for 24 hours at 25 °C. One hundred µl aliquots of the mixtures were precipitated and run on RP-HPLC and on SDS-PAGE.

Desorption of TLP and chitinase from *B. cinerea* mycelium

After 5 days of growth on 2 mL of Czapek-Dox medium with 150 µg mL^{-1} of grape proteins, PDA disks colonized by *B. cinerea* were collected on sterile gauze and gently squeezed. According to Klis et al. (2007) with some modifications, the cell surface-associated proteins were removed by suspending each colonized disk for 8 hours at 37 °C in 1 mL of 0.2 M sodium phosphate buffer pH 8.0 containing 1% SDS (w/v). The proteins released in this alkaline buffer were recovered in deionized water after desalting with a PD-10 column (GE Healthcare) and analyzed by RP-HPLC as above reported for the quantification of TLP and chitinase.

To remove proteins more strongly bound to the fungal cell wall, the mycelium was suspended in 1 mL of 20 mM sodium citrate buffer pH 5.0 and incubated for 24 hours at 30 °C with 0.065 units of laminarinase (L5272, Sigma), an enzyme preparation containing β-1,3 glucanase. After centrifugation and filtration, these mixtures were analyzed by RP-HPLC for determining TLP and chitinase released from *B. cinerea* cell wall. The experiments were repeated 2 times.

Polysaccharides absorption experiments

Ten mg of chitin (C-7170, Sigma Aldrich), chitosan (419419, Sigma Aldrich), laminarin (L9634, Sigma), pachyman (P-PACHY, Megazyme) and scleroglucan (Actigum^{TM} CS, Cargill) were suspended in 1 mL of 20 mM sodium citrate buffer pH 3.5 and incubated at 25 °C with 150 µg mL^{-1} of grape proteins. After 16 h, the mixtures were centrifuged and the supernatants were analyzed by RP-HPLC for TLP and chitinase quantification. For the scleroglucan mixture, both the gelatinous pellet and the viscous supernatant obtained after centrifugation were incubated with 0.065 units of β-1,3-glucanase at 30 °C. After 24 h, the two mixtures were filtered and analyzed by RP-HPLC as above reported. The level of scleroglucan degradation was estimated at the end of the experiment by measuring the reducing-ends groups released with the Nelson-Somogyi method (Nelson, 1944) using D-glucose as a standard. Aliquots of the incubation mixture were also analyzed by SDS-PAGE.

Localization of the *B. cinerea* polysaccharide component in cell wall hyphae and infection cushions

To obtain hyphae and infection cushions of *B. cinerea,* a $2 \cdot 10^6$ conidia mL^{-1} were diluted in a potato dextrose broth (PDB) medium. One hundred µl of this preparation were spread on a cellophane sheet covering a modified PDA medium (PDA diluted in water 1:4 with 25 g L^{-1} of agar) in a Petri dish. After 48 h of incubation at 21°C, portions of cellophane recovered from the PDA plate colonized by *B. cinerea* mycelium were cut and fixed with 3% (v/v) formaldehyde solution in distilled water at 65°C for 30 min. The fixed samples were washed three times in PBS buffer (137 mM NaCl, 2.7 mM KCl, 8.1 mM Na$_2$HPO$_4$, 1.5 mM KH$_2$PO$_4$, pH 7.4) before being infiltrated with

1% (v/v) Tween 20 in PBS buffer. To observe the localization of polysaccharides on the *B. cinerea* cell wall, several fluorescent compounds and epifluorescence microscope Leica DM2500 were used.

To detect chitin, fixed samples were incubated overnight in the dark with 20 µl of Calcoflour white stain (18909; Sigma Aldrich). Calcofluor white (excitation ~350 nm; emission spectrum ~ 450 nm) binds nascent microfibrils of chitin. The stained samples were rinsed with PBS before microscopic observations (epifluorescence microscope Leica DM2500).

To detect chitosan fixed samples were incubated 30 minutes in the dark with 20 µl of Eosin Y (230251; Sigma Aldrich). Eosin Y specifically binds to chitosan (the deacetylated form of chitin) of living fungal cells and can be visualized by fluorescence microscopy (excitation ~510; emission spectrum ~535 nm). The stained samples were washed with ethanol (10% v/v) 3 times for ten minutes and rinsed with PBS before microscopic observations.

To detect β glucan, Aniline Blue (B8563; Sigma Aldrich) solution (2.5% v/v in acetic acid 2%) was used. Aniline blue reacts with β-1,3 glucans of cell wall giving a brilliant yellow fluorescence in UV light (excitation ~390 nm, emission spectrum ~500 nm). The stained samples were rinsed with PBS before microscopic observations.

Gene expression analysis

To ascertain if the grape proteins can induce glucan, chitin or chitosan synthesis, an expression analysis of β-glucan synthase, three chitin synthases and three chitin deacetylase genes of *B. cinerea* (Supplementary Tab 1), was performed. In parallel, also the expression of the α-glucan synthase of *B. cinerea* was monitored.

After 3 days of growth in 1 mL Czapek Dox medium containing 111 mM fructose as carbon source, one PDA disk colonized by *B. cinerea* mycelium was transferred in 1 mL Czapek Dox medium supplemented with 150 µg of grape proteins or BSA. After 6 hours, about 100 mg of mycelium were frozen with liquid nitrogen and RNA was extracted with the RNeasy Plantmini kit (Qiagen GmbH, USA) according to the manufacturer's instruction. RNAs of three extractions were mixed together and precipitated with one volume of cold isopropanol overnight at -20 °C. The extraction was repeated on three different biological replicates.

Reverse transcription was performed by mixing 0.5 µg of an oligo-dT (15/18 thymine) reverse primer with about 1 µg of RNA and by using the ImPromII reverse transcriptase (Promega, Milano, Italy), following manufacturer's instructions. Primers for real-time expression analysis were designed by using Perl Primer v.1.1.17 programs based on the transcript sequences of *B. cinerea* genes (Supplementary Table 1).

The gene sequences of *B. cinerea* (B05.10), were obtained from Fungi Ensembl database (http://fungi.ensembl.org/Botrytis_cinerea/Info/Index).

The amplification of the above reported genes was performed by qPCR (Rotor-Gene Q 2plex, Qiagen GmbH). The 20 µl reaction mixture contained 10 µl of 2X Brilliant III Ultra-Fast SYBR Green QPCR MasterMix (Agilent Technologies, Milano, Italy), 0.4 µM of each specific primer and 3 µl of cDNA as template. The qPCR was performed by repeating 40 times the following cycle: 20 s at 95 °C; 20 s at 56 °C; 30 s at 72 °C. Relative expression results were analyzed by using the Rotor-Gene 2.0.3.2 Software version (Qiagen GmbH). The gene Bcin01g08040 (β tubulin) was selected as housekeeping.

Molecular Modeling

The X-ray crystal structure of grape TLP was retrieved from the Protein Data Bank (PDB ID: 4MBT) (Berman et al., 2000; Marangon et al., 2014). The structural of class IV chitinase was carried out by homology modeling using the homology tool of MOE (CCG Inc.) based on the deposited structure of the *Bryum coronatum* chitinase co-crystallizated with chitin (PDB ID: 3WH1) (Ohnuma et al., 2014). The coordinates of polysaccharides (chitin, chitosan, laminarin, pachyman and scleroglucan) were retrieved from POLYSAC3DB database (http://polysac3db.cermav.cnrs.fr/) (Sarkar and Pérez, 2012). Molecular docking studies were carried out using SwissDock (http://www.swissdock.ch/), a web service to predict the molecular interactions that may occur between a target protein and a small molecule, and the analysis of results were performed with Chimera 1.0.10.

Statistical

Data obtained from experimental were subjected to t-test according to experimental design.

Results

TLP and chitinase are the main proteins extracted from grapes

The grape proteins show a simplified chromatographic pattern on RP-HPLC. The retention times of the two main peaks correspond to those of a purified TLP (UniProtKB: O04708_VITVI) and class IV chitinase (UniProtKB O24530_VITVI), which were used as external standards (Fig. Suppl. S1) (Vincenzi et al., 2014 and unpublished results). TLP and chitinase were estimated at a weight ratio of 1.3:1 and at a similar molar concentration according to their mass of about 24 and 31 kDa, respectively (Tattersall et al., 1997; Vincenzi et al, 2014). These proteins are structurally very similar to PR-proteins present in other plants (Supplementary Tab. 1 and 2). Together the two proteins represent more than 90% of total protein extracted from grape juice. This protein preparation was used in the following experiments as a source of TLP and chitinase.

B. cinerea remove TLP and chitinase from the culture

B. cinerea was grown in a culture medium containing 150 μg mL^{-1} of the grape proteins or 35 mM of NaNO$_3$ as nitrogen sources. After 5 days of culture, at stereomicroscopy inspection, the fungus growth in presence of grape proteins showed a mycelium development not dissimilar to that observed with inorganic nitrogen (Fig. 1). At this time, an aliquot of the fungal culture was analyzed by RP-HPLC to determine the residual amount of TLP and chitinase. Evident changes in the chromatographic profiles of residual chitinase and TLP were observed (Fig 2): *B. cinerea* reduced the level of chitinase and TLP in the medium by 50.1±2.5% and by 36.4±11.6%, respectively. Moreover, an aliquot of the cultural medium was also analyzed by SDS-PAGE. A band corresponding to TLP was still clearly visible while a band corresponding to chitinase was no more evident (Supplementary Fig. 2).

B. cinerea protease cleaves chitinase but not TLP

In order to establish the involvement of fungal protease activity in the removal of TLP and chitinase, the protease activity secreted by *B. cinerea* grown in the presence of grape proteins was determined. When chitinase and TLP are present in the medium, *B. cinerea* produces about 0.05 U mL^{-1} of protease activity. The same level of protease activity was detected in the medium containing hydrolyzed casein (5 g L^{-1}) or BSA (5 g L^{-1}), notoriously good inductor of protease. To characterize the type of protease activity produced by *B. cinerea*, a protease inhibition assay was performed. Among the inhibitors assayed, only the inhibitors pepstatin and PMSF were able to reduce the protease activity (Supplementary Fig. 3). Either pepstatin or PMSF inhibited the protease activity by

about 40%. Based on these results, aspartic and serine proteases are the main proteolytic enzymes secreted by *B. cinerea* grown on grape proteins.

To determine the contribution of the protease activity to TLP and chitinase degradation, grape proteins and the *B. cinerea* fungal filtrate containing protease activity produced on hydrolyzed casein were incubated together. No significant reduction of TLP was noticed by RP-HPLC analysis and only about a 30% decrease of the chitinase peak was measured (Fig 3). However, when the proteolytic preparation of *B. cinerea* was incubated with the grape purified chitinase, a SDS-PAGE analysis showed a reduction of the size of the chitinase (Fig. 4). This cleaved chitinase, at RP-HPLC analysis, showed the same retention time of the undigested chitinase (not shown)
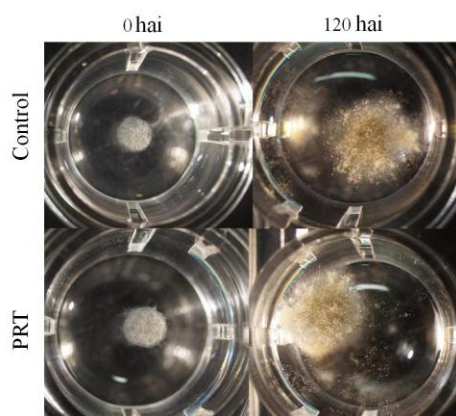
Figure 1. Growth of *B. cinerea* in presence of 150 µg mL^{-1} of grape proteins (PRT) or 35 mM of NaNO$_3$ (Control). The cultures were grown for 120 hours on the Czapek-Dox medium.
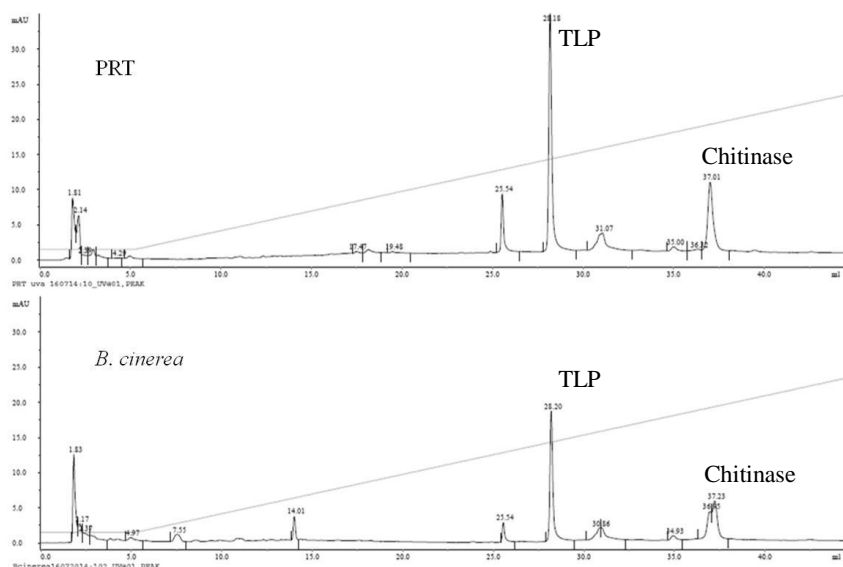
Figure 2. Typical RP-HPLC patterns of the proteins present in 100 µl of the culture filtrates of *B. cinerea* after five days of growth on Czapek-Dox medium supplemented with 150 µg mL^{-1} of grape proteins (PRT). As a control, 150 µg mL^{-1} of grape proteins were incubated for five days.
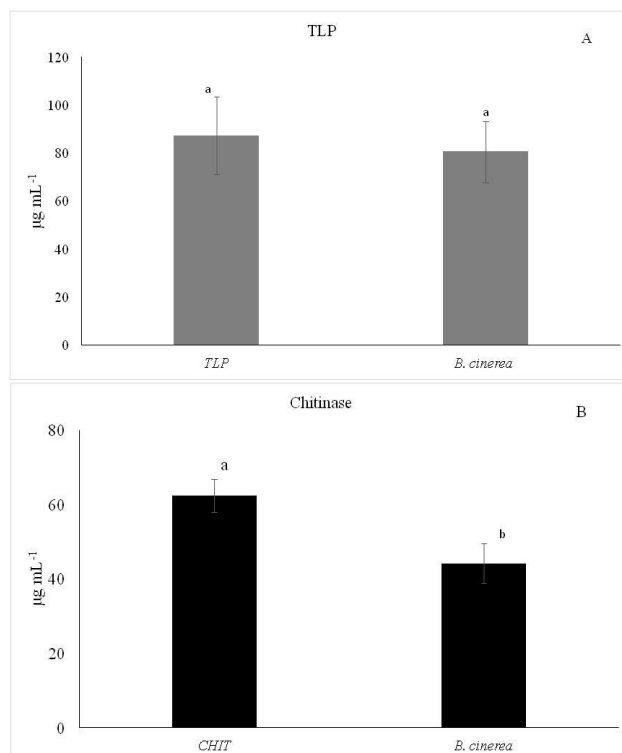
Figure 3. Residual grape proteins after incubation with the *B. cinerea* culture filtrates. **(A)** and **(B)**: 100 µl aliquots of the mixtures were analyzed by RP-HPLC and residual TLP **(A)** and chitinase **(B)** was determined. Five-day-old culture filtrates of the *B. cinerea* containing the protease activity induced with 0.5% (w/v) of casein hydrolysate were incubated for three days with 150 µg mL^{-1} of grape proteins. Controls without culture filtrates were performed and TLP and chitinase (CHIT) determined after three days. Values are the average of three experiments ±SD. Data were statistically analyzed by applying the t-test using the Student's t-distribution. Different letters indicate significant differences at P < 0.05.
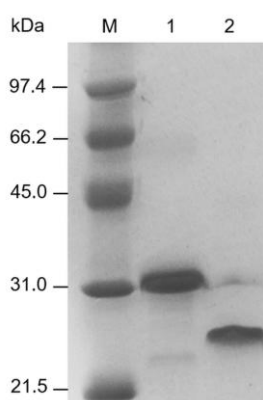
Figure 4. SDS-PAGE of the incubation mixture of the grape chitinase with the fungal culture filtrate of *B. cinerea*. One hundred µg mL^{-1} of purified chitinase were incubated for 24 hours with or without the culture filtrate of *B. cinerea* obtained on Czapek-Dox medium supplemented with 0.5% (w/v) of casein hydrolysate. One hundred µl aliquots of the mixtures were precipitated and loaded. The gel was stained with a colloidal Coomassie G250 protocol. Lane 1: purified chitinase. Lane 2: digested chitinase. M = Molecular weight markers (Low range, Bio-Rad Laboratories, Milano, Italy).

The cell wall of *B. cinerea* interacts with TLP and chitinase

To check if a passive mechanism is in some way involved in the protein removal by *B. cinerea*, a heat inactivated three days-old mycelium of this fungus grown in the presence of $NaNO_3$ was mixed with the grape proteins. After 16 h of incubation, about one-half of TLP and chitinase was removed from the medium (data not shown). Therefore, the capacity of the *B. cinerea* cell wall to absorb chitinase and TLP in culture was more deeply investigated. To this aim, after 5 days of growth in presence of the grape proteins, mycelium was washed with an alkaline solution to detach the fungal surface-associated proteins (Klis et al., 2007). The chromatographic analysis of this washing solution showed that two small peaks, apparently corresponding to those of TLP and chitinase, were recovered from the mycelium of *B. cinerea* (Supplementary Fig. 4). In a further experiment, the mycelium of the fungus grown in the presence of grape proteins was treated with laminarinase (an enzymatic preparation containing β-1,3-glucanase activity) and protein peaks corresponding to those of TLP and chitinase were clearly recovered after this treatment (Fig. 5).

From the above results it appears that protease digestion and adsorption by cell wall polysaccharides are two possible mechanisms capable to explain the decrease of TLP and chitinase observed in the culture filtrates of *B. cinerea*.
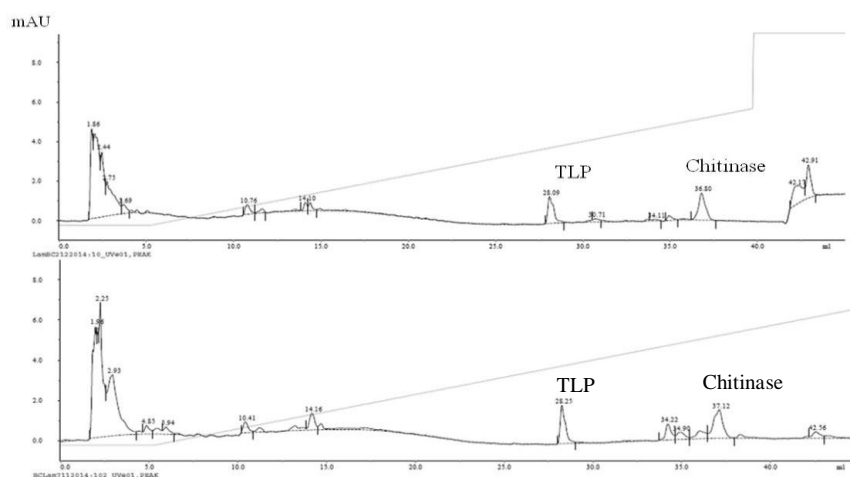

Figure 5. RP-HPLC patterns of proteins released following treatment of *B. cinerea* mycelium with laminarinase (containing β-1,3-glucanase activity). The mycelia were obtained after growing the fungi for five days on the Czapek-Dox medium with 150 μg mL^{-1} of grape proteins. One hundred μl aliquots of each incubation mixture were analyzed. The experiment was repeated twice and the chromatograms of the two replicates obtained is reported in the figure.

Polysaccharides absorption experiments

To clarify if chitin, chitosan or β-glucan produced by *B. cinerea* are responsible for this passive absorption, ten mg of commercial chitin (β 1→4 N-acetyl D - glucosamine), chitosan (β 1-4 D-glucosamine), laminarin (a β 1→3 glucan with several β 1→6 glucan side-chains), pachyman (a β 1→ 3 glucan) or scleroglucan (a β [1→3]₃, 1→6 glucan) were incubated for 16 h with the grape proteins. After incubation, these mixtures were centrifuged, filtered and aliquots were analyzed chromatographically. Both TLP and chitinase disappeared almost completely from the medium with scleroglucan while the other glucan polymers were ineffective to remove TLP and were variably effective in removing chitinase. Chitinase level was reduced by about 50% in the sample mixture with laminarin, by about 70% in presence of chitin, it was not reduced in the mixture with chitosan and not at all by pachyman (Fig. 6).

To verify that the subtracted proteins are actually absorbed by the polymers, an experiment was performed with scleroglucan to possibly recover the absorbed TLP and chitinase. To this aim the scleroglucan incubated with TLP and chitinase was centrifuged before the chromatographic analysis. A gelatinous pellet and a viscous supernatant were obtained. An aliquot of the supernatant was loaded onto the RP-column and neither TLP nor chitinase peaks were detected (data not shown). Therefore, both the pellet and the supernatant were digested with β-1,3-glucanase (laminarinase) but none of these mixtures revealed the presence of TLP and chitinase peaks (Supplementary Fig. 5.). However, TLP and chitinase bands were recovered when aliquots of these two fractions were run in SDS-PAGE (Fig. 7). The level of glucan hydrolysis after β-1,3-glucanase activity was about 58% as estimated by a reducing-end groups assay.
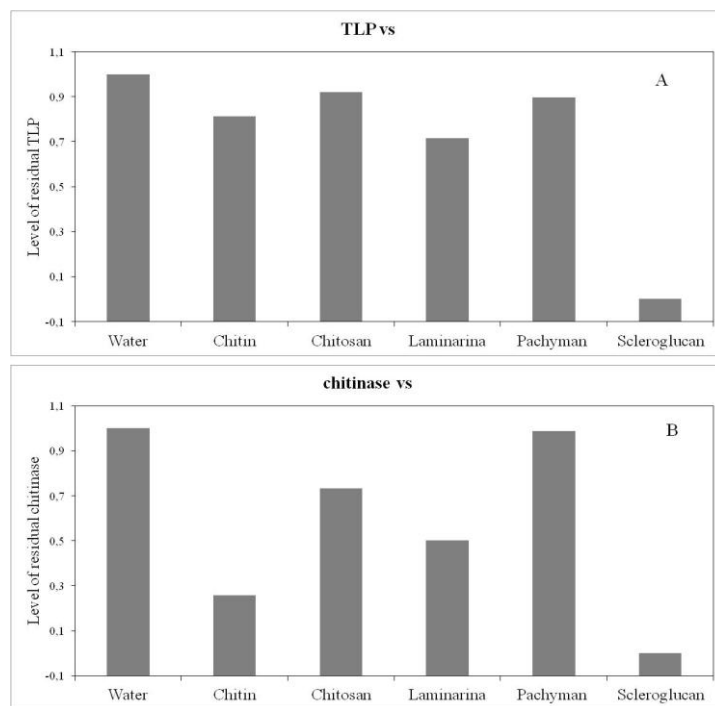
Figure 6. Relative amounts of residual TLP (A) and chitinase (B) after incubation with several polysaccharides. Ten mg of commercial chitin (β 1→4 N-acetyl D - glucosamine), chitosan (β 1-4 D-glucosamine), laminarin (a β 1→3 with several β 1→6 glucan), pachyman (a β 1→ 3 glucan) or scleroglucan (a β [1→3]$_3$, 1→6 glucan) were incubated for 16 h with the grape proteins (150 μg mL^{-1}). After centrifugation the supernatant (100 μl) of each mixture were analyzed on RP-HPLC.
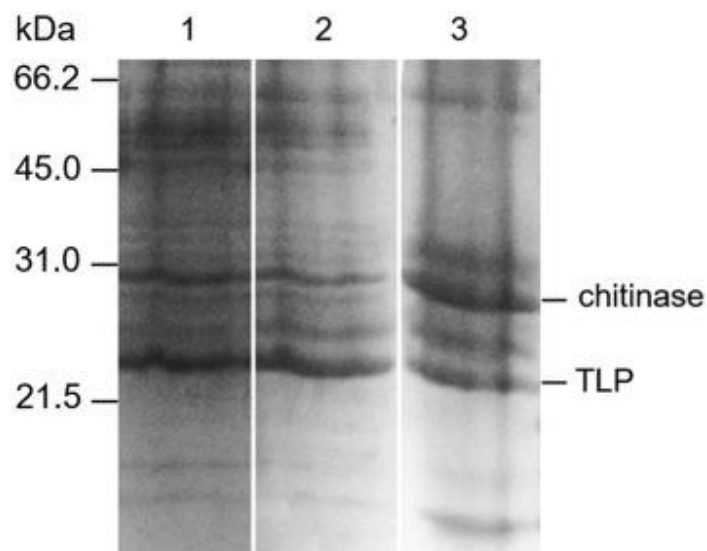

Figure 7. SDS-PAGE of grape proteins absorbed by scleroglucan. Scleroglucan (5 mg mL^{-1}) was incubated for 16 h with grape proteins (150 μg mL^{-1}). After centrifugation, the gelatinous pellet and the viscous supernatant were treated with laminarinase (containing β-1,3-glucanase activity) for 24 h. One hundred μl aliquots of the β-1,3-glucanase treated pellet (lane 1) and supernatant (lane 2) were precipitated and loaded on the gel. A 100 μl aliquot of grape proteins (150 μg mL^{-1}) was also precipitated and loaded on the gel (lane 3). The gel was stained with a colloidal Coomassie G250 protocol. Molecular weight markers (Low range, Bio-Rad Laboratories) are indicated on the left.

Localization of the *B. cinerea* polysaccharide in the hyphae cell wall and infection cushions

Since the *B. cinerea* cell wall is an important barrier against plant PR-proteins, the immunological characterization of cell walls polysaccharides (β-glucan, chitin and chitosan) of *B. cinerea* was performed studying the difference among vegetative hyphae and infection cushions. The results obtained showed that β-glucan seems present in all parts of hyphae and in the infection cushion (supplementary Fig. 6), differently from chitin and chitosan. In fact, the infection cushions of *B. cinerea* are stained by eosin Y that labels chitosan, as opposed to vegetative hyphae that seem refractory to this staining. Differently, using the chitin stain calcofluor, hyphae exhibited a continuous fluorescence along the longitudinal walls often more marked at the apex and septa while infection cushions seemed refractory to this staining (Fig. 9). Therefore, chitin and chitosan seem to have a complementary localization: chitin is present prevalently along the longitudinal hyphae walls (often more marked at the apex and septa), while chitosan (the deacetylated form of chitin) is prevalent in infection cushion structures.
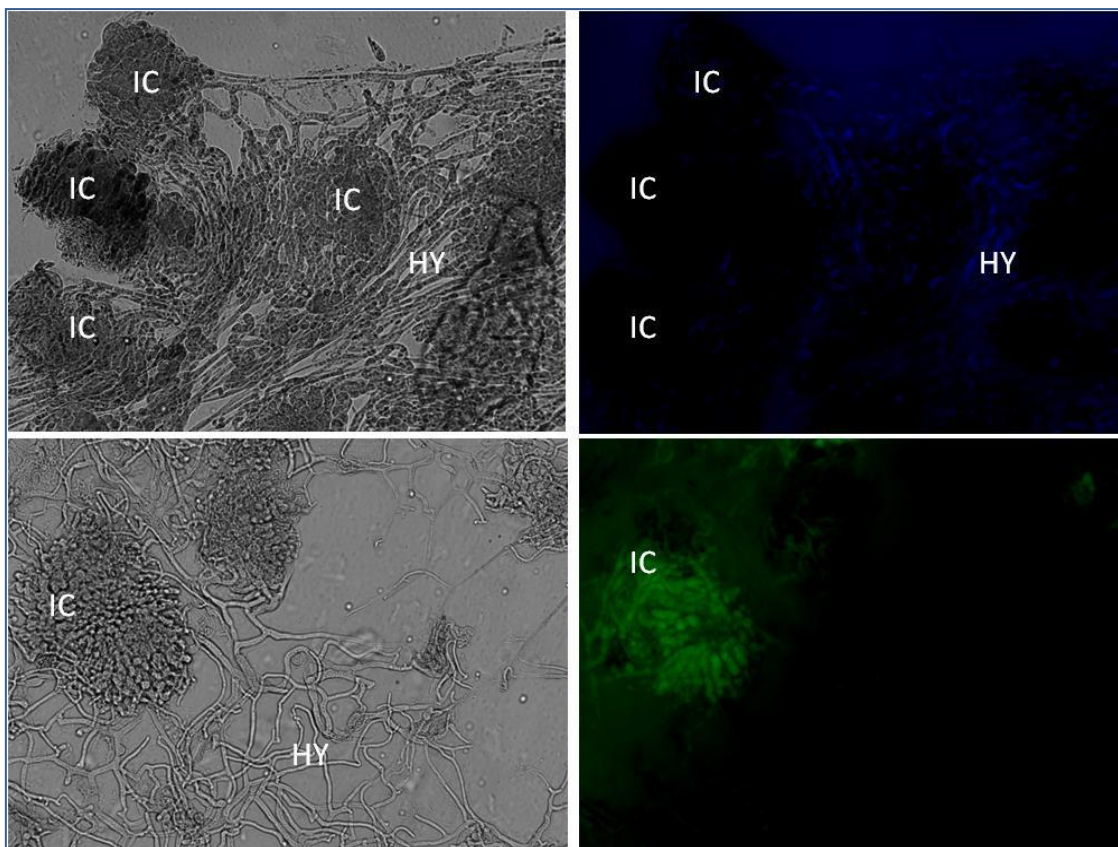

Figure 9. Epifluorescence localization of chitin (blu) and chitosan (green) in *B. cinerea* hyphae (HY) and infection cushions (IC). Chitin is present prevalently along the longitudinal hyphae walls and is abundant at the apex and septa, while chitosan is prevalent in the infection cushion structure.

Genes expression involved in fungal cell wall formation in presence of grape TLP and chitinase

As above reported, the fungal *B. cinerea* cell wall matrix seems to entrap TLP and chitinase. To ascertain if the grape proteins can induce glucan, chitin or chitosan synthesis, an expression analysis of *B. cinerea* α-glucan and β-glucan synthase gene, three chitin synthase genes and three chitin deacetylase genes (Supplementary Tab 1), was performed by RT-qPCR on RNA extracted from mycelia 6 h after treatment with grape proteins. Treatments of fungi with BSA were also performed as a control.

In comparison to the housekeeping gene (β tubulin), expression levels of the α- and β- glucan synthases genes (Bcin08g02140 and Bcin02g06930, respectively) are comparable in *B. cinerea* mycelium treated or not with grape proteins (Fig. 8 A). On the contrary, two chitin synthase genes (Bcin09g01210 and Bcin12g05370) and two chitin deacetylase genes (Bcin11g04800 and Bcin03g05710) were down regulated after treatment with grape proteins (Fig 8 B and C).
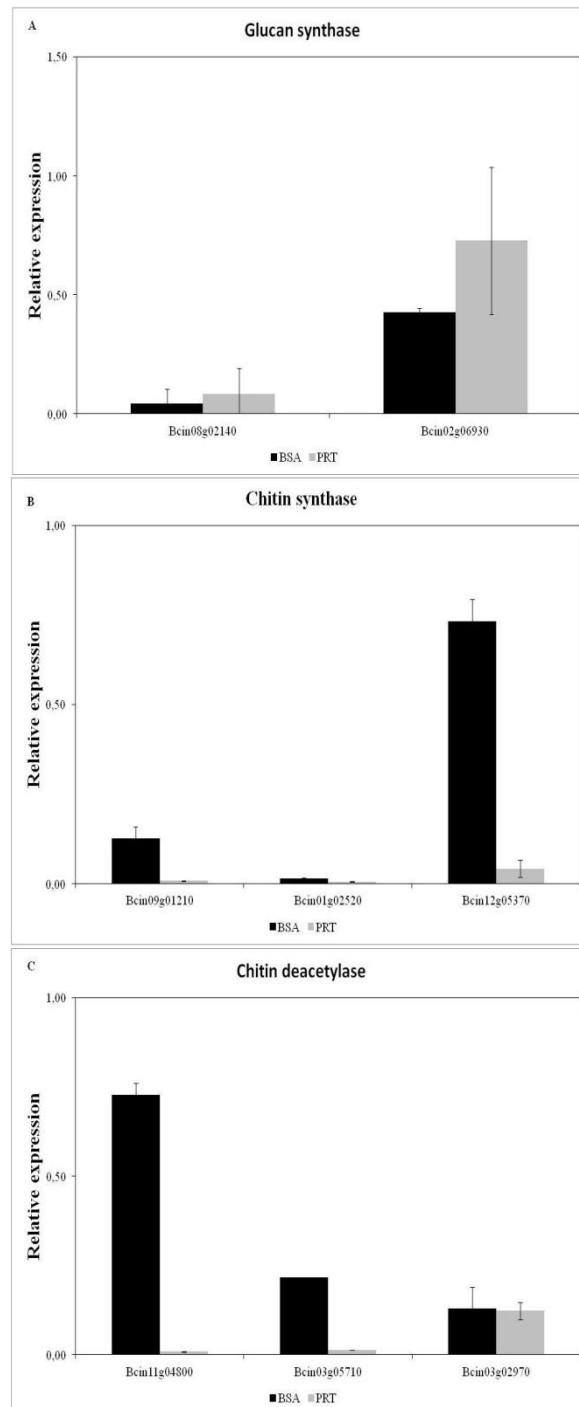
Figure 8. Relative expression level of α and β-glucan synthase genes (A), chitin synthase genes (B) and chitin deacetylase genes (C) of *B. cinerea* after addition of BSA (black) or grape proteins (grey, PRT) to the cultures. The *B. cinerea* mycelium obtained after three days of growth in the Czapek-Dox medium with $NaNO_3$, was transferred into a fresh medium containing 150 μg mL^{-1} of BSA or grape proteins only. After 6 h the RNA was extracted from each mycelium and qPCR was performed with Rotor-Gene Q 2plex (Qiagen GmbH). Each transcript was normalized with the corresponding β-tubulin genes and the relative expression was analyzed by using the Rotor-Gene 2.0.3.2 Software version (Qiagen).

Molecular modeling

Molecular modeling studies were performed to predict a possible interaction of TLP and chitinase with the cell wall polysaccharides of *B. cinerea*. To this aim, the X-ray structure of grape TLP (PDB ID: 4 MBT) was used, while the 3D model of grape type IV chitinase was obtained by homology modeling using the *Bryum coronatum* chitinase (PDB ID 3WH1) co-crystallized with chitin as template. Chitin, chitosan, laminarin, pachyman and scleroglucan were used as ligand.

Docking analysis revealed that all β-glucans polymers (scleroglucan, laminarin and pachyman) can possibly interact with the TLP cleft establishing a network of hydrogen bonds with Gly99, Glu107, Asp120, Cys172. Molecular docking showed that the oligomers of β-glucan tested could also interact with the catalytic domain of chitinase, interacting with the same residues involved in the binding with chitin (His126; Ile137; Asn173; Phe206; Glu231).

When docked to grape chitinase, chitin adopts the same conformation observed in the *Bryum coronatum* chitinase–chitin complex (PDB ID 3WH1). The free energy calculated of chitinase interaction with chitin or with oligomers of β-glucan suggests a stronger binding for chitin.

Interestingly, based on the results of free energy calculated with a docking experiment performed with chitosan or chitin as ligand and chitinase as receptor, the interaction between chitin and chitinase has higher affinity compared to the chitosan-chitinase interaction (Fig. 9).
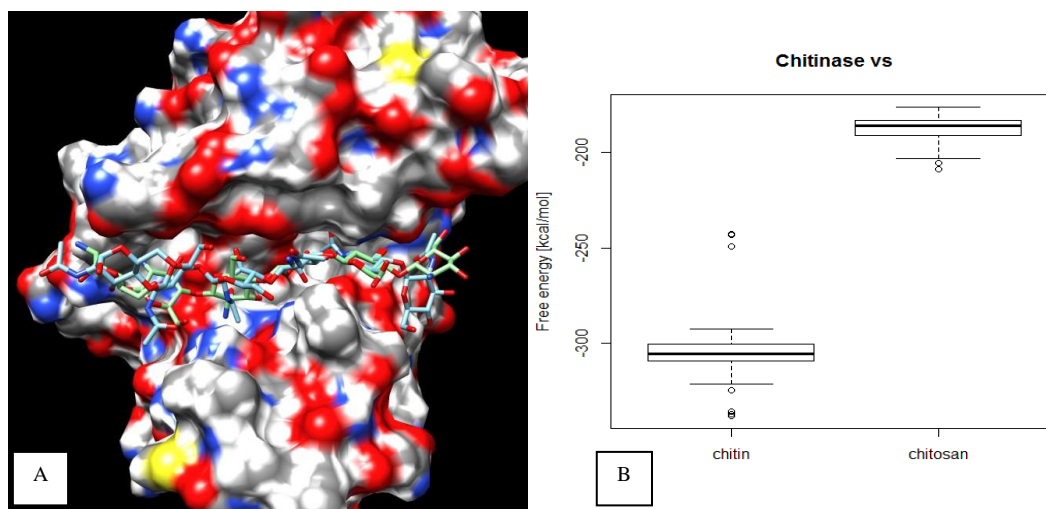

Fig. 9. (A) Binding mode for chitin (β-(1→4) N-acetyl D – glucosamine) and chitosan (β 1-4 D-glucosamine) to chitinase (O24530_VITVI) obtained with docking modeling. The three-dimensional representation is reported; the protein surface is colored according to the lipophilicity propensity given by the atoms forming the surface (magenta, green and white represent respectively hydrophilic, lipophilic and neutral regions) and the chitin and chitosan are represented as sticks. (B) Boxplot of free-energy (kcal/mol) calculated for docking models between chitinase and chitin or chitosan.

Discussion

TLPs and chitinase are the major PR proteins present in grape berries (Vincenzi et al., 2011). However, the *in vitro* growth of *B. cinerea*, one of the main pathogens of grape, is unaffected when these proteins are supplemented to the medium. This result could be explained by the observation that *B. cinerea* is able to remove these proteins from the culture broth. Moreover, TLP and chitinase do not seems to inhibit *B. cinerea* growth since its mycelium development was similar to that obtained in the medium with inorganic nitrogen.

Aim of this work was to identify the mechanisms exploited by the fungus to escape from PR proteins effect. We first investigated the contribution of the protease activity that was induced by grape proteins added to the growth medium. TLP appears completely refractory to fungal proteolysis while grape chitinase is degradable by *B. cinerea* proteases. In particular, when the grape chitinase was incubated with *B. cinerea* proteases a new band of lower size was formed in SDS-PAGE likely due to a protease cleavage. Several authors have suggested a role of fungal proteases in the cleavage of PR proteins (Manteau et al., 2003; Poussereau et al., 2001a and 2001b; ten Have et al., 2004 and 2010) and, recently, several metalloproteases of fungal plant pathogens (fungalysin family), possibly with the contribution of a serine protease (Karimi Jashni et al., 2015), were reported as enzymes that cleave class IV of plant chitinases (Naumann et al., 2011; Naumann and Price, 2012; Karimi Jashni et al., 2015). However, no gene orthologous to fungalysin was found in the genome of *B. cinerea* and metalloprotease activity was not produced by *B. cinerea* in presence of grape proteins as shown by a specific enzyme inhibition assays. Instead, the inhibition assay showed that aspartic protease and serine protease activities were present in the fungal medium containing grape proteins. In agreement with this result, previous work showed that the major protease secreted in the medium containing hydrolyzed casein by *B. cinerea* is an aspartic protease (BC1G_03070) and a tripeptidyl protease (serine-like) (BC1G_02944) (da Chuna, 2012). The aspartic protease was previously designated as Bcap8 and described as the most expressed *B. cinerea* protease both *in vitro* and *in planta* (ten Have et al., 2010; Billon-Grand et al., 2012; Li et al., 2012). Van Sluyter et al. (2013) documented that the heterologously expressed Bcap8 can degrade the class IV grape chitinase. Conversely, it has been reported that Bcap8 is not responsible for the cleavage of class I and IV of tomato chitinases. For that reason, the actual contribution to grape class IV chitinase degradation by *B. cinerea* proteases needs to be still clarified.

Since the activity of the secreted fungal protease does not explain the amount of protein subtracted by *B. cinerea* from the culture, we hypothesized that the fungal mycelium could passively contribute to the removal of TLP and chitinase. Fungi possess a complex and dynamic multilayered

cell wall (Cantu et al., 2009) composed of α- and β-glucans, chitin, chitosan and glycomannoproteins (Bowman and Free, 2006). As shown by desorption experiments, the polysaccharides composing the *B. cinerea* cell wall showed a capacity to absorb the grape TLP and chitinase, since significative amount of TLP and chitinase was released after fungal cell wall digestion with β-1,3-glucanase. To identify which type of polysaccharide could possess binding capacity for TLP and chitinase, several polysaccharides were incubated with the grape proteins and the residual proteins remaining in solution were evaluated. Pachyman (a β 1→ 3 glucan) did not show any ability to bind TLP and chitinase, while chitin and to a lesser extent chitosan (a β 1-4 D-glucosamine) showed the capacity to bind chitinase as expected considering that chitin is the substrate of chitinase while chitosan is structurally modified chitin. Laminarin (a β 1→3 with sporadically β 1→6 glucan) and scleroglucan (β [1→3]₃, 1→6 glucan) showed the ability to interact with TLP and chitinase. Scleroglucan is particularly efficient in the binding of the grape proteins and the binding was maintained even after a prolonged treatment with β-1,3-glucanase. This indicates a strong interaction between the two PR-proteins and the scleroglucan backbone or its fragments, probably for the regular spacing of the β 1→6 side chains of this polysaccharide. In fact, pachyman that have a linear glucan structure was completely ineffective in binding the two proteins. Instead, a binding between barley TLP (Trudel et al., 1998) and some fruit TLPs with linear β-1,3-glucan was demonstrated and molecular models able to explain this interaction was constructed (Osmond et al., 2001; Menu-Bouaouiche et al., 2003). Therefore, the absence of binding between pachyman and the grape proteins may depend by the different characteristics of the PR proteins analyzed. However, a docking analysis did not show any particular difference between the interaction of grape TLP and chitinase with scleroglucan or other glucan polymers.

The observation that the fungal glucan matrix sequesters the TLP and chitinase suggested verifying whether these proteins induce the expression of glucan synthase genes, thus increasing the glucan formation. β-glucan synthase gene of *B. cinerea* was previously demonstrated as constitutively expressed during the vitro growth, during the formation and maturation of infections cushions (Choquer, personal communication) and during grape tissue infection (data not shown). Following grape proteins treatments *B. cinerea* did not show a significant up-regulation of the β glucan synthase gene indicating that the synthesis of new glucan is likely independent from the presence of stressful plant proteins. Whether the binding of TLP and chitinase with this glucan matrix of the fungal cell wall is a requisite for its activity or, on the other hand, interferes with its activity (Trudel et al., 1998), still remains to be clarified. Recently, α-glucan, which cannot be hydrolyzed by β-1,3-glucanase, has been reported as a fungal defense mechanism involved in the

protection of β-glucan degradation and necessary for expression of fungal virulence (Fujikawa et al., 2012). However, also in this case, *B. cinerea* did not show a significant up-regulation of putative α glucan synthase gene following grape proteins treatments.

The fluorescence localization of polymeric compounds on the fungal cell wall provided interesting features on the modification of cell wall during the formation of infection cushions. While the β-glucan matrix surrounds both hyphae and infection cushions of *B. cinerea*, a different localization of chitin and chitosan were observed. In particular, chitin is present prevalently along the longitudinal hyphae walls while chitosan (the deacetylated form of chitin) is localized prevalently in infection cushions. Molecular docking studies showed that the affinity of chitinase for chitosan is lower than that for chitin. Based on this observation, it is possible that the removal of acetyl groups of chitin may result in the reduction of plant chitinase activity and thus of the lysis of the fungal cell wall. Besides, the reduced chitinase activity can also prevent the release of lower size oligomers that can be recognized by plant chitin receptors activating plant defence responses. Therefore, *B. cinerea* can escape plant recognition changing the composition of its cell wall and the deacetylation of chitin observed in *B. cinerea* infection cushion could protect the fungus from the action of plant chitinase.

The genome of *B. cinerea* contains three genes encoding putative chitin deacetylase enzymes (Bcin03g02970, Bcin11g04800 and Bcin03g05710). These genes are up-regulated during the maturation of *B. cinerea* infection cushions (Choquer, personal communication). The conversion of surface-exposed chitin to chitosan in cell walls of in vitro- and in vivo- differentiated infection structures of other plant pathogenic fungi has already been demonstrated (El Gueddari et al., 2002). It is worth noting that two of these chitin deacetylase genes (Bcin11g04800 and Bcin03g05710) are drastically down regulated after grape proteins treatment. A down regulation was also observed studying the expression of three genes coding for chitin synthase after treatment of *B. cinerea* mycelium with grape proteins. These three chitin synthase genes are essential for full virulence of *B. cinerea* (Choquer et al. 2004; Soulié et al., 2003 and 2006). The role of fungal chitin synthase was generally associated with hyphae development, growth and conidiation (Ichinomiya et al., 2002; Roncero, 2002). Therefore, the alteration of expression of fungal chitin synthase and deacetylase points out a new effect of these PR proteins that deserve further insights. It is possible, however, that *B .cinerea,* during the interaction with the host, reduces its levels of chitin in an attempt to reduce the release of biologically active oligomers produced by plant chitinase.

In conclusion, results obtained depict some mechanisms carried out by *B. cinerea* to escape the action of grapeTLP and chitinase:

a) *B. cinerea* can cleave the plant chitinase by its protease activity. However, protease activity is ineffective against TLP.

b) β glucan sheath, a layer surrounding vegetative hyphae and infection cushion, absorbing plant TLP and chitinase could avoid these proteins reach their targets, i.e. the fungal plasma membrane and the chitin fibers, respectively.

c) *B. cinerea* can escape plant recognition changing its cell wall structure. Specifically, the reduction of expression of chitin synthase genes after PR proteins treatment and the deacetylation of chitin observed in *B. cinerea* infection cushion could mask the fungus surface avoiding or delaying the recognition by the host.

Supplementary Materials

Table S1. Oligonucleotide primers used in this study.

Gene Code	Protein	Forward	Reverse
Bcin09g01210	Chitin synthase I	CTTACTACCAACCTCCATACCA	TACCGTCCCGTCATAACCA
Bcin01g02520	Chitin synthase II	GTGAATCTGAAGAAGTCCAGGT	CTGTCGTCTCCTTTCCATCC
Bcin12g05370	Chitin synthase III	GTAACGACAGACCTACTCCAC	TACCAACTTTCACAACGACCA
Bcin11g04800	Chitin deacetylase I	GTCCTTACATCTACACCTCCC	AATGCCATCTCGTTCTTCCA
Bcin03g05710	Chitin deacetylase II	AACAGCCGTTCAAGACTACC	TACTTTGAGATGAGACTGCGA
Bcin03g02970	Chitin deacetylase III	ATCATCCACATCCTCAACCAG	CAAAGCCCTTCTGACATCCA
Bcin08g02140	α glucan synthase	TTCCAACCAGACAATCGAAATCC	GACCTGCTTTCAATGTAACCTCC
Bcin02g06930	β glucan synthase	TTGACCAGAGAATTGAAGACCGA	TCACAGAATGAAGCTTGTCAACC
Bcin01g08040	β tubulin*	GTCTCAAGATGTCCTCCACC	ACTCCATCTCGTCCATACCT
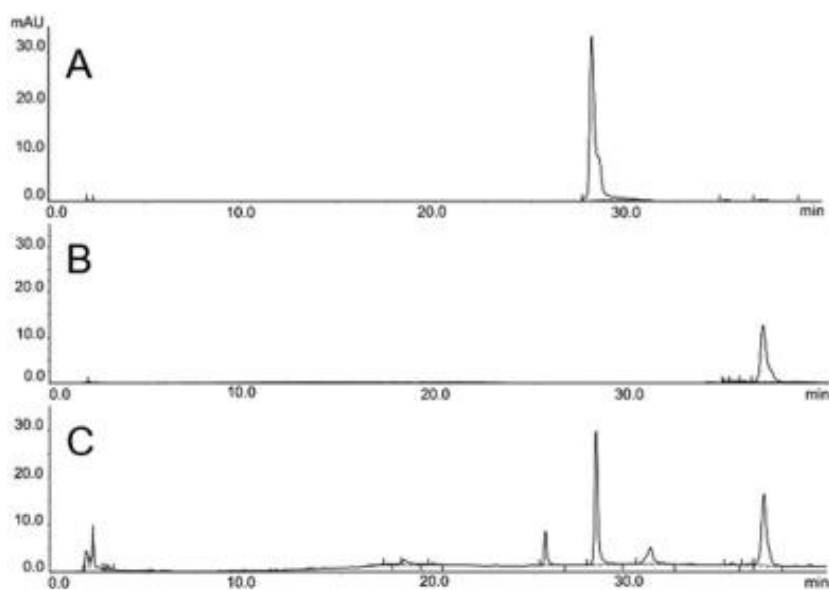
Figure S1. RP-HPLC patterns of 6 μg of purified TLP (A) and chitinase (B) or 15 μg of grape proteins (C). Elution conditions are reported in Material and methods.

Table S1. Amino acid identity of class IV chitinase (O24530_VITVI) with other plant chitinases performed by BLAST (http://blast.ncbi.nlm.nih.gov) analysis using default parameters.

Description (Name Protein and Organism)	Identity	Accession
class IV endochitinase precursor [*Vitis vinifera*]	100%	NP_001268031.1
class IV chitinase precursor [*Vitis vinifera*]	98%	NP_001268075.1
class IV endochitinase [*Vitis vinifera*]	96%	AAB65777.1
endochitinase PR4-like [*Nelumbo nucifera*]	75%	XP_010268233.1
chitinase 6-like [*Phoenix dactylifera*]	71%	XP_008801201.1
unknown [*Populus trichocarpa*]	72%	ABK95688.1
Endochitinase EP3 [*Ananas comosus*]	71%	OAY71751.1
hypothetical protein TSUD_111210 [*Trifolium subterraneum*]	69%	GAU21981.1
basic endochitinase CHB4-like [*Populus euphratica*]	71%	XP_011002656.1
endochitinase EP3-like [*Prunus mume*]	69%	XP_008222565.1
class IV chitinase [*Pyrus x bretschneideri x Pyrus pyrifolia*]	69%	ACM45716.1
hypothetical protein POPTR_0019s12360g [*Populus trichocarpa*]	71%	XP_002326040.2
endochitinase PR4-like [*Fragaria vesca subsp. vesca*]	69%	XP_004309832.1
hypothetical protein POPTR_0013s12880g [*Populus trichocarpa*]	69%	XP_006376419.1
endochitinase EP3-like [*Malus domestica*]	68%	XP_008369197.1
homolog of carrot EP3-3 chitinase [*Arabidopsis thaliana*]	70%	NP_191010.1
unnamed protein product [*Coffea canephora*]	70%	CDP10828.1
endochitinase EP3 [*Daucus carota subsp. sativus*]	70%	XP_017219532.1
hypothetical protein AMTR_s00066p00199930 [*Amborella trichopoda*]	71%	ERN20351.1
hypothetical protein PRUPE_ppa009844mg [*Prunus persica*]	68%	XP_007222515.1
endochitinase PR4-like [*Eucalyptus grandis*]	69%	XP_010055735.1
class IV chitinase [*Corylus heterophylla*]	69%	AEM97876.1
class IV chitinase [*Nicotiana tabacum*]	68%	BAF44533.1
hypothetical protein POPTR_0019s12390g [*Populus trichocarpa*]	70%	XP_002326042.1
unnamed protein product [*Coffea canephora*]	70%	CDP21309.1
hypothetical protein POPTR_0019s12380g [*Populus trichocarpa*]	70%	XP_002326041.2
endochitinase PR4-like [*Nicotiana sylvestris*]	68%	XP_009777773.1

Table S2. Amino acid identity of mature sequence without signal peptide of grape TLP (O04708_VITVI) with other plant chitinase performed by BLAST (http://blast.ncbi.nlm.nih.gov) analysis using default parameters.

Description (Name Protein and Organism)	Identity	Accession
VVTL1 [*Vitis vinifera*]	100%	AAB61590.1
thaumatin-like protein [*Actinidia eriantha*]	84%	AGC39182.1
thaumatin-like protein [*Actinidia chinensis*]	83%	AGC39181.1
thaumatin-like protein [*Actinidia deliciosa*]	83%	AGC39180.1
hypothetical protein [*Eucalyptus grandis*]	82%	KCW77573.1
thaumatin-like protein [*Sambucus nigra*]	81%	AAK59278.1
PR- proteins [*Solanum tuberosum*]	81%	XP_006364119.1
protein NP24-like [*Eucalyptus grandis*]	82%	XP_010053305.1
PR – proteins [*Capsicum annuum*]	79%	XP_016551680.1
thaumatin-like protein [*Sesamum indicum*]	79%	XP_011072290.1
putative thaumatin-like protein [*Solanum tuberosum*]	80%	AAU95246.1
PR - protiens [*Capsicum annuum*]	79%	XP_016551686.1
Thaumatin-like protein [*Camellia sinensis*]	78%	ABE01396.1
thaumatin-like protein 1 [*Ricinus communis*]	80%	XP_002509748.1
thaumatin-like protein [*Sambucus nigra*]	80%	AAK59276.1
PR – proteins [*Nicotiana tabacum*]	79%	NP_001312145.1
thaumatin-like protein 1 [*Ricinus communis*]	78%	XP_002509749.1
hypothetical protein [*Manihot esculenta*]	78%	OAY56579.1
PR protein [*Solanum tuberosum*]	80%	XP_006364121.2
unnamed protein product [*Coffea canephora*]	78%	CDP16242.1
PR protein [*Nicotiana tabacum*]	77%	NP_001311972.1
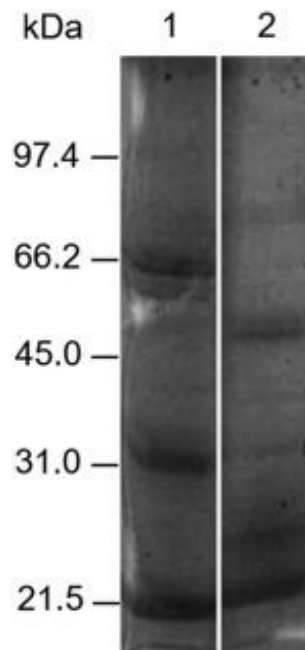
Figure S2. SDS-PAGE of 100 µl aliquot of *B. cinerea* (2) culture grown for five days in the Czapek-Dox medium containing 150 µg mL^{-1} of grape proteins. As a control, a 100 µl aliquot containing 15 µg of grape proteins was loaded on lane 1. The gel was stained with a colloidal Coomassie G250 protocol. Molecular weight markers (Low range, Bio-Rad Laboratories) are indicated on the left.
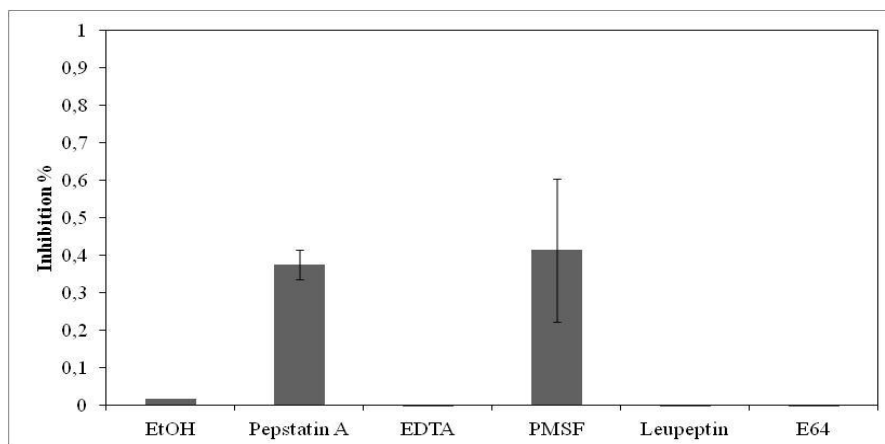
Figure S3. Effects of specific protease inhibitors on protease activity produced by *B. cinerea* grown on grape proteins. The percentage of inhibition is reported on y-axis. The values are average of three experiments ±SD.
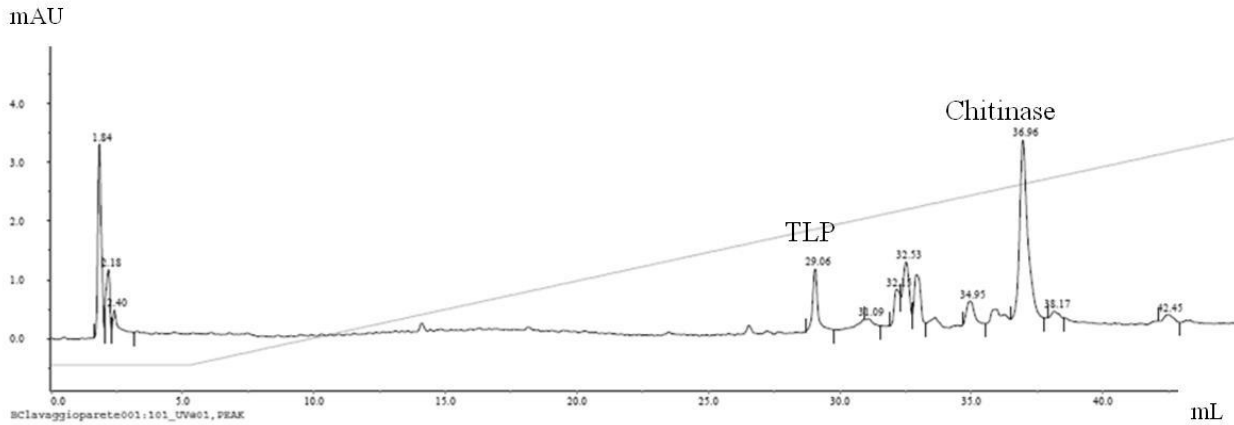
Figure S4. RP-HPLC patterns of 100 µl aliquots of SDS-alkaline washing fluids of *B. cinerea* mycelia collected after five days of growth in presence of 150 µg mL^{-1} of grape proteins. This experiment was replicated three times obtaining similar results. The labels correspond to elution times of TLP and chitinase.
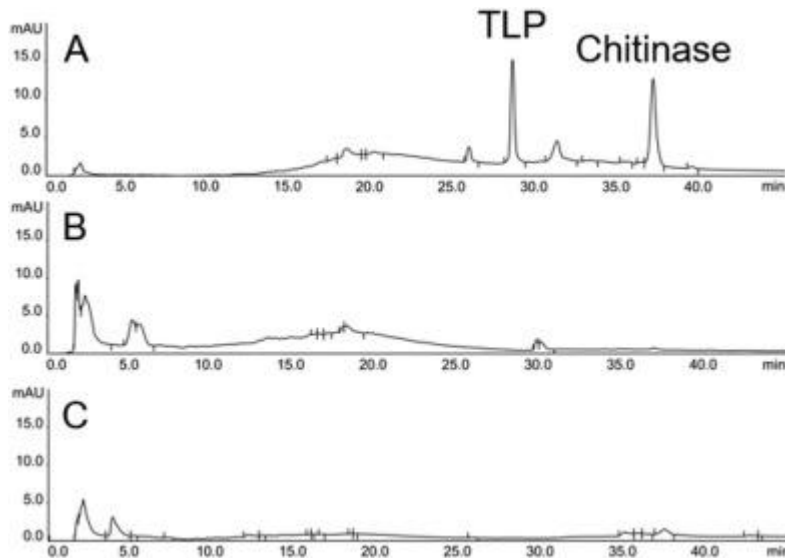
Figure S5. RP-HPLC patterns of a grape protein sample (150 µg mL-1) after 16 h of incubation with scleroglucan (5 mg mL^{-1}). After centrifugation the mixture separated into two fractions: a gelatinous pellet and a viscous supernatant. Both fractions were treated with laminarinase (containing β-1,3-glucanase) for 24 h. One hundred µl aliquots of the β-1,3-glucanase treated supernatant (B) and pellet (C) were loaded on the RP-column. A 100 µl aliquot of the same protein sample without scleroglucan was loaded as a control (A). This experiment was replicated two times obtaining similar results.
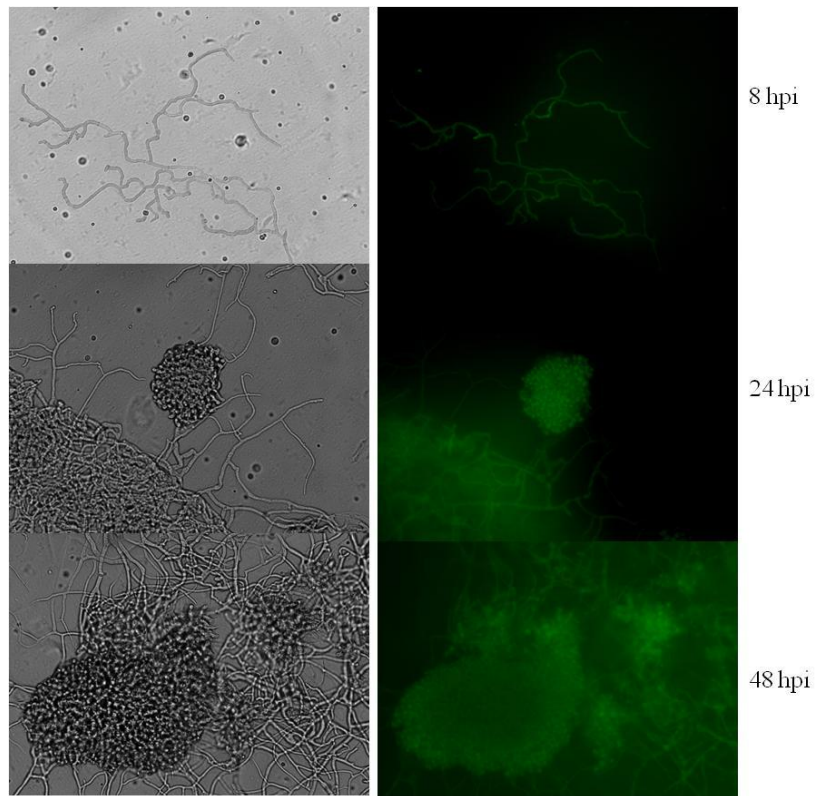
Figure S6. Epifluorescence localization of β-glucan in *B. cinerea* hyphae and infection cushions stained with Aniline Blue fluorochrome. β glucan is present long the longitudinal hyphae walls and infection cushion structure. The three pictures were taken 8, 24 and 48 hours post inoculation. All pictures were taken using the same magnification.

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CHAPTER 2. Inhibitory effect and quantitative-structure-activity relationship analysis of sixteen plant secondary metabolites against *Botrytis cinerea*.

ABSTRACT

Botrytis cinerea is a necrotrophic fungal plant pathogen responsible of the gray mold disease. To prevent gray mold several Plant Secondary Metabolites (PSMs), an important plant defence molecules against fungal infection, are possibly used as alternative to synthetic fungicides. The goal of this work is to evaluate the antifungal activity of sixteen PSMs against *B. cinerea* and analyse the chemical features related to their activity. The antifungal activity of PSMs was determined in agarized cultures with *B. cinerea*. Among PSMs tested, only thymol, eugenol, cinnamaldehyde, isoeugenol and carvacrol were strong inhibitors of *B. cinerea* growth. These five compounds caused also release of cellular material from *B. cinerea* mycelium, indicating possible cell membrane damages, and inhibited *B. cinerea* conidia germination. QSAR (Quantitative Structure–Activity Relationship) study reveals that Hy (Hydrophilic factor), tPSA (polar surface area), AMR (molar refractivity) and HBD (hydrogen bond donors) are correlated to biological activity of compounds. Eugenol, thymol, cinnamaldehyde, isoeugenol, and carvacrol have negative Hy value, relative low tPSA value and one HBD count. These parameters suggest that the more inhibiting compounds have affinity for the lipophilic structure of fungal cell and they might have the cell membrane as their principal target. Furthermore, the absence of synergistic effects between these five compounds and the similar values of AMR suggest that eugenol, thymol, cinnamaldehyde, isoeugenol, carvacrol, could have a common targets. According to the descriptors obtained in this QSAR study, the anti-*Botrytis* activity of PSMs can be predicted by their molecular properties and structural characteristics. These results could be employed to predict the anti-*Botrytis* activity of other PSMs in the search for new alternatives or complementary strategies to combat gray mold disease.

RIASSUNTO

Botrytis cinerea è un patogeno necrotrofo agente eziologico della malattia nota come muffa grigia. Per contrastare le perdite causate da questo fungo, numerosi composti del metabolismo secondario delle piante (PSM) sono stati proposti come alternativa ai fungicidi di sintesi. Inoltre, queste molecole sono considerate una importante barriera di difesa delle piante contro le infezioni fungine. L'obiettivo di questo lavoro è valutare l'attività antifungina di sedici PSM contro *B. cinerea* e analizzare le caratteristiche chimiche legate alla loro attività tramite uno studio QSAR (Quantitative Structure-Activity Relationship). L'attività antifungina di PSM è stata determinata studiando l'effetto di questi composti sulla crescita in vitro del micelio di *B. cinerea*. Tra i PSM testati, solo il timolo, eugenolo, cinnamaldeide, isoeugenolo e carvacrolo si sono dimostrati significativi inibitori della crescita miceliare di *B. cinerea* e della germinazione dei suoi conidi. Inoltre, questi cinque composti hanno causato anche un rilascio di materiale cellulare dal micelio di *B. cinerea*, indicando eventuali danni alla membrana cellulare. Lo studio QSAR ha rivelato che caratteristiche chimiche come Hy (fattore idrofilo), tPSA (superficie di area polare), AMR (refrattarietà molare) e HBD (donatori legame idrogeno) sono correlati all'attività biologica dei composti. Eugenolo, timolo, cinnamaldeide, isoeugenolo, e carvacrolo hanno valore negativo di Hy, un valore relativamente basso di tPSA e valori di uno o zero di HBD. Questi parametri suggeriscono che i composti più attivi nell'inibire la crescita di *B. cinerea* hanno affinità per le strutture lipofiliche della cellula fungina e potrebbero avere il loro target nel plasmalemma. Inoltre, l'assenza di effetti sinergici tra questi cinque composti e i valori simili di AMR suggeriscono che eugenolo, timolo, cinnamaldeide, isoeugenolo, carvacrolo, potrebbero interagire con gli stessi bersagli. Secondo i descrittori ottenuti in questo studio QSAR, l'attività anti-botritica dei PSM può essere predetta dalle loro proprietà chimiche. Questi risultati potrebbero essere impiegati per predire l'attività botriticida di altri PSMs nella ricerca di nuovi composti alternativi di lotta.

Introduction

Botrytis cinerea is a necrotrophic fungal pathogen attacking important crops at all latitudes worldwide. This pathogen is widely studied for its ability to cause rot on a number of fruits and vegetables in open field, greenhouse and during storage (Nicot et al., 2016). Diseases by *B. cinerea* are described as gray mold on vegetables or bunch rot on grapevine. In general, synthetic fungicides are used for the control of *B. cinerea* but eco-toxicological considerations have generated interest in the prevention of gray mold by using eco-friendly, non-toxic and not persistent compounds (Wilson et al., 1997). The European Union directive gives priority to non-chemical methods and encourages the use of natural and non-toxic substances (Sustainable Use Directive 2009/128/EC). Among these alternative compounds, several Plant Secondary Metabolites (PSMs) have been proposed for the control of gray mold (Archbold et al., 1997; Romeo et al., 2015; Couderchet, 2015). Recently, some of these compounds have been authorized for field utilization in EU (Registration n. 16480/PF). PSMs are important not only for their possible use in crop protection, but also because these types of compounds have a crucial role in the innate immunity of plants against fungal pathogens (Kim et al., 2014). Indeed, PSMs (or their precursors) are present in high concentrations in skin of fruits and they are considered barrier against fungal infection (Pierpoint, 2000; Pusztahelyi et al., 2015). Therefore, these two aspects, i.e. the relevance in plant defence and practical exploitation in plant protection, makes these compounds particularly interesting for studying the chemical characteristics related to their biological activity.

Biocidal activity of PSMs has been determined against various plant pathogens, including *B. cinerea* (Bishop et al., 1997; Daferera et al., 2003; Tripathi et al., 2008; Romanazzi et al., 2016) and molecular properties of some PSMs related to their activity against fungal pathogens such as *Fusarium verticillioides* and *Aspergillus parasiticus* have been identified (Dambolena et al., 2012; Pizzolitto et al., 2015). Biological effects of PSMs may be predicted by QSAR (Quantitative Structure–Activity Relationship) modelling as reported for various biological systems (Dambolena et al., 2011, 2012; Pizzolitto et al., 2015; Greenberg et al., 2008) but QSAR studies of PSMs against *B. cinerea* have not yet been performed.

The aim of this work was to determine the effect of sixteen PSMs (caffeic acid, carvacrol, catechin, cinnamaldehyde, emodin, estragole, eugenol, ferulic acid, isoeugenol, p-coumaric acid, p-hydroxybenzoic acid, protocatechuic acid, quercetin, thymol, vanillin and veratric acid) on *B. cinerea* mycelium growth and conidia germination and to evaluate the molecular descriptors (*logarithm of the octanol/water partition coefficient* -logP-, *apolar desolvation* –APD-, *polar desolvation* –PD-, *hydrogen bond donors* –HBD-, *hydrogen bond acceptors* –HBA-, *polar surface area* –tPSA-, *rotatable bonds* –RB-, *Unsaturation index* –Ui-, *Hydrophilic factor* –Hy-, *Ghose-*

Crippen molar refractivity –AMR-, *3D-Wiener* -3D-W-) which better explain their antifungal activity.

Materials and methods

Plant Secondary Metabolites

Eugenol (4-Allyl-2-methoxyphenol, ≤99% purity), isoeugenol (2-methoxy-4-(prop-1-en-1-yl)phenol, ≤98% purity, cis- and trans- mixture), thymol (5-Methyl-2-(propan-2-yl)phenol, ≤99% purity), carvacrol (2-Methyl-5-(propan-2-yl)phenol, ≤98% purity), cinnamaldehyde (3-Phenylprop-2-enal, ≤95% purity), vannilin (4-Hydroxy-3-methoxybenzaldehyde, ≤98% purity), quercetin (2-(3,4-dihydroxyphenyl)-3,5,7-trihydroxy-4H-chromen-4-one, ≤95% purity), catechin ((2R,3S)-2-(3,4-dihydroxyphenyl)-3,4-dihydro-2H-chromene-3,5,7-triol, ≤98% purity), emodin (1,3,8-trihydroxy-6-methylanthracene-9,10-dione, ≤95% purity), caffeic acid (3-(3,4-Dihydroxyphenyl)-2-propenoic acid, ≤98% purity), ferulic acid ((E)-3-(4-hydroxy-3-methoxy-phenyl)prop-2-enoic acid, 99% purity, trans-), p-hydroxybenzoic acid (≤99% purity), protocatechuic acid (3,4-Dihydroxybenzoic acid, ≤97%), p-cumaric acid ((E)-3-(4-hydroxyphenyl)-2-propenoic acid, ≤98% purity), veratric acid (3,4-Dimethoxybenzoic acid, ≤99% purity**)** and estragole (1-Methoxy-4-(prop-2-en-1-yl)benzene) were purchased from Sigma-Aldrich (NY, USA).

Fungal strain, plate antifungal assay and conidia germination

The fungal pathogens *B. cinerea* (strain B05.10) was grown on Petri dishes containing potato dextrose agar (PDA, BD, Difco, USA) at 25 °C. For culture inoculation, PDA disks (0.4 cm of diameter) were taken from the marginal zone of actively growing colonies.

For spore production, completely colonized plates were incubated under near UV light for 16 h per day. 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 and counted using a haemocytometer.

To evaluate the antifungal activity of compounds against *B. cinerea* mycelium growth, experiments were performed using a modified semisolid agar antifungal susceptibility method (Provine et al., 2000). Briefly, 10 ml of PDA containing the selected compounds, pre-suspended in dimethyl sulfoxide (DMSO) and diluted to a final concentration ranging from 10 to 500 μg mL^{-1}, were poured onto sterile Petri plates (9 cm diameter). As a control, PDA with 0.05% (v/v) DMSO was used. Forty-eight hours after inoculation (hai) with the *B. cinerea* mycelium, the diameters of colonies were measured. The data were obtained from four independent experiments. Forty-eight

hours after inoculation (hai) with the *B. cinerea* mycelium, the diameters of colonies were measured. The data were obtained from four independent experiments.

To evaluate a possible synergistic effect of the molecules, 100 μg mL^{-1} of the most active compounds (eugenol, isoeugenol, thymol, carvacrol, cinnamaldehyde) were assayed together in different combinations against *B. cinerea* growth (Table S1). Data were obtained from two independent experiments.

To study the inhibitory effect on spore germination, conidia of *B. cinerea* were suspended in a Czapek-Dox medium (6.7 mM KCl, 2 mM MgSO$_4$·7 H$_2$O, 7.3 mM K$_2$HPO$_4$, 0.03 mM FeSO$_4$, 10 mM citric acid, 111 mM glucose, pH 5.0±0.1) to obtain a final concentration of 10^6 conidia mL^{-1}. The compounds dissolved in 10% DMSO were added to obtain a final concentration of 500 μg mL^{-1}. Controls were performed with 10% DMSO. After 48 hai at 25°C in the dark, conidia germination was evaluated with a light microscope.

Molecular parameters

To perform QSAR study chemical descriptor parameters (logP, APD, PD, HBD, HBA, tPSA, RB, Ui, Hy, AMR and 3D-W) were selected from MOLE db database (http://michem.disat.unimib.it/mole_db/).

Statistical analysis

The data of mycelia radial growth obtained by plate antifungal assays were elaborated using logit transformation and the hypothetical concentrations that reduce the growth by 50% (Ec50) and 100% (Ec100) were calculated and used in a Multiple Linear Regression (MLR).

MLR analysis was performed in order to examine the quantitative relationships between linear combinations of the Ec50 and Ec100 values, considered as the dependent variables, and the molecular properties of the substances. The resulting MLR models (QSAR models) were checked for linearity, normality and homoscedasticity. In addition, the analysis of variance (ANOVA) was conducted and the model was validated with the root mean square prediction error (RMSPE) obtained by a cross validation leave-one-out procedure. All statistical analyses were calculated by using the R software (https://www.r-project.org/).

Furthermore, a Principal Component Analysis (PCA) was performed in order to establish if significant molecular properties were able to discriminate the most active compounds.

Release of cellular material

Damage to the *B. cinerea* cell membrane was determined after treatment of the fungal mycelium with the PSMs by measuring the release of cytosolic material in the medium (Wang et

al., 2010). Briefly, 50 mL of Czapek-Dox medium in 250 mL Erlenmeyer flasks were inoculated with three PDA disks (0.4 cm diameter) colonized by *B. cinerea* mycelium. The flasks were incubated at 25°C in an orbital shaker (140 rpm) and after 5 days mycelia were harvested by sterile gauze, washed twice with water and re-suspended in 15 mL of sterile water. Eugenol, isoeugenol, thymol, carvacrol, and cinnamaldehyde (at 500 µg mL^{-1}) were added and after 6 hours the samples were centrifuged at 10,000 g at 4°C for 10 min. The release of cellular materials was determined in the supernatants by UV (Absorbance 260 nm) spectroscopy as reported by Lunde et al. (2000). The release of extracellular K$^+$ was determined by atomic absorption spectroscopy (AA-7000, Shimadzu) using KCl as a standard.

Results

Antifungal effects of PSMs against *B. cinerea*

The 16 PSMs tested showed varying levels of antifungal activity against *B. cinerea* (Tab. 1). The hierarchical clustering (Fig. 1 B1) obtained on the basis of Ec50 and Ec100 values, identified three distinct groups of PSMs. Eugenol, isoeugenol, thymol, carvacrol and cinnamaldehyde (with Ec50 comprised between 0.53 and 1.80 mM and Ec100 values comprised between 2.11 and 6 mM) were the most active inhibitors (Table 1). Estragole (Ec50 3.56 mM; Ec100 6.78 mM) and vanillin (Ec50 3.81 mM; Ec100 6.04 mM) were slightly less effective. All these seven compounds formed the most effective group of inhibitors (Fig. 1.B1) and, when tested at the concentration of 500 µg mL^{-1} inhibited completely the *B. cinerea* conidia germination (not shown). Quercetin, emodin and catechin (Fig. 1.B) also affected the fungal growth showing Ec50 values comparable to those of the first group of molecules, but had much higher Ec100 values (Table 1). Instead, caffeic acid, p-cumaric acid, ferulic acid, p-hydroxybenzoic acid, protocatechuic acid and veratric acid (Fig. 1.B) were much less effective against mycelia growth and at 500 µg mL^{-1} the conidia still germinated (not shown). The most active compounds eugenol, isoeugenol, thymol, carvacrol and cinnamaldehyde were also evaluated for a possible synergistic effect. However, for all the pair combinations only additive effects against *B. cinerea* radial growth were recorded (not shown). The additive effect observed means that the combined effect produced by the action of each couple of compounds tested did not exceed the sum of the effects measured using separately the compounds.

Quantitative structure/activity relationships (QSAR)

To perform QSAR study several descriptors (logP, APD, PD, HBD, HBA, tPSA, RB, Ui, Hy, AMR and 3D-W) were selected and the values for each molecule were obtained from MOLE db database (http://michem.disat.unimib.it/mole_db/). The hierarchical clustering based on these

selected chemical features separated the PSMs into three groups (Fig. 1 B2). The analysis groups together molecules with very different inhibitory activity against *B. cinerea*. For example, the most active molecules eugenol and thymol, and their respective isomers isoeugenol and carvacrol, are grouped together with the less active estragole and the poorly active ferulic acid and veratric acid.

MLR analysis were then performed in order to find out the quantitative relationships between the antifungal activities of the PSMs (Ec50 and Ec100 values) and the structural and molecular properties of the compounds tested. Since Ec50 and Ec100 have a relatively low positive correlation each other (R^2=0.804), these two parameters were treated separately and two QSAR models were obtained. Equation (1), obtained considering the Ec50 as dependent variable, produces a model in which 94.43% of total variance ($R^2 = 0.9443$) (Fig. 2 A, Tab. S2) is explained by the three molecular predictors HBD, tPSA and Hy. Equation (2), obtained considering the Ec100 as dependent variable, produces a model in which 85.65% of total variance ($R^2 = 0.8565$) (Fig. 2 B, Tab. S2), was explained by HBD, Hy and AMR.

(1) $[Ec50] = -4.85716 \times [HBD]^a + 0.06371 \times [tPSA]^b + 4.61009 \times [Hy]^c + 5.73684$

(2) $[Ec100] = -15.1088 \times [HBD]^a + 17.8439 \times [AMR]^b + 0.51341 \times [Hy]^c - 1.1791$

The same parameters were obtained also in a MRL model calculated with Ec50 and Ec100 variables transformed in a logarithmic function (log and 1/log) (Tab. S3).
Therefore, the Ec50 and Ec100 equations share the two variables HBD and Hy but differ in the third significant predictor, i.e. tPSA and AMR, respectively. As expected the clustering obtained with the four selected parameter correspond to that obtained on the basis of the antifungal activity (c.f. Fig. 1 B1 with Fig. B2)

In order to demonstrate if the identified molecular properties HBD, AMR, Hy, and tPSA are able to discriminate the more active compounds, a Principal Component Analysis (PCA) was performed (Fig. S2). The plot of this analysis showed that thymol, carvacrol, isoeugenol, eugenol, estragole and cinnamaldehyde are separated from the other less active molecules.

Eugenol, isoeugenol, thymol, carvacrol and cinnamaldehyde release cellular material from fungal cell

Since QSAR analysis of the most inhibiting compounds (eugenol, isoeugenol, thymol, carvacrol, and cinnamaldehyde) of *B. cinerea* growth have selected chemical features related to lipophylicity (specifically Hy), the cell membrane of the fungal cell was regarded as a possible target of these molecules.

To ascertain possible damage to *B. cinerea* plasma membrane following the treatment with these effective inhibitory compounds, the leakage into the medium of K^+ and of intracellular UV-absorbing material was determined after treating the *B. cinerea* mycelium with 500 μg ml^{-1} of each PSM. These assays were used to verify the presence of abnormal material derived from fungal cells exposed to antimicrobial compounds (Wang et al., 2010). Compared to untreated control, all the assayed molecules increased significantly the level of K^+ released in the medium (Fig. 3). All these molecules, except isoeugenol, also induced an abnormal release of intracellular material as determined by measuring the increase of absorbance at 260 nm (data not shown)..

Discussion

In recent years, the search for alternative antifungal compounds has been a major concern and numerous researches are conducted to identify natural compounds active against fungal plant pathogens such as *B. cinerea* (Brito Gamboa et al., 2006; Neppelenbroek et al., 2006, Feliziani et al., 2013). Several of these compounds are PSMs initially studied by plant pathologists as defence molecules produced by plants to counteract fungal pathogens' infection (Dixon et al., 1986; Hain et al., 1993; Curtis et al., 2004; Jeandet et al., 2013). Now some PSMs are proposed and authorized to control *B. cinerea* (Romanazzi et al., 2016). Further research is needed to identify the chemical properties of PSMs underlying their biological activity and to detect their molecular targets on fungal cells.

The first goal of this work was to study the biological activity of sixteen PSMs (caffeic acid, carvacrol, catechin, cinnamaldehyde, emodin, estragole, eugenol, ferulic acid, isoeugenol, p-coumaric acid, p-hydroxybenzoic acid, protocatechuic acid, quercetin, thymol, vanillin and veratric acid) on *B. cinerea* mycelium growth and conidia germination. To evaluate the biological activity of the selected compounds, Ec50 and Ec100 values were obtained for each molecule. Based on Ec50 and Ec100 values, the compounds can be divided into three groups of decreasing inhibitory activity against *B. cinerea* growth and conidia germination. The first group comprises eugenol, thymol, cinnamaldehyde, isoeugenol, carvacrol and, with a lesser activity, vanillin and estragole. These seven PSMs are components of many essential oils and according to their antifungal activities these extracts were successfully experimented and proposed for fungal control in postharvest (Valero et al., 2006; Martínez-Romero et al., 2007; Elshafie et al., 2015). Emodin, quercetin and catechin form a second group of molecules which were unable to inhibit the conidia germination at the maximum concentration tested and were weaker inhibitors of *B. cinerea* mycelium growth. These three compounds are considered important antifungal molecules in plants (Treutter et al., 2006) and this contrast with their low activity in vitro. However, it should be considered that in plant tissue, catechin, emodin and quercetin are present in a glycosylated form (Catechin-7-O-glucoside, emodin glucoside or rutin) (Koyama et al., 2003; Iacopini et al., 2008 and Ojwang et al., 2013) with the glycosidic moiety possibly increasing their biological activity against fungal pathogens. Consistently, we observed that the flavonoid rutin is ten folds more active against *B. cinerea* than its aglycon quercetin (unpublished results). The remaining tested molecules, such as protocatechuic acid, coumaric acid, ferulic acid, caffeic acid, veratric acid and hydroxybenzoic acid, have negligible effects on *B. cinerea* growth. However, these compounds could play other roles in plant defense system. For example, coumaric acid is modified several times in plants and it is the precursor of other PSMs such as lignin related compounds (Sakakibara et al., 2007), while ferulic

acid may interfere with fungal secondary metabolite biosynthetic pathway as demonstrated for the synthesis of the virulence factor deoxynivalenol by some *Fusarium* species (Boutigny et al., 2009).

Among the PSMs chemical features selected for studying the activity of the tested PSMs against *B. cinerea* growth, Ec50 and Ec100 values were correlated only with some descriptors analyzed by QSAR model. To perform QSAR study logP, APD, PD, HBD, HBA, tPSA, RB, Ui, Hy, AMR and 3D-W were selected as chemical descriptors. These parameters are easily found in several free libraries of compounds (for example in Zinc database - http://zinc.docking.org/ -, PubChem database - https://pubchem.ncbi.nlm.nih.gov/ -, or ChEMBL database - https://www.ebi.ac.uk/chembl/ -) or easily calculated with appropriate commercial software. A hierarchical clustering based on selected chemical features was obtained and represented in Fig. 1 B3. These cluster analyses identified three different groups: (G1) emodin, quercetin and catechin; (G2) veratric acid, estragole, eugenol and isoeugenol (isomers), ferulic acid, thymol and carvacrol (isomers), (G3) cinnamaldehyde, vanillin, p-hydroxybenzoic acid, protocatechuic acid, p-cumaric acid and caffeic acid.

Based on results obtained with QSAR analysis, the predictors Hy, tPSA, AMR and HBD resulted significantly correlated with the antifungal activity and a hierarchical clustering based on this activity is consistent with the properties of the identified PSMs (Fig 1B2).

The most active compounds (eugenol, thymol, cinnamaldehyde, isoeugenol, carvacrol, vanillin and estragole) have negative value of Hy, a parameter measuring the level of hydrophilicity (Todeschini et al., 1997) and dependent from the number of hydroxyl groups. Thus, the most active compounds, having none or at most one hydroxyl groups, are quite hydrophobic (Fig. 1). This characteristic determines the ability to penetrate into the plasma membrane (Knobloch et al., 1987; Rasooli et al., 2005). This relationship among antifungal activities of some PSMs and the number of hydroxyl groups attached to benzene rings has also been observed in studies with the fungal pathogens *Sclerotium cepivorum* and *Fusarium verticillioides* (Minambres et al., 2010; Dambolena et al., 2011). Similarly, pterostilbene, a stilbenoid molecule containing an hydroxyl group, is suggested as more active against *B. cinerea* than the analogous resveratrol that, having three hydroxyl groups, is more hydrophilic and less diffusible through the cell membranes (Caruso et al., 2011). It was concluded that there is a positive correlation among antifungal activity of natural and synthetic stilbenes and their hydrophobicity (van Barlen et al., 2004; Caruso et al., 2011). tPSA of a molecule is defined as the surface sum over all polar atoms and it is commonly used to describe the ability of compounds to cross the plasma membrane, then tPSA is considered a good predictor for drug transport inside the cell (Österberg et al., 2000; Ertl et al., 2000) and molecules with a relatively high tPSA can easily permeate the cell membrane entering rapidly into

the cytosol (Ertl, 2008). Our results showing tPSA values of the most active molecules 2-6 fold lower than those of the less active, indicate that an easily permeation of fungal cell membrane is not favourable for an effective activity of the PSMs. A low HBD count is another common element among PSMs with a strong inhibitory activity against *B. cinerea*. This parameter corresponds to value of 1 for eugenol, thymol, carvacrol, isoeugenol and vanillin and 0 for cinnamaldehyde and estragole. Several hydrogen bonds make molecules overly-polar, preventing their spread in a lipophilic environment (Lipinski et al., 1996).

In summary, negative values of Hy, relatively low values of tPSA and of HBD are common features of the most active compounds overall indicating an affinity of these compounds for hydrophobic layer of cell membranes.

AMR is a measure of the total polarizability of a mole of a substance (Padrón et al., 2002) and has been used to explain the electronic effects in chemical–biological interactions and it has been reported to be valuable in the correlation of the allosteric effects in enzyme-ligand interactions (Hansch et al., 2003). AMR was correlated with the antifungal activity of plant phenolic compounds (O'Brien et al., 2003; Voda et al., 2004). The significance of AMR descriptor in our Ec100 model suggests the involvement of the most active compounds in specific interactions with target enzymes. Since eugenol, thymol, cinnamaldehyde, isoeugenol, and carvacrol did not show any synergistic effect against *B. cinerea* mycelium growth and have similar value of AMR, the target enzymes of these compounds might be the same.

Finally, the mathematical expression obtained by the QSAR analysis could be useful for predicting the antifungal activity of other structurally related PSMs. These findings could provide an important contribution in the search for new compounds with antimicrobial activity and in the cataloguing of PSMs activity against the plant pathogenic fungus *B. cinerea*.

Table 1. Experimental values of efficiency of sixteen PSMs tested against *B. cinerea* and deposited molecular descriptors used in QSAR analysis.

	Ec50 (mM)	Ec100 (mM)	xlogP	Apolar desolvation (kcal/mol)	Polar desolvation (kcal/mol)	H-bond donors[1]	H-bond acceptors	tPSA (Å²)	Mol. weight	Rotatable bonds	Unsaturation index	Hydrophilic factor	Ghose-Crippen molar refractivity	3D-Wiener index
Eugenol	0.72	2.11	2.10	3.62	-5.44	1	2	29	164.20	3	3.00	-0.24	45.50	1084.41
Isoeugenol	1.00	5.97	2.38	3.61	-5.57	1	2	29	164.20	2	3.00	-0.24	49.58	1113.31
Thymol	0.53	2.60	3.34	4.34	-3.43	1	1	20	150.22	1	2.81	-0.29	46.98	1173.23
Carvacrol	1.48	3.16	3.82	4.30	-3.43	1	1	20	150.22	1	2.81	-0.29	46.98	1182.78
Cinnamaldehyde	1.79	2.97	2.48	5.49	-7.64	0	1	17	132.16	2	3.17	-0.86	42.21	568.82
Vannilin	3.81	6.04	1.07	1.39	-8.79	1	3	47	152.15	2	3.00	-0.12	40.81	621.53
Caffeic acid	7.39	13.80	0.94	1.43	-49.59	2	4	81	179.15	2	3.17	1.46	46.52	872.67
p-Hydroxybenzoic acid	8.54	12.58	1.37	2.22	-47.43	1	3	60	137.11	1	3.00	0.74	34.51	413.61
Protocatechuic acid	7.98	20.17	0.88	0.14	-48.46	2	4	81	153.11	1	3.00	1.66	36.21	481.57
p-Cumaric acid	7.98	19.23	1.43	2.50	-50.43	1	3	60	163.15	2	3.17	0.59	44.83	753.40
Veratric acid	8.16	17.85	1.50	4.26	-48.19	0	4	59	181.17	3	3.00	-0.13	45.74	996.63
Estragole	3.56	6.78	2.82	5.79	-3.32	0	1	9	148.21	3	3.00	-0.88	46.81	1021.72
Ferulic acid	9.32	15.69	1.25	3.50	-48.23	1	4	70	193.00	3	3.17	0.55	51.29	1179.06
Quercetin	2.62	9.68	1.68	-2.90	-13.58	5	7	131	302.24	1	3.91	2.78	75.43	2605.87
Catechin	1.70	12.60	1.37	-4.94	-11.51	5	6	110	290.27	1	3.70	2.80	72.85	2783.64
Emodin	2.10	12.50	3.01	1.74	-10.81	3	5	95	270.24	0	3.91	1.11	71.28	2106.27
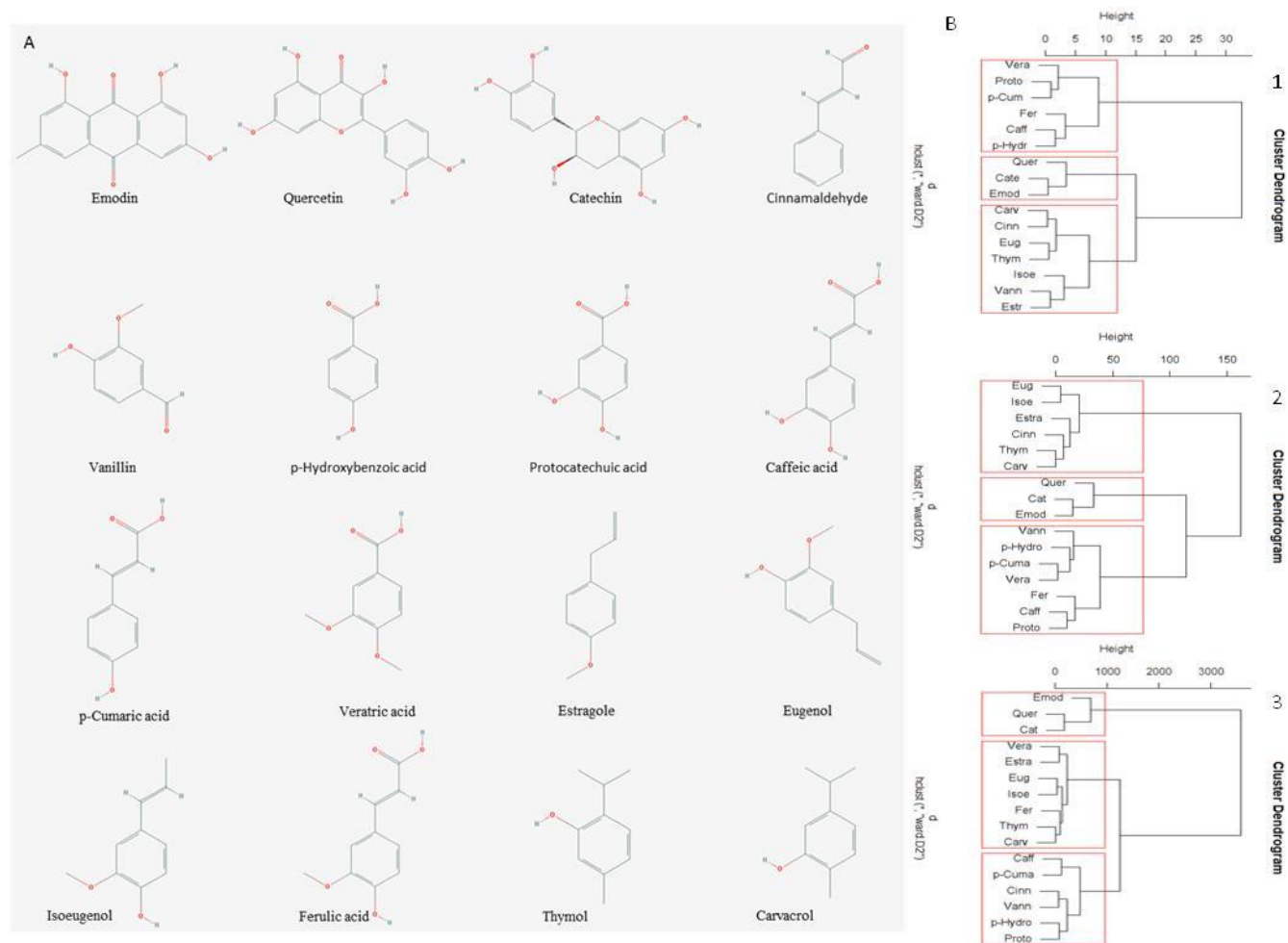
Figure 1. Chemical structures of PSMs tested for their antimicrobial activity against *B. cinerea* (A) and hierarchical clustering (B) based on their radial growth inhibition capacity (B1), on chemical features revealed significant by QSAR study (B2) and based on all chemical features of compounds selected (B3).
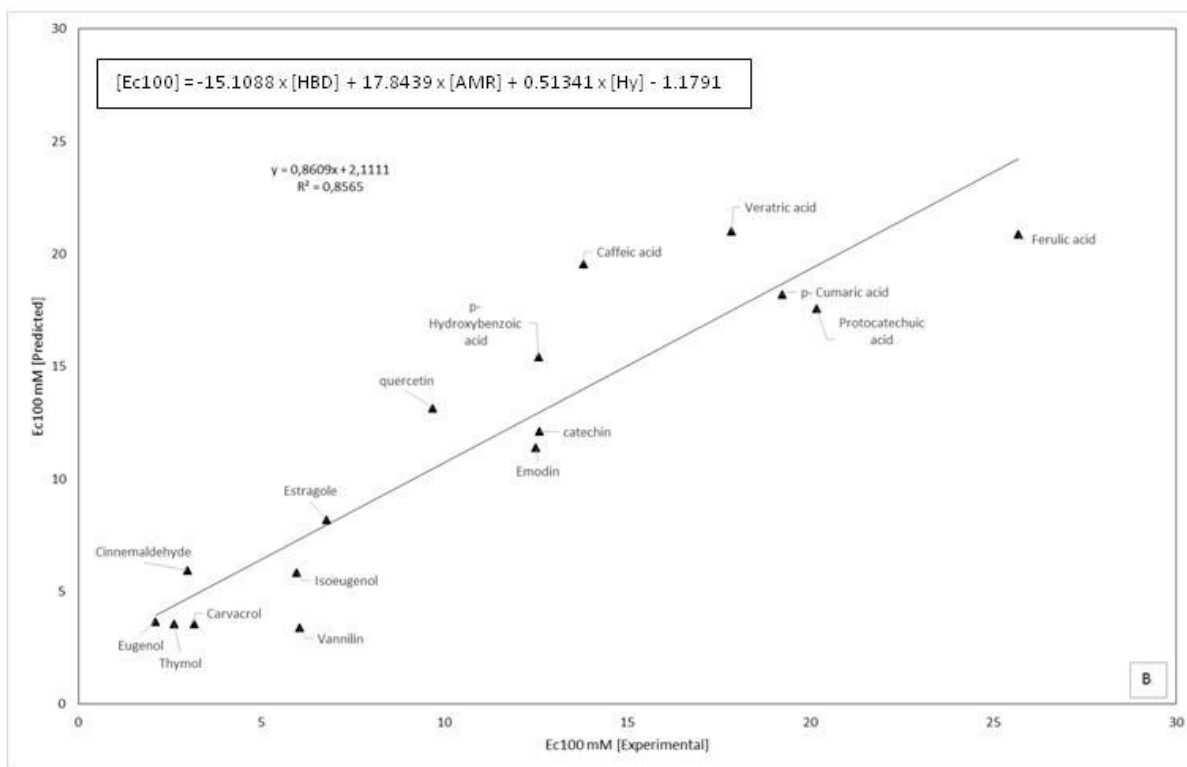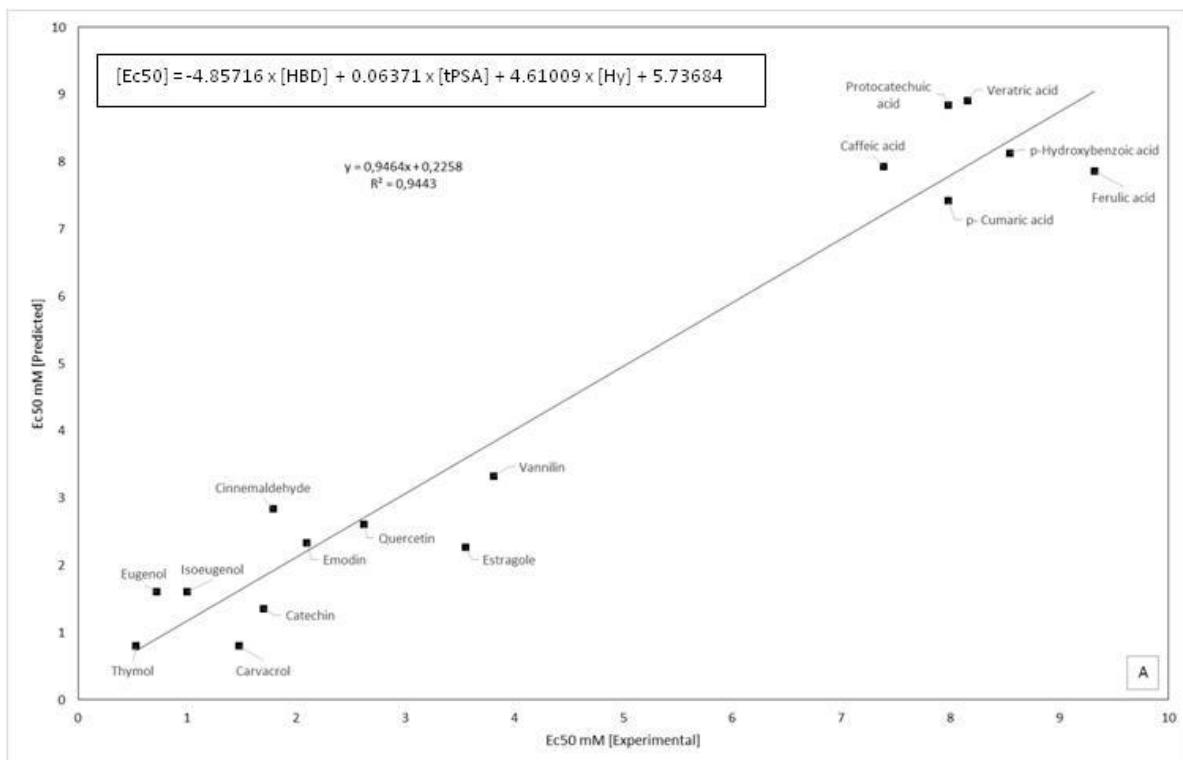
Figure 2. Plot of calculated versus experimental Ec50 (A) and Ec100 (B) of the sixteen PSMs on *B. cinerea* growth. Multiple linear regression analyses (MLR) calculated to examine quantitative relationships between linear combinations of the dependent variables (Ec50 or Ec100) and the physicochemical descriptors (Tab. 1). The obtained QSAR models were checked for linearity, normality and homoscedasticity, and the analysis of variance was conducted.

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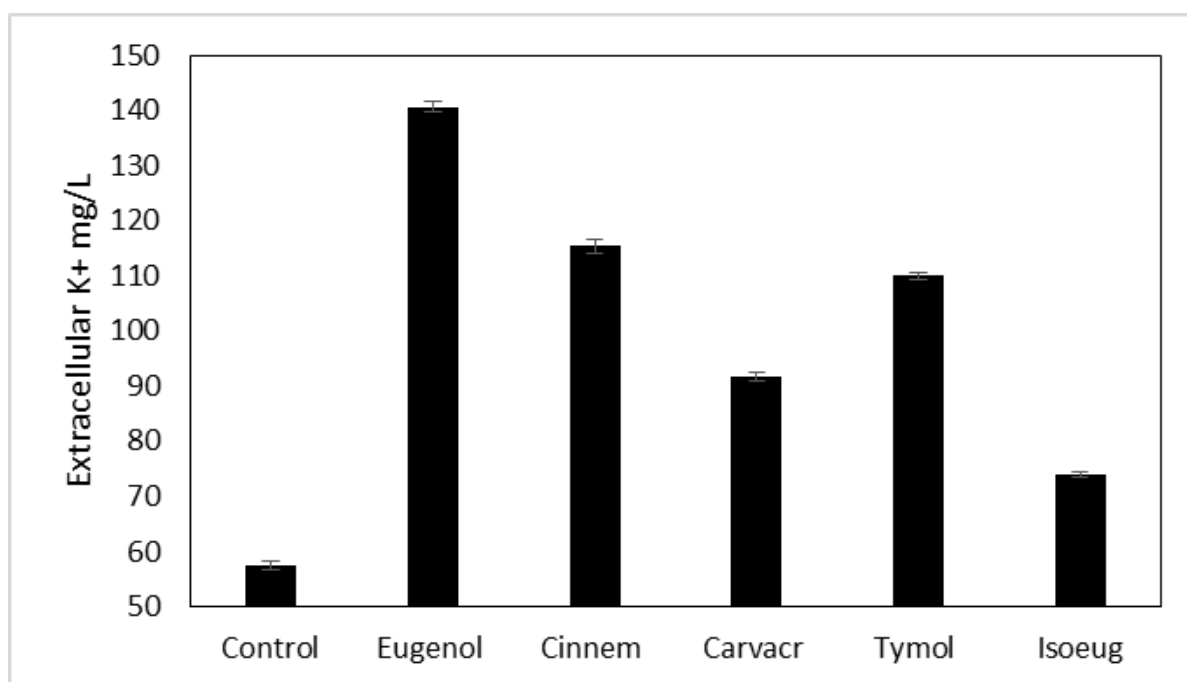
Figure 3. Effects of treatments with the most active PSMs on the release of cytosolic K$^+$ from fungal hyphae. The mycelium of *B. cinerea* was treated with 500 μg/ml of each compound for 6 hours and the relative amount of K$^+$ released was measured and compared with the value of untreated control. Each bar represents the average values from two experiments. The errors bars show the standard deviation.

SUPPLEMENTAL MATERIALS

Tab S1. Experimental design of semisolid agar antifungal susceptibility test used to evaluate synergistic effects of the five more active PSMs tested (eugenol, isoeugenol, thymol, carvacrol, cinnamaldehyde) against *B. cinerea* strain B05.10 growth. The concentrations used were 100 μg mL^{-1} for each compounds. The experiments were repeated twice. In all combination tested no synergistic effect was observed.

	Eugenol	Isoeugenol	Carvacrol	Thymol	Cinnamald.
Control	100 μg mL^{-1}	100 μg mL^{-1}	100 μg mL^{-1}	100 μg mL^{-1}	100 μg mL^{-1}
Eugenol		100 +100 μg mL^{-1}	100 +100 μg mL^{-1}	100 +100 μg mL^{-1}	100 +100 μg mL^{-1}
Isoeugenol			100 +100 μg mL^{-1}	100 +100 μg mL^{-1}	100 +100 μg mL^{-1}
Carvacrol				100 +100 μg mL^{-1}	100 +100 μg mL^{-1}
Thymol					100 +100 μg mL^{-1}

Tab S2. R results obtained after Multiple regression linear (MRL) analyses calculated to examine the quantitative relationships between variables (Ec50 and Ec100) and the predictors with respective analysis of variance (anova).

```
Call:
lm(formula = Ec50 ~ HBD + tPSA + Hy)
Residuals:
   Min    1Q Median    3Q    Max
-1.0653 -0.6499 -0.1144 0.5062 1.4449
Coefficients:
            Estimate Std. Error t value Pr(>|t|)
(Intercept) 5.73684   0.96338   5.955 6.66e-05 ***
HBD        -4.85716   0.37159 -13.071 1.85e-08 ***
tPSA        0.06371   0.02001   3.185 0.007855 **
Hy          4.61009   0.81517   5.655 0.000106 ***
---
Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
Residual standard error: 0.8706 on 12 degrees of freedom
Multiple R-squared: 0.9439,   Adjusted R-squared: 0.9299
```

F-statistic: 67.28 on 3 and 12 DF, p-value: $8.936e^{-08}$

```
> anova(model)
Analysis of Variance Table
Response: Ec50
          Df  Sum Sq Mean Sq  F value   Pr(>F)
HBD        1   6.507   6.507   8.5848 0.0126044 *
tPSA       1 122.244 122.244 161.2681 2.567e-08 ***
Hy         1  24.244  24.244  31.9832 0.0001064 ***
Residuals 12   9.096   0.758
---
Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
> coefficients(model)
(Intercept)       HBD       tPSA          Hy
 5.73683956 -4.85715834 0.06371282 4.61008720
> confit(model, level=0.95)
Errore: non trovo la funzione "confit"
> confint(model, level=0.95)
                  2.5 %     97.5 %
(Intercept)  3.63782569  7.835853
HBD         -5.66677717 -4.047540
tPSA         0.02012168  0.107304
Hy           2.83398468  6.386190
Call:
lm(formula = Ec100 ~ HBD + Hy + AMR)
Residuals:
   Min    1Q Median    3Q    Max
-3.8044 -1.7969 0.0447 1.2647 3.4314
Coefficients:
            Estimate Std. Error t value Pr(>|t|)
(Intercept)  2.9287     4.0616   0.721 0.48468
HBD        -11.7201     1.8029  -6.501 2.93e-05 ***
Hy          14.8614     1.7641   8.424 2.21e-06 ***
AMR          0.3529     0.1124   3.140 0.00854 **
---
Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
Residual standard error: 2.486 on 12 degrees of freedom
Multiple R-squared: 0.8702,   Adjusted R-squared: 0.8377
```

F-statistic: 26.81 on 3 and 12 DF, p-value: $1.324e^{-05}$

```
> anova(model)
Analysis of Variance Table
Response: Ec100
          Df Sum Sq Mean Sq F value   Pr(>F)
HBD        1  22.43   22.43  3.6280 0.081059 .
Hy         1 413.91  413.91 66.9463 2.984e-06 ***
AMR        1  60.94   60.94  9.8566 0.008539 **
Residuals 12  74.19    6.18
---
Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
```

Tab S3. R results obtained after Multiple regression linear (MRL) analyses calculated to examine the quantitative relationships between logarithm of depend variables (Ec50 and Ec100) and the predictors.

Call: lm(formula = log(Ec50) ~ HBD + tPSA + Hy)	Call: lm(formula = log(1/Ec50) ~ HBD + tPSA + Hy)
Residuals:	Residuals:
Min 1Q Median 3Q Max	Min 1Q Median 3Q Max
-0.66263 -0.19849 0.00992 0.15810 0.85836	-0.85836 -0.15810 -0.00992 0.19849 0.66263
Coefficients:	Coefficients:
Estimate Std. Error t value Pr(>|t|)	Estimate Std. Error t value Pr(>|t|)
(Intercept) 1.029757 0.477274 2.158 0.0519 .	(Intercept) -1.029757 0.477274 -2.158 0.0519 .
HBD -1.235226 0.184091 -6.710 2.17e-05 ***	HBD 1.235226 0.184091 6.710 2.17e-05 ***
tPSA 0.025654 0.009912 2.588 0.0237 *	tPSA -0.025654 0.009912 -2.588 0.0237 *
Hy 0.965050 0.403850 2.390 0.0342 *	Hy -0.965050 0.403850 -2.390 0.0342 *
---	---
Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1	Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
Residual standard error: 0.4313 on 12 degrees of freedom	Residual standard error: 0.4313 on 12 degrees of freedom
Multiple R-squared: 0.8368, Adjusted R-squared: 0.7959	Multiple R-squared: 0.8368, Adjusted R-squared: 0.7959
F-statistic: 20.5 on 3 and 12 DF, p-value: 5.147^{-05}	F-statistic: 20.5 on 3 and 12 DF, p-value: 5.147e-05
Call:	all:
lm(formula = log(Ec100) ~ HBD + Hy + AMR)	lm(formula = log(1/Ec100) ~ HBD + Hy + AMR)
Residuals:	Residuals:
Min 1Q Median 3Q Max	Min 1Q Median 3Q Max
-0.53478 -0.27086 0.02173 0.25043 0.53430	-0.53430 -0.25043 -0.02173 0.27086 0.53478
Coefficients:	Coefficients:
Estimate Std. Error t value Pr(>|t|)	Estimate Std. Error t value Pr(>|t|)
(Intercept) 0.86871 0.56660 1.533 0.151157	(Intercept) -0.86871 0.56660 -1.533 0.151157
HBD -1.39870 0.25150 -5.561 0.000124 ***	HBD 1.39870 0.25150 5.561 0.000124 ***
Hy 1.77781 0.24609 7.224 1.05e-05 ***	Hy -1.77781 0.24609 -7.224 1.05e-05 ***
AMR 0.04919 0.01568 3.137 0.008584 **	AMR -0.04919 0.01568 -3.137 0.008584 **
---	---
Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1	Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
Residual standard error: 0.3469 on 12 degrees of freedom	Residual standard error: 0.3469 on 12 degrees of freedom
Multiple R-squared: 0.8348, Adjusted R-squared: 0.7935	Multiple R-squared: 0.8348, Adjusted R-squared: 0.7935
F-statistic: 20.21 on 3 and 12 DF, p-value: 5.528^{-05}	F-statistic: 20.21 on 3 and 12 DF, p-value: 5.528e-05

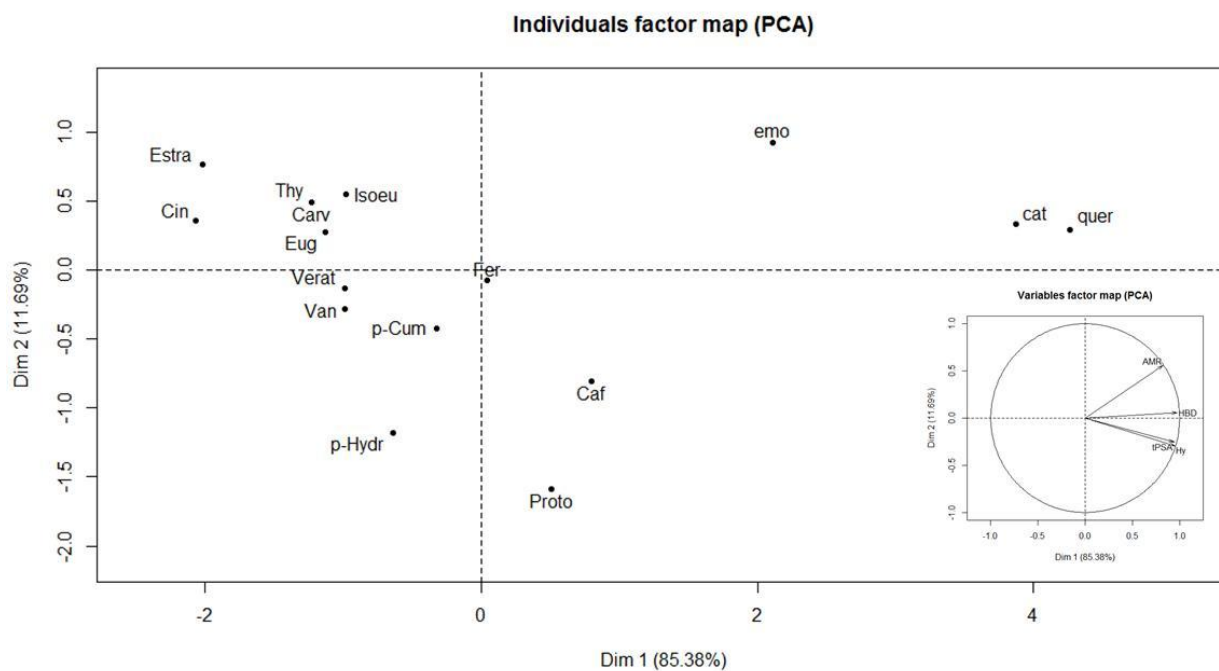**Individuals factor map (PCA)**

Fig S1. Principal component analysis (PCA) based on selected properties of 16 natural compounds. Score plot of the significant molecular parameters (tPSA, Hy, HBD, AMR) and tested compounds into the plane defined by the first two principal components (Dim2 against Dim1).

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RINGRAZIAMENTI

14/11/2016
Riccardo Marcato

"Quando ci preparavamo per venire a Cuba, ci dicevamo sempre che l'uomo più lento della guerriglia era quello che stabiliva la velocità. Dovevamo marciare tutti al passo del più lento, perché certamente non potevamo abbandonarlo. Ed è per questo che ognuno di noi cercava di rafforzare le proprie gambe per non restare ultimo."

Ernesto Guevara

Discorso della riunione plenaria dell'industria zuccheriera, 13 aprile 1962