THE ROLE OF GRAPE POLYPHENOLS ON *TRANS*-RESVERATROL ACTIVITY AGAINST *BOTRYTIS CINEREA* AND OF FUNGAL LACCASE ON THE SOLUBILITY OF PUTATIVE GRAPE PR PROTEINS

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SUMMARY

The necrotrophic fungus *Botrytis cinerea* is the causal agent of grey mould disease on grapevine. In contact with mature grape berries, the fungus encounters an environment particularly rich in polyphenols and proteins, where the stilbenic phytoalexin *trans*-resveratrol may accumulate. Some grape proteins are structurally and functionally related to plant pathogenesis-related proteins. To mimic conditions similar to those found in grape berries, *B. cinerea* was grown *in vitro* with proteins and polyphenols extracted from mature grapes, and with *trans*-resveratrol. Results showed that in the presence of highly toxic amounts of *trans*-resveratrol, grape polyphenols allowed total recovery of fungal growth, and proteins allowed partial recovery. These resveratrol-polyphenol or resveratrol-protein combinations also induced a strong release into the medium of laccase activity, which is likely to be involved in *trans*-resveratrol detoxification. The protein pattern changed during fungal growth; most grape proteins quickly disappeared from the culture when polyphenols and *trans*-resveratrol were present together. Similar protein patterns were obtained *in vitro* by incubating grape proteins with grape polyphenols and/or *trans*-resveratrol with a purified *B. cinerea* laccase. Under these conditions, most proteins became insoluble. The grape protein pattern obtained from grape berries infected by *B. cinerea* strongly resembled that obtained *in vitro* by incubating grape proteins and polyphenols with fungal laccase. It seems that *B. cinerea*, through laccase secretion and activity and by exploiting the berry polyphenols, easily neutralizes the toxicity of grape stilbenic phytoalexins and makes the grape pathogenesis-related proteins insoluble.

Key words: stilbenic phytoalexins, grey mould, PR proteins, polyphenols.

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INTRODUCTION

Botrytis cinerea causes grey mould disease on grapevine, resulting in loss of grape production and wine quality. *B. cinerea* kills the host plant tissue to acquire the nutrients necessary for growth and reproduction. This process is assisted by the secretion of cell-wall-degrading enzymes and phytotoxin compounds (Williamson *et al.*, 2007). However, to colonize the plant tissue successfully, *B. cinerea* must also neutralize several plant anti-fungal compounds; in the case of grape berries, these compounds are primarily the stilbenic phytoalexins and pathogenesis-related (PR) proteins.

Stilbenic phytoalexins accumulate primarily in the skin of grape berries (Jeandet *et al.*, 1991; Montero *et al.*, 2003; Fornara *et al.*, 2008) and *trans*-resveratrol, a 3,5,4'-trihydroxystilbene is the predominant compound. The amount of *trans*-resveratrol is largely influenced by the grapevine genotype (Li *et al.*, 2006) and is induced by *B. cinerea*, among other fungi (Montero *et al.*, 2003). *Trans*-resveratrol is the precursor of *trans*-dehydrodimers and *trans*-ε-viniferin, both obtained through a coupling reaction catalyzed by plant peroxidase and phenoloxidases, or by fungal laccase (Pezet, 1998; Breuil *et al.*, 1999; Nicotra *et al.*, 2004). In *B. cinerea* cultures, *trans*-resveratrol induces a specific *B. cinerea* laccase gene, and *trans*-ε-viniferin is believed to be the toxic conversion product of *trans*-resveratrol in infected grapevine (Pezet, 1998; Schouten *et al.*, 2002). Thus, by way of laccase activity, *B. cinerea* would, paradoxically, self-intoxicate (Schouten *et al.*, 2002). In contrast, other research has suggested that *B. cinerea*, through laccase activity, degrades *trans*-resveratrol (Sbaghi *et al.*, 1996; Adrian *et al.*, 1998), and the role of *B. cinerea* laccase in grape infection thus remains undecided.

Grapevine proteins may also contribute to protecting berries from fungal infection. Proteome and transcriptome analyses have shown that ripening grape berries constitutively contain large amounts of chitinase, β-1,3-glucanase, osmotin, and thaumatin-like proteins, normally considered PR proteins (Pocock *et al.*, 2000; Sarry *et al.*, 2004; da Silva *et al.*, 2005; Monteiro *et al.*, 2007). These proteins inhibit conidial germination and mycelial growth (Derckel *et al.*, 1998; Monteiro *et al.*,

2003a). During host infection, *B. cinerea* expresses several proteases thought to be involved in virulence by degrading plant PR proteins (Schulze Gronover *et al.*, 2004), although *B. cinerea* is reported to degrade grape proteins with difficulty (Marchal *et al.*, 2006); grape proteins are considered to be particularly resistant to enzymatic proteolysis (Waters *et al.*, 1992).

Several studies have investigated the interactions of some plant proteins and polyphenols. Both groups of compounds may interact, forming non-covalent and covalent bonds, a process influenced by the properties of specific proteins and polyphenols, by the level of oxidation of polyphenols, and by the pH (Siebert *et al.*, 1996; Charlton *et al.*, 2002; Kroll *et al.*, 2003; Rawel *et al.*, 2005; Prigent *et al.*, 2007). Grape berries contain large amounts of polyphenolic compounds, such as catechins, flavonols, and hydroxybenzoic and hydroxycinnamic derivatives (Borbalan *et al.*, 2003); in grape juice, some of these may interact with grape proteins to form a colloidal complex (Siebert, 2006).

During berry infection, it is likely that *B. cinerea* develops in an environment in which PR proteins, *trans*-resveratrol, and other polyphenols come into contact with each other. The effects on fungal growth of the interaction among these three factors remain to be investigated.

The aim of this research was to study whether proteins, *trans*-resveratrol, and the other polyphenols extracted from grape berries and used separately or concurrently in fungal cultures affect *B. cinerea* growth and laccase production. Possible alterations in the grape protein pattern in *B. cinerea* cultures were also investigated and compared with those obtained during grape berry infection and *in vitro* with a purified *B. cinerea* laccase.

MATERIALS AND METHODS

***B. cinerea* cultures.** *B. cinerea* strain PM-10 isolated from grape was kindly provided by Prof. Giuseppe Firrao, University of Udine (Italy). The fungus was grown on Petri dishes on potato dextrose agar (PDA, Difco, USA) at 24°C. For spore production, completely colonized plates were incubated under near UV light for 16 h as reported by Schouten *et al.* (2002). After 15 days, conidia were collected into 5 ml of sterile water by gently scraping the plates with a glass rod. Conidia were filtered through sterilized gauze and counted using a haemocytometer. Liquid cultures were produced in a modified Czapek-Dox medium (2 g l^{-1} $NaNO_3$, 0.5 g l^{-1} KCl, 0.5 g l^{-1} $MgSO_4 \cdot 7H_2O$, 1 g l^{-1} K_2HPO_4, 0.01 g l^{-1} $FeSO_4$, 1 ml of a solution of 1% $ZnSO_4$ plus 0.5% $CuSO_4$, and 20 g l^{-1} glucose), adjusted to pH 3.5 with tartaric acid (about 10 mM), which was sterilized by autoclaving. The medium was prepared at twice working concentration, and 2 ml aliquots were plated onto Petri dishes (3 cm diameter) and diluted 1:2 with filter-sterilized stock solutions of grape proteins, grape polyphenols, *trans*-resveratrol (Sigma-Aldrich, USA. Purity >99%), and/or water, and with the concentrated spore suspension to obtain a final concentration of 10^4 conidia ml^{-1}.

Absence and presence of each factor (proteins, polyphenols, and *trans*-resveratrol) were compared in a factorial design. Proteins, polyphenols, and *trans*-resveratrol were supplied at 100 μg ml^{-1}, 200 μg ml^{-1}, and 200 μg ml^{-1}, respectively. The *trans*-resveratrol stock solution (25 mg ml^{-1}) was in 95% ethanol, and the same amount of ethanol (0.8%, v/v) was added to the cultures not treated with *trans*-resveratrol. Crystals formed when *trans*-resveratrol was added at the beginning of the experiment, but the compound dissolved during the course of culturing. A higher concentration of ethanol (4%, v/v) has been recommended to increase the solubility of *trans*-resveratrol (Adrian *et al.*, 1998), but was not used because this concentration reduced growth of the fungus. Each treatment was replicated three times.

The cultures were maintained in the dark at 24°C for 4 days. Aliquots of 100 μl were harvested daily from each culture to assess laccase activity and the protein pattern. At the end of the experiment, the content of each culture medium was transferred into 5-ml preweighed tubes and centrifuged at 12,000 × *g* for 30 min. In addition, each plate was rinsed with 3 ml of water, which was then added to each tube, and the mycelium mat was briefly vortexed and centrifuged again. The supernatant was discarded, and the tubes were oven dried at 80°C for 3 days and then weighed.

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

For inoculation, the berries were detached from the bunch and surface-sterilized with 99% ethanol for 1 min, then rinsed with sterile water. A 3×2 mm piece of PDA, cut from the margin of an actively growing fungal colony, was placed on a small wound created on the berry surface with a razor blade. The berries were placed on a moist filter paper and closed in a plastic bag at about 22°C. After 7 days, the inoculated berries appeared extensively brownish, and were stored at -20°C until extracted. Berries inoculated with non-colonized PDA were incubated in a similar way and used as the control. Healthy berries and naturally infected ones with grey mould symptoms were harvested from the vineyard at the beginning of November 2008.

Grape protein extraction and analysis. Proteins were extracted from about 1 l of grape juice obtained by

hand crushing about 1.6 kg of berries at 4°C in a beaker. The grape juice was immediately filtered through a nylon gauze, and cysteine was added to give a final concentration of 4 mM to prevent polyphenol oxidation. All successive operations were also performed at 4°C. The juice was centrifuged at 30,000 × *g* for 30 min, and the supernatant was filtered in succession through glass-microfibre discs (GMD) and cellulose acetate filters (0.8 µm) (Sartorius, Germany) and dialyzed overnight (membrane cut off =10 kDa) against 10 mM potassium tartrate buffer, pH 3.5. The dialyzed material was adjusted to 20% saturation (106 g l^{-1}) with $(NH_4)_2SO_4$ and stirred for 1 h. After centrifugation at 30,000 × *g* for 30 min, the supernatant was filtered in succession through 0.8, 0.45, and 0.2 µm membranes, concentrated 8 times with a VivaFlow 5000 apparatus (Sartorius, Germany), and loaded on a Sephadex G-25 column (PD-10, GE Healthcare, UK). The protein was eluted with water and further concentrated with a VivaFlow 5000 apparatus and then assayed for protein (Bradford, 1976) and phenol (Folin-Ciocalteau assay) amounts using BSA and gallic acid as the standards, respectively. The protein recovered, about 20 µg g^{-1} of berry fresh weight, was approximately one-third that measured initially in the grape juice. The estimated weight ratio between protein and phenols in the final protein preparation was about 5:1. This protein was used for *B. cinerea* cultures and *in vitro* assays.

The protein profile was analyzed after precipitation of the protein with four volumes of cold ethanol at -20°C for 2 h. The pellet was washed once with cold 70% ethanol. After centrifugation at 12,000 × *g* for 15 min, the precipitated protein was air dried and resuspended in the sample buffer (Laemmli, 1970), and separated by sodium dodecyl sulphate gel electrophoresis on 16% (w/v) polyacrylamide gels (SDS-PAGE). The gel was stained with the colloidal Coomassie G-250 blue silver method (Candiano *et al.*, 2004).

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

Grape phenol extraction. Once crushed and divested of seeds, the solid grape residue was mixed with potassium metabisulphite (0.5 g kg^{-1} of fresh grape) and stored at -20°C. Polyphenols were extracted by the method of Kammerer *et al.* (2004) with some modifications. A total of 50 g of the stored material was stirred with four volumes of methanol/0.1% HCl (v/v) for 2 h under nitrogen at room temperature. The extract was filtered through filter paper and vacuum dried using a Rotavapor at 30°C. The residue was dissolved in 100 ml of acidified methanol, centrifuged at 8,600 × *g* for 20 min, and dried again. The residue was dissolved in 40 ml of deionized water brought to pH 3.5 with HCl. The aqueous suspension was centrifuged at 8,600 × *g* for 15 min. The supernatant was filtered through 0.45 µm membranes, and 10 ml aliquots were loaded onto SPE DSC-18/6 ml tubes (Supelco, USA) equilibrated with deionized water. After washing with 5% methanol in water, the phenols were eluted with absolute methanol and concentrated using the Rotavapor, as described above. The residue was dissolved in 2 ml of deionized water, and the phenol concentration was determined by the Folin-Ciocalteau assay, using gallic acid as a standard. About 500 µg of phenols per gram of grape fresh weight were obtained, and stored at -20°C until required.

***B. cinerea* laccase production, purification, and assay**. *B. cinerea* laccase was extracted as described by Slomczynski *et al.* (1995). After culturing for 7 days, the 50-ml contents of several 250 ml Erlenmeyer flasks were pooled and filtered through GMD and then 0.8 and 0.45 µm cellulose acetate membranes (Sartorius, Germany). The filtrates were dialysed against deionized water, concentrated to 40 ml using a VivaFlow 5000 apparatus, adjusted to pH 6.0 with 10 mM potassium tartrate, and loaded onto a Q-sepharose column (16×120 mm, GE Healthcare, UK). Bound protein was eluted with a 60 min linear gradient of 0-0.5 M NaCl dissolved in 10 mM Potassium tartrate buffer.

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

Laccase activity was determined spectrophotometrically as described by Wolfenden and Willson (1982) in a volume containing 0.7 ml of 0.1 M acetate buffer, 0.1 ml of 2 mM 2,2'-azinobis-(3-ethylbenzthiazoline-6-sulphonate) (ABTS) substrate, and variable volumes of samples (1-30 µl). The assay was performed at 30°C by monitoring the A_{420}. One laccase unit (U) was defined as the amount of enzyme that oxidizes 1 µmol ABTS per min.

Polyphenols, protein and *trans*-resveratrol interactions. Grape proteins, grape polyphenols, and *trans*-resveratrol were mixed in the same weight ratio of 1:2:2 as used for the *B. cinerea* cultures. Grape proteins (50 µg ml^{-1}), *trans*-resveratrol (100 µg ml^{-1}), and polyphenols (100 µg ml^{-1}) were dissolved in 0.1 M potassium-tartrate buffer pH 3.5 in the presence or absence of purified laccase (0.02 U ml^{-1}). After 24 h of incubation at 24°C, 100 µl aliquots of the mixtures were centrifuged at 16,000 × *g* for 20 min, and the pellet containing the precipitated protein was washed once with cold 70%

ethanol. The protein in the supernatant (soluble protein) was precipitated with 4 volumes of cold ethanol at -20°C for 2 h and then washed once with cold 70% ethanol. Both soluble and insoluble proteins were recovered by centrifugation and air dried. Proteins were run on SDS-PAGE gels and stained as described above.

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

RESULTS

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

Mycelia were collected and dried at day 4 of culture. In both experiments, the dry weights were higher than in untreated controls (zero level of each factor) when proteins were supplied alone, in combination with polyphenols, or with both polyphenols and *trans*-resver-

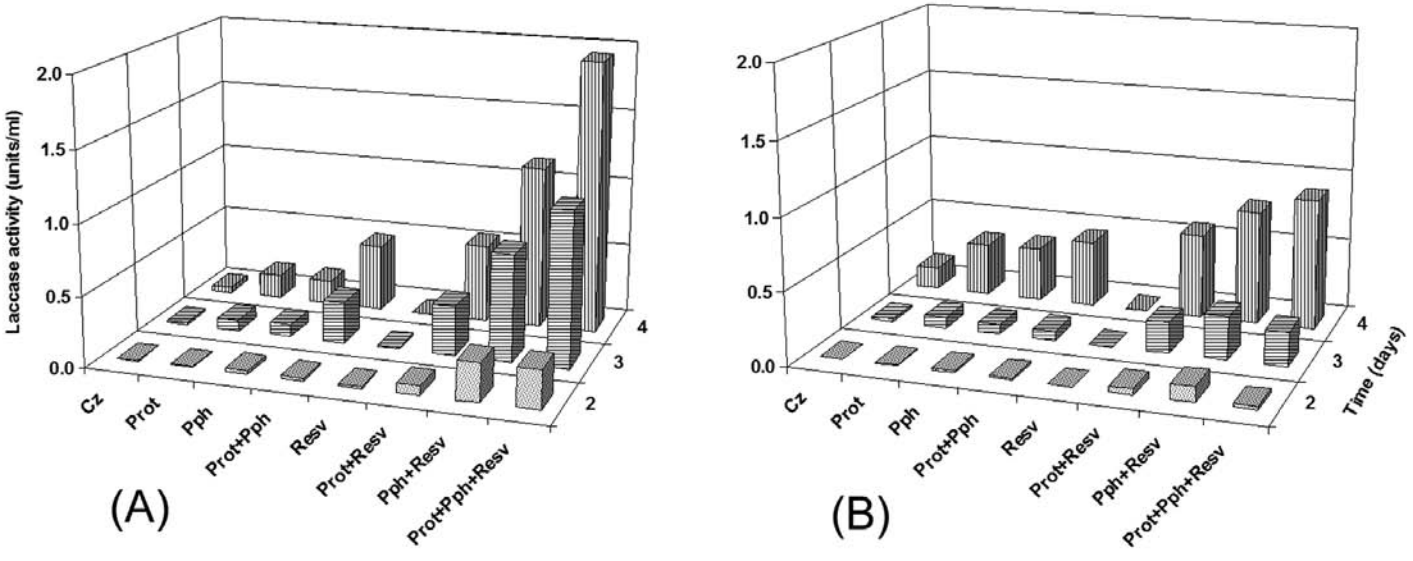

Fig. 1. Laccase activity detected in *B. cinerea* cultures at days 2, 3, and 4 after spore inoculation (10^4 spores ml^{-1}). In a factorial design, the 4-ml cultures were incubated in Czapek-Dox medium at pH 3.5 and supplied without (Cz) or with different combinations of grape proteins (Prot), grape polyphenols (Pph), and *trans*-resveratrol (Resv) at 100 µg ml^{-1}, 200 µg ml^{-1}, and 200 µg ml^{-1}, respectively. At each data point, 1–30 µl aliquots of each culture were assayed for laccase activity. Data from two experiments performed with different batches of proteins, polyphenols, and spore preparations are presented (A and B). Each data point was obtained from three replicated cultures. The main effects and the first- and second-order interactions of the three factors were significant at $P<0.01$ at all data points except in experiment (A), in which the interaction of proteins × polyphenols and the second-order interaction were not significant at day 3 of the culture.

atrol (Fig. 2). A few sparse mycelium flakes were observed in the culture treated with *trans*-resveratrol alone, and the mycelium dry weight was almost negligible. Mycelium growth was low when *trans*-resveratrol was supplied with proteins, and the detrimental effect of *trans*-resveratrol on mycelium growth was completely abolished when polyphenols were present (Fig. 2). The main effects of the three factors and first-order interactions of protein × *trans*-resveratrol and polyphenols × *trans*-resveratrol were significant at $P<0.01$. Overall, the growth data indicated that grape polyphenols abolish the toxicity of *trans*-resveratrol and that grape proteins promote fungal growth but mostly in the absence of *trans*-resveratrol.

Alteration of grape protein in *B. cinerea* cultures. Estimation of the total grape protein present in the cultures during fungal growth was impaired because polyphenols and *trans*-resveratrol interfere with standard protein assays. Therefore, aliquots of the *B. cinerea* cultures containing the grape proteins were analyzed by SDS-PAGE. Control samples incubated without the fungus showed the typical grape protein pattern of three main broad bands at about 25, 31, and 66 kDa and a few bands of minor intensity (Fig. 3, controls). The pattern changed with the type of treatment in the *B. cinerea* cultures. In the culture treated with protein only, the intensity of the band at 31 kDa gradually attenuated and a new band of lower size was formed. This band is probably the degradation product of the 31 kDa protein, which finally, after 4 days, localized at about 27 kDa (Fig. 3A). A similar pattern was obtained in the culture with *trans*-resveratrol, except that the 25 kDa band was also less intense at the end of the culture, and

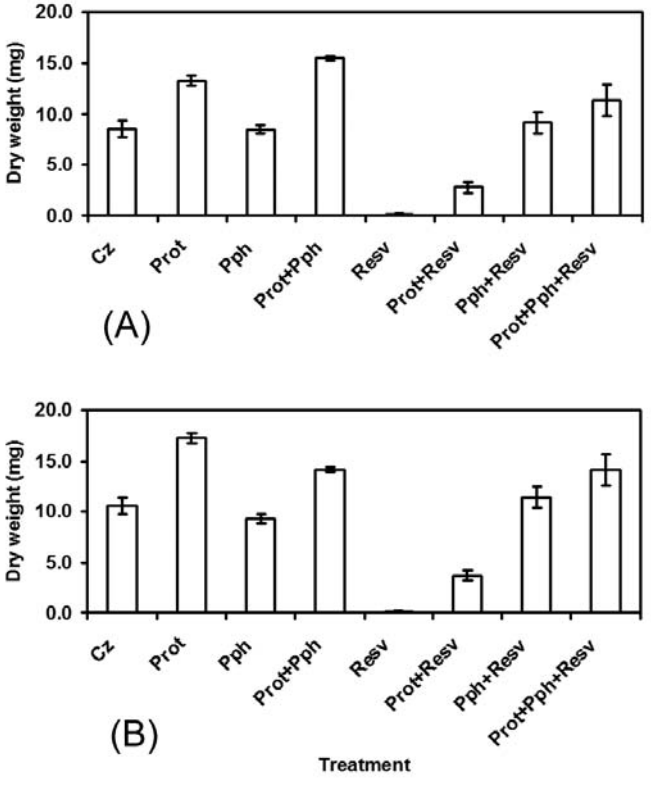

Fig. 2. *B. cinerea* mycelium dry weight harvested at day 4 after spore inoculation (10^4 spores ml^{-1}) from the two experiments (A and B) described in Fig. 1. In a factorial design, the cultures were incubated in 4 ml Czapek-Dox medium at pH 3.5 supplied without (Cz) or with different combinations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

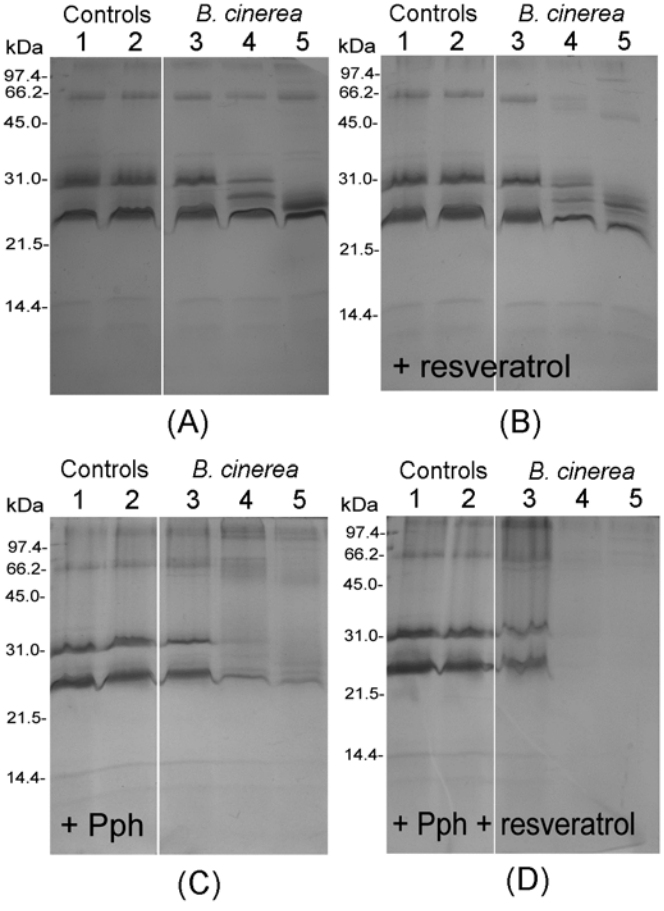

Fig. 3. SDS-PAGE patterns of proteins collected from the *B. cinerea* cultures described in Fig. 2A. Only the cultures supplied with the grape proteins (100 µg ml^{-1}) alone (A) or in combination with resveratrol (B) or grape polyphenols (Pph) (C), or both resveratrol and Pph (D) were analyzed. Samples of 40 µl of inoculated cultures collected at 1, 2, and 4 days (lanes 3, 4 and 5, respectively), and of culture media, incubated without the fungus, collected at 1 and 4 days (lanes 1 and 2, respectively), were centrifuged, precipitated and analyzed as reported in Materials and Methods. The gels were stained with the Coomassie G-250. The low resolution of several bands was probably the result of salts in the medium contaminating the proteins during ethanol precipitation. Similar results (not shown) were obtained with the culture indicated in Fig. 2B.

the 66 kDa band disappeared (Fig. 3B). In the culture supplied with grape polyphenols, the 31 kDa band gradually disappeared and the 25 kDa band faded, becoming poorly visible by the end of the culture period (Fig. 3C). Protein bands almost completely disappeared in the culture containing both polyphenols and *trans*-resveratrol (Fig. 3D).

Alteration of grape protein pattern by *B. cinerea* laccase. From the above results, it seems that polyphenols and *trans*-resveratrol alter the grape protein pattern, mostly when both compounds are added concurrently to the culture. Strong laccase activity was also induced in these cultures. To establish whether *B. cinerea* laccase can mimic these protein alterations, incubations were performed by mixing together proteins with polyphenols and/or *trans*-resveratrol and the fungal laccase. A low laccase concentration (0.02 U ml^{-1}) was used that was less than or comparable to the values measured at the second day of the *B. cinerea* cultures when proteins were mixed with polyphenols and/or *trans*-resveratrol. After a 24 h incubation, these mixtures were centrifuged to separate soluble and insoluble components which were analyzed by SDS-PAGE (Fig. 4). In the supernatants of samples treated with laccase, the major proteins bands became much less intense in the mixture with polyphenols, became weakly visible in the mixtures containing *trans*-resveratrol, and were undetectable in the sample with *trans*-resveratrol and polyphenols (Fig. 4A). These proteins were at least partially recovered from the precipitated materials (Fig. 4B). However, it is worth noting that in the mixture containing polyphenols, the 31 kDa band, which was weak in the soluble fraction (Fig. 4A), was almost undetectable in the insoluble fraction (Fig. 4B). In addition, in the mixture with polyphenols and resveratrol, the 31 kDa band, which had disappeared from the soluble fraction (Fig. 4A), was strongly reduced in the insoluble fraction (Fig. 4B); a smear of protein was clearly visible above 66.2 kDa, probably indicating the formation of high molecular weight aggregates (Fig. 4B). Controls without laccase or with protein plus laccase showed that most protein remained in solution (Fig. 4A). In the control with protein and laccase, a band at 28 kDa was more intense, possibly as a consequence of proteolytic activity in the laccase preparation (Fig. 4A).

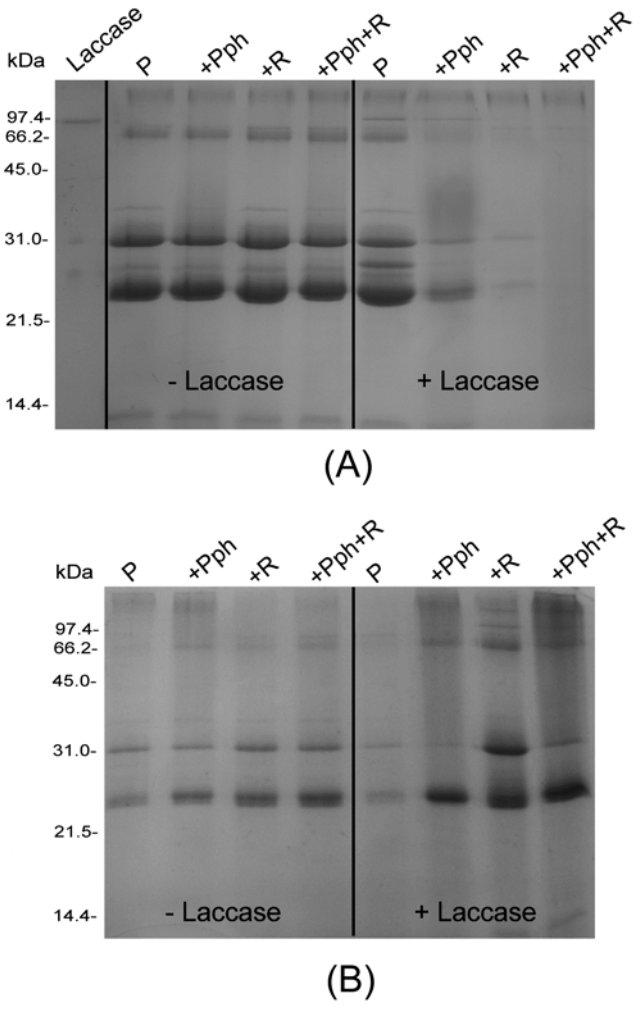

Fig. 4. SDS-PAGE of grape proteins (P) incubated with 0.02 U ml^{-1} of *B. cinerea* laccase activity and with grape polyphenols (Pph), *trans*-resveratrol (R), or polyphenols plus *trans*-resveratrol (Pph+R). Grape proteins, grape polyphenols, and *trans*-resveratrol were supplied at 50, 100, and 100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

Protein pattern in grapes infected by *B. cinerea*. The protein pattern in grape berries infected with *B. cinerea* was analyzed 7 days after inoculation. At this time, the berries appeared completely brown. Grape berries showing grey mould disease symptoms in the vineyard were also analyzed.

Compared with healthy grapes (Fig. 5A, lane 1 and Fig. 5B, lane 2), both artificially or naturally infected berries did not show the 31 kDa band and the 25 kDa band showed strongly reduced intensity (Fig. 5A, lane 2 and Fig. 5B, lane 3). These protein patterns resemble those obtained in *B. cinerea* cultures treated with proteins and polyphenols or with incubation of these compounds with *B. cinerea* laccase. A new band at about 45 kDa was observed in the healthy control berries harvested from the field (Fig. 5B, lane 2). This band was not visible in the control grapes used for artificial inoculation, and is possibly linked to the different harvesting dates of the two control samples.

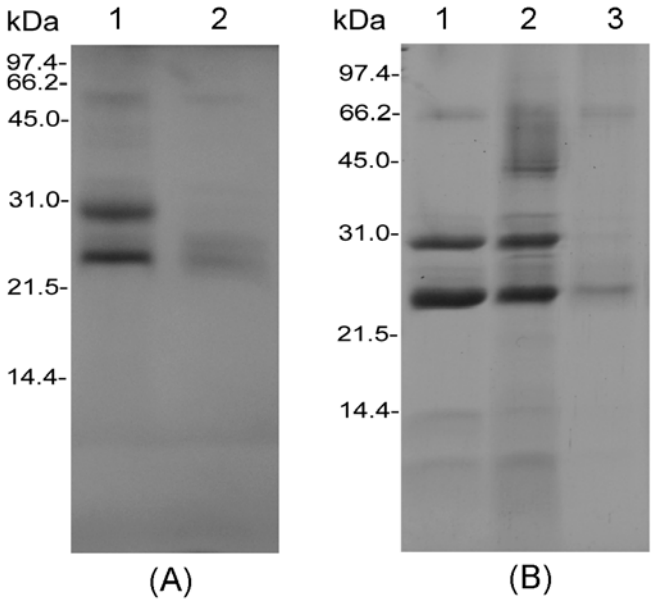

Fig. 5. SDS-PAGE of protein extracted from grape berries either healthy or infected with *B. cinerea*. A volume of extract of healthy berries containing 5 µg of protein and an identical volume from the infected berries were precipitated with four volumes of cold ethanol and analyzed. (A) Protein extracted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

DISCUSSION

During grape infection, the necrotrophic fungus *B. cinerea* encounters an environment rich in phenols and proteins. Among the phenols, the stilbenic *trans*-resveratrol is a phytoalexin precursor toxic to several fungal pathogens, including *B. cinerea* (Adrian *et al.*, 1997). Other grape phenols, which here we generically designated as polyphenols, have no reported effect against pathogenic fungi. In addition, some grape berry proteins have been reported to be toxic against *B. cinerea* and belong structurally and functionally to the PR proteins (Derckel *et al.*, 1998; Monteiro *et al.*, 2003).

To mimic conditions similar to the natural infection of grape berries, the combined effect of *trans*-resveratrol, grape proteins, and grape polyphenols on the growth of *B. cinerea* was studied. It is possible that the grape polyphenol preparations used (about 500 µg g^{-1} of grape fresh weight) could have contained minimal amounts of *trans*-resveratrol. However, the concentration is estimated to be less than 2 µg g^{-1} of skin fresh weight in healthy *Vitis vinifera* grapes (Li *et al.*, 2006). In our experiments, *trans*-resveratrol (200 µg ml^{-1}) was added to cultures at concentration considered lethal for *B. cinerea* (Adrian *et al.*, 1997). An equal amount of polyphenols was also applied. In the literature, there is no clear determination of the *trans*-resveratrol content in grape berries infected by *B. cinerea*, but it is likely to be exceeded by that of polyphenols.

The selected *trans*-resveratrol concentration almost completely prevented fungal growth and laccase activity was negligible in these cultures. In contrast to these findings, Schouten *et al.* (2002) observed only a reduction in fungal growth and a strong induction of the fungal laccase that converts *trans*-resveratrol into the more toxic dimer *trans*-ε-viniferin. The discrepancy between these results may be explained by the lower *trans*-resveratrol concentration (50 µg ml^{-1}) and the high titre of spore suspension (5×10^5 ml^{-1}) used by Schouten *et al.* (2002). Induction of laccase activity and partial fungal growth recovery was also observed after lowering the *trans*-resveratrol concentration to 100 or 50 µg ml^{-1} and increasing the spore concentrations to 10^5 ml^{-1} (data not shown). Hoos and Blaich (1990) previously showed that the effect of *trans*-resveratrol largely depends on the ratio between this molecule and the spore titre. Membrane-bound ATP-binding cassette (ABC) transporters are likely involved in protecting *B. cinerea* germlings from the effects of sub-lethal concentrations of *trans*-resveratrol (Schoonbeek *et al.*, 2001).

Trans-resveratrol became completely ineffective in reducing fungal growth when grape polyphenols where added to the culture, and laccase activity released into the culture was greatly stimulated by this combination of compounds. Thus it can be inferred that under these conditions laccase activity is involved in neutralizing *trans*-resveratrol toxicity. In the presence of grape polyphenols, fungal laccase, which catalyzes a number of phenol coupling reactions (Baldrian, 2006; Riva, 2006), could lead to the formation of more complex compounds, hampering the formation of the toxic *trans*-ε-viniferin. Further work should clarify the role of laccase in the *trans*-resveratrol detoxification process as well as the phenol species involved. Recently, Schouten *et al.* (2008) invoked a similar mechanism for the tannic acid-mediated degradation of the phenolic antibiotic 2,4-diacetylphloroglucinol and also suggested that, at the developing stage, the ABC efflux pump could give *B. cinerea* spores sufficient time to initiate the degradation process.

Grape proteins may also contribute towards making the grape environment inhospitable to fungal attack because they have been described mostly as PR or PR-like proteins. The most highly-expressed proteins in the skin of mature berries are chitinases and β-1,3-glucanase (Deytieux *et al.*, 2007), which together with thaumatin-like proteins are also largely represented in the mesocarp of mature berries (Sarry *et al.*, 2004), and particularly enriched in grape juice (Tattersall *et al.*, 1997). The protein pattern obtained from grape juice in this study is similar to that obtained in previous studies and is

characterized by three major bands at about 25, 31, and 66 kDa, tentatively identified as thaumatin-like proteins, chitinase, and invertase, respectively (Tattersall *et al.*, 1997; Davies and Robinson, 2000).

Grape thaumatin-like proteins and chitinases have significant antifungal activity against *B. cinerea* (Derckel *et al.*, 1998; Monteiro *et al.*, 2003). In contrast, this current study showed an increase in mycelium growth upon supplying *B. cinerea* cultures with the grape proteins at a concentration comparable to that measured in grape juice. The discrepancy with the results of other studies may depend on differences in the bioassay used, including protein concentrations and purity, and the sensitivity of the fungal strain. Indeed, protein preparations used in this work were not pure, and phenol contamination may have played a role in diminishing the effect of grape proteins against the fungus.

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

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

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

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

In general, grape proteins are considered resistant to fungal digestion (Waters *et al.*, 1992; Ferreira *et al.*, 2002). However, this contrasts with the observation that *B. cinerea*–infected grape berries showed a simplified protein pattern with bands of reduced intensity. The pattern observed in infected grape berries (Marchal *et al.*, 1998; this paper) resembles that obtained with protein-polyphenol mixtures after addition of laccase. Laccase activity is usually produced by *B. cinerea* in infected berries (Dewey *et al.*, 2008), and we propose here that this interaction is responsible for the protein pattern observed in the grey mould-rotted grape.

B. cinerea laccase has been suggested as a possible virulence factor during grape infection, causing direct *trans*-resveratrol degradation (Sbaghi *et al.*, 1996). On the other hand, Schouten *et al.* (2002) suggested that laccase increases the toxicity of *trans*-resveratrol by producing *trans*-ε-viniferin, and also showed that knock-out mutants in the *Bclcc2* gene, a laccase gene induced *in vitro* by tannic acid and *trans*-resveratrol, were as virulent as wild type on peanut and grapevine leaves.

The data presented here support the conclusion that in a grape environment characterized by an abundance of polyphenols, *B. cinerea* laccase not only detoxifies the *trans*-resveratrol phytoalexin but also modifies the solubility of grape proteins. Future research will address the kind of interaction taking place between *trans*-resveratrol and polyphenol components and between grape polyphenols and proteins. The effect of this interaction on the degradability of the proteins by fungal proteases will also be considered. Because most of the grape proteins are PR-like proteins, it will be interesting to establish whether other laccase-producing fungi use a similar avoidance mechanism to escape the effect of such proteins.

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