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Influence of *Botrytis cinerea* infection on Champagne wine proteins (characterized by two-dimensional electrophoresis/immunodetection) and wine foaming properties

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Abstract

Proteins are implicated in the foam stabilization of Champagne wines. They may have a grape, yeast, bacteria or fungal origin. *Botrytis cinerea* is a widespread fungal pathogen, which is the causal agent for gray mold. The first part of this work showed the deleterious effect of the presence of this fungus on the foaming properties of a champenois base wine. Foamability and foam stability were reduced, respectively, by 47.7% and 33.3% in the botrytized wine, as compared to the healthy wine. In a second part, SDS–PAGE and two-dimensional electrophoresis (2-DE), coupled with immunodetection, were used to study (thoroughly) the protein patterns of both wines. With 2-DE and silver-staining detection, the disappearance of numerous spots, located in an acidic pH range, was observed. Indeed, the number of spots detected was about two times more abundant in the healthy wine than in the botrytized one, suggesting that a proteolysis occurred. On the other hand, the presence of new proteins, likely fungal proteins, proteins secreted by the plant as a response to *B. cinerea* infection, or even protein fragments resulting from partial proteolysis, was detected in the botrytized wine. All these modifications of the wine protein content were undoubtedly due to the presence of *B. cinerea* and this might be a reason for the loss of foaming properties of Champagne base wines, though no relationship between these two phenomena can be established from the results obtained.

Keywords: Proteins; Wine; Champagne; Foam; *Botrytis cinerea*; Gray mold; 2-DE; Immunoblotting

1. Introduction

In sparkling wines, effervescence and foam are two undissociable criteria and their appearance and behaviour are considered as one of the most important characteristics for consumers. Accordingly, these ephemeral phenomena have been studied in recent years in a number of sparkling wines, particularly in Champagne (Andrés-Lacueva, López-Tamames, Lamuela-Raventós, Buxaderas, & de la Torre-Boronat, 1996; Gallart, Tomás, Suberbiola, López-Tamames, & Buxaderas, 2004; Liger-Belair, 2005; Marchal et al., 2001; Maujean, Poinsaut, Dantan, Brissonnet, & Cossiez, 1990; Pueyo, Martín-Alvarez, & Polo, 1995).

Champagne wines contain surface-active compounds, which have significant effects on bubble stability (Péron et al., 2001), and contribute to the stabilization of foam (Brissonnet & Maujean, 1991; Malvy, Robillard, & Duteurtre, 1994; Senée, Robillard, & Vignes-Adler, 2001).

Abbreviations: SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; MW, molecular weight.

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In this sense, Maujean et al. (1990) reported a positive correlation between the protein concentration and the foamability, which was confirmed by Malvy et al. (1994). Since proteins are present at low levels in sparkling wines, it is of major interest to preserve them from factors that may cause their degradation during the winemaking process. Indeed, fermentations and some enological treatments can lead to a decrease in the protein content of wines (Luguera, Moreno-Arribas, Pueyo, Bartolomé, & Polo, 1998; Marchal, Chaboche, Douillard, & Jeandet, 2002; Martínez-Rodriguez & Polo, 2003), as well as protein degradation, by the action of yeast enzymes (Bayly & Berg, 1967; Moreno-Arribas, Pueyo, & Polo, 1996).

In the Champagne vineyard, *Botrytis cinerea* is a widespread fungal pathogen, responsible for the gray mold disease, which causes considerable economic losses for winemakers. Depending on the vintage, fungal infection rates can reach 15–25% and wines prepared from infected grapes usually exhibit organoleptic defaults, such as an oxidation of the colour or the appearance of typical aromatic notes (''moldy'', ''rotten''), which are not appreciated by consumers (Bocquet, Moncomble, & Valade, 1995, 1996). Marchal et al. (2001) examined the effects of *B. cinerea* infection on the foaming properties of Champagne wines obtained from Chardonnay, Pinot noir and Pinot meunier grape berries. From 20% of infection, foam characteristics of the three Champagne wines were dramatically altered, whereas total protein contents were very similar. These authors concluded that the higher the degree of rot, the more are foaming properties altered. However, they gave no information about the modifications undergone by proteins, and no electrophoretic approach was undertaken in this work.

In a previous study (Marchal et al., 1998), it was also reported that the presence of fungal proteins, namely proteases, in a highly botrytized must of Pinot noir, resulted in the complete degradation of the initial protein fraction, as shown by SDS–PAGE and immunodetection analyses. This study gave information about the electrophoretic characteristics of must proteins but no data were presented about what occurs during the winemaking process; moreover, this work did not afford any data concerning wine foaming properties.

According to these results and because proteins have been proven to play a role in wine foaming properties, we have undertaken a thorough study of the proteinic content of two wines prepared with healthy and botrytized grapes.

Proteins, from grape berries, musts, base wines or sparkling wines, have previously been analyzed using various analytical methods (Moreno-Arribas, Pueyo, & Polo, 2002), including chromatographic techniques (Berthier et al., 1999; Luguera et al., 1998; Monteiro, Piçarra-Pereira, Teixeira, Loureiro, & Ferreira, 2003; Waters, Peng, Pocock, & Williams, 1995), polyacrylamide gel electrophoresis (PAGE) (Dambrouck et al., 2003; Marchal, Bouquelet, & Maujean, 1996; Marchal et al., 1998; Moreno-Arribas, Cabello, Polo, Martín-Alvarez, & Pueyo, 1999; Waters, Wallace, & Williams, 1992) and isoelectric focussing (IEF) (Anelli, 1977; Luguera et al., 1998). However, only a few papers concern the use of 2-DE (IEF followed by SDS–PAGE or LDS-PAGE) (Hsu & Heatherbell, 1987a, 1987b, 1987c; Marshall & Williams, 1987; Sarry et al., 2004; Tesnière & Robin, 1992).

In this study, electrophoretic techniques (SDS–PAGE and 2-DE), combined with protein blotting and immunodetection, were applied to study the impact of *B. cinerea* on the protein content of Chardonnay base wines.

2. Materials and methods

2.1. Musts

Musts were prepared from grape berries of the Chardonnay variety. Healthy grapes and grapes infected by *B. cinerea* were hand-harvested in the same vineyard in the Champagne area (France), in September 2003. The rate of infection was expressed as numbers of infected grape berries as a percentage of the total number of grape berries on a bunch. Grapes were pressed using a pneumatic press (pressure between 1.5 and 2 bar). Sulphur dioxide was added to the free run juice as follows: 150 mg/l for polyclonal antiserum production and 60 mg/l for wine production. After static settling at 12 °C for 24 h, musts were centrifuged at 8000*g* for 10 min. Supernatants were separated, filtered through a 3 μm membrane (Schleicher & Schuell) and then through a 0.45 μm membrane (Alltech), and stored at 4 °C prior to use.

2.2. Wines

Wines were made from musts prepared from either healthy grapes or grapes naturally infected with *B. cinerea*. Settled Chardonnay musts were racked and chaptalized with sucrose. The alcoholic fermentation was achieved by *Saccharomyces bayanus* at 18 °C. Malolactic fermentation did not take place in these wines. Wines were centrifuged and filtered through a 0.45 μm membrane and stored at 4 °C.

2.3. Enological analyses

Several parameters, including pH, total acidity, potassium, calcium, gluconic acid content and wine colour, were determined for both healthy and infected wines. An Orion 420A pH meter (Fischer Scientific, Elancourt, France) was used to determine the pH. Total acidity was determined by M/64 NaOH titration, using bromothymol blue as an indicator, and results were expressed as g/l of sulphuric acid. The potassium and calcium contents were determined by atomic absorption spectrophotometry (Varian Spectra 640, Melbourne, Australia). The gluconic acid content, a chemical index currently used to estimate the level of *B. cinerea* infection, was enzymatically assayed and results

were expressed as g/l. The alcohol content was determined after distillation with a Dujardin-Salleron class II alcoholmeter 9–16% volume (accuracy 0.1% v/v). The wine colour was measured using a colorimeter (Lovibond PFX 190, Tintometer Series II, UK) and a 50 mm glass cell. Three parameters were obtained: L^* (lightness), a^* (from green to red), b^* (from blue to yellow).

2.4. Protein concentration

The wine protein content was determined according to the Bradford method (Bradford, 1976), with some modifications to avoid interferences due to ethanol and phenolic compounds (Marchal, Seguin, & Maujean, 1997). Results were expressed in mg/l and bovine serum albumin (BSA) was used as a standard. Each value was the average of three independent measurements. The standard coefficient of correlation was $r^2 = 0.9924$.

2.5. Foaming properties

Foam measurements were carried out using the Mosalux apparatus and performed as described by Maujean et al. (1990). All measurements were carried out in triplicate. Two parameters were obtained: (1) foamability (H_M), which corresponds to the maximum height reached by the column of foam, and (2) foam stability (H_S), which represents the persistence of the foam collar.

2.6. Viscosity

Viscosity was measured using an Ubbelohde capillary viscometer (Schott – Geräte, GmbH, Germany) with a calibration constant (K) of 0.003107 mm^2/s^2. Wines were analyzed in triplicate at a constant immersion temperature of 20 °C. The kinematic viscosity (v) was determined by measuring the time (t) required for a fixed volume of sample (15 ml) to flow through the capillary with a Schott-Geräte ViscoClock measuring unit, and using the equation $v = K \cdot t$.

2.7. Protein sample preparation

The healthy must and the two wines were centrifuged at 9500*g* for 10 min and filtered through a 0.45 μm membrane (Alltech). Resulting samples were concentrated six times and dialyzed three times against distilled water (0.2 l of concentrate plus 1.3 l of water). A polyethersulfone membrane with a 5000 MW cutoff was used (Minisette Omega, screen channel membrane, Pall Filtron, France). The ultrafiltration module (tangential membrane, manifold and side plates) was connected to a pumping system. Ultrafiltrations were carried out at room temperature. Dialyzed retentates were aliquotted (15 ml) and stored at −20 °C before being used for alcoholic precipitation. Each sample aliquot (15 ml) was diluted with eight volumes of an ice-cold ethanol solution containing 15% (w/v) of trichloroacetic acid (TCA). Proteins were then allowed to precipitate at 4 °C for 1 h. After centrifugation at 9500*g* for 8 min at 4 °C, pellets were washed in 60 ml of ice-cold ethanol, as described above. Samples were washed again in 30 ml of ice-cold ethanol and supernatants were removed. Resulting pellets were air-dried and then resolubilized in 1 ml of a sample buffer consisting of 7 M urea, 2 M thiourea, 4% (w/v) 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), 0.5% (v/v) IPG buffer 3–10, 60 mM 1,4-dithiothreitol (DTT) and traces of bromophenol blue.

2.8. Botrytis cinerea cultures

The strain of *B. cinerea* (i.e. *B. cinerea-616*) used in this study, was provided by Dr. Brygoo (INRA Versailles, France). This strain was isolated from infected grape berries cultivated in the *Montagne de Reims* area (Trépail, France). The fungus was cultured on an agar-based medium [potato extract (4 g/l) + glucose (20 g/l) + bacteriologic agar (20 g/l) adjusted to pH 5.5]. Conidia were collected from 15-day-old cultures, placed into 3 l conical flasks containing 1 l of Morquer liquid medium (Morquer, 1931), and incubated on a rotary shaker at 150 rpm at 20 °C for 3 weeks, with a 12-h day/night cycle. Fungal cultures were centrifuged at 10,000*g* for 10 min and filtered through a 0.45 μm membrane (HA Millipore), before being ultrafiltered as described above, lyophilized, and stored at −20 °C. Lyophilisates were used as fungal antigens.

2.9. Polyclonal antibody preparation

Preimmune sera were obtained from two rabbits before immunization. Soluble antigens (healthy must proteins and *B. cinerea-616*-secreted proteins) were diluted in 0.3 ml of 3% polyacrylamide and mixed with 0.9 ml of Freund's complete adjuvant (Sigma–Aldrich, France) (Freund, 1956) to give a final protein concentration of approximately 1 mg/ml. The two rabbits were intradermally immunized on the back at six sites, one rabbit with healthy must proteins and one rabbit with *B. cinerea-616*-secreted proteins (6×0.15 ml of the immunogen). Subsequent injections with the same antigen preparations were made at weeks 3 and 6 in Freund's incomplete adjuvant. Each rabbit was bled after 5 and 8 weeks from the first injection. Blood samples were left at 30 °C for 1 h before being centrifuged at 6000*g* for 10 min. Antiserum supernatants containing polyclonal antibodies were stored at −20 °C prior to use.

2.10. Cross-reactivity and Western blotting staining

The specificity of polyclonal antibodies raised against both *B. cinerea-616* and must proteins was first tested using a non-competitive dot-blot technique. Must and wine proteins, as well as pre-immune sera and *B. cinerea-616* proteins, were spotted (3 μl), in duplicate, onto nitrocellulose

membranes and air-dried at room temperature. Non-specific binding sites were blocked using a solution containing 2% (w/v) skim milk powder (Merck, Germany) in TBS (Tris buffer saline). Membranes were rinsed three times in TBS and incubated for 3 h with the appropriate primary antibody diluted 1/1500 in TBS and 1% (w/v) skim milk powder. They were then successively washed in TBS, TBS plus 0.05% (v/v) Tween 20, and TBS, before incubation with a goat anti-rabbit IgG peroxidase conjugated antibody (Sigma–Aldrich, USA), diluted 1/1500 in TBS plus 1% (w/v) skim milk powder at 12 °C overnight. After a further rinse in PBS, peroxidase activity was developed with the 4-chloro-1-naphthol (1.2 g/l) substrate. The ability of the antiserum to bind to the spots (after dot-blots) was assessed by visually comparing the colour of each spot stained with the antiserum test, with that of the non-immune serum.

2.11. Electrophoretic analyses

2.11.1. SDS–PAGE

Vertical SDS–PAGE was performed in a Mini-Protean II electrophoresis cell (Bio-Rad, USA) according to the method of Laemmli (1970) (stacking gel = 5% T and 2.7% C; separating gel = 13.5% T and 2.7% C). Wine total proteins were separated under a constant voltage of 150 V until the bromophenol blue tracker dye reached the bottom of the gel (usually 65 min at room temperature). Standard protein markers (LMW electrophoresis calibration kit, Amersham Pharmacia biotech) were simultaneously loaded at each run. The protein amounts loaded in each lane were, respectively, 1.5 μg for silver stained gels and 5 μg for western blot analyses. The relative molecular mass (M_r) of proteins of interest was calculated from the linear regression equation of log M_r vs. mobility. After electrophoresis, the separated proteins were either silver-stained (Rabilloud, Vuillard, Gilly, & Lawrence, 1994) or were transferred to a nitrocellulose membrane (Sartorius, Germany), using a Bio-Rad electroblotting apparatus, under a constant current at 100 V for 1 h. Composition of the transfer buffer was 25 mM Tris, 190 mM glycine, and 20% (v/v) methanol. Prestained markers (Bio-Rad, USA) were used in this case.

2.11.2. 2-DE

For analytical 2-DE analysis, precast dry polyacrylamide 18 cm length gels (ReadyStrip IPG [pH 3-6], Bio-Rad, USA) were rehydrated with a mixture containing approximately 50 μg of total proteins diluted in sample buffer to a final volume of 350 μl. IEF was conducted at 20 °C in an IPGphor unit (Amersham Pharmacia, Sweden) as follows: a linear increase from 50 V to 8000 V to give a total of 70,000 V h. Focussed proteins were reduced and subsequently alkylated according to Görg et al. (1987). Gels were then placed on the top of vertical slabs of polyacrylamide (12% T, 2.6% C). The stacking gel was replaced by a layer of 1% (w/v) low melting point agarose, 0.15 M Bis–Tris/ 0.1 M HCl and 0.2% (w/v) SDS. Electrophoretic migration along the second dimension was performed using the Laemmli buffer in a Protean II xi Cell (Bio Rad) at 10 °C under a constant current of 20 mA per gel for 1 h, followed by 40 mA per gel for 3 h. After completion of SDS–PAGE, gels were either silver-stained, as described by Rabilloud et al. (1994), or were electrophoretically transferred to nitrocellulose membranes in a Hoefer Transphor unit (Amersham Pharmacia Biotech, Sweden) at 80 V for 1 h. Blotted membranes were then incubated with different polyclonal antibodies, following the protocol described above. For detection, membranes were incubated with equal parts of luminol enhancer and peroxide solutions (SuperSignal West Pico Chemiluminescent Substrate, Pierce, USA) for 8 min in the dark. Membranes were then placed in a plastic sheet protector and exposed to an autoradiographic film (Biomax ML, Kodak), which was fixed and developed using GBX solutions (Kodak, France).

2.12. Image analysis

Digitized images at 84.7 μm resolution were obtained using the GS-710 scanner and Quantity One 4.0.3 software (Bio-Rad, USA). Computerized 2-D gel analysis, including spot detection and quantification, were performed using the Melanie II software (Bio-Rad, USA). Reproducibility of 2-D gels was assayed by running all samples at least 3 times. The relative molecular mass was calibrated with internal markers (2 D Standards, Bio-Rad, USA) after co-electrophoresis. Selected protein features were modelled as Gaussians and the relative optical densities (OD); i.e. the feature OD, divided by the total OD over the whole image, were computed. Means ± SD ($n = 3$) were finally calculated for each sample. The ratio of expression level for each protein in the botrytized wine over the healthy one was also estimated. Values above 1.5 or below 0.5 were considered as significant.

3. Results and discussion

3.1. Enological parameters

The effect of *B. cinerea* infection on wines was first evaluated by measuring various enological parameters. Results of the different chemical variables determined for both base wines are presented in Table 1. A higher pH (+0.33 unit) and a lower acidity (−2.0 g/l H_2SO_4) were observed in the botrytized wine than in the healthy one. According to Donèche (1993), the development of gray mold on grape berries usually leads to an important degradation of the main organic acids of grapes (tartaric and malic), and hence a weak concentration of these acids in the musts and wines, a rise of pH and a low total acidity. Potassium concentration was much more important in the botrytized wine (+50%) than in the healthy wine whereas the calcium concentration remained stable, thus confirming previous studies (Bocquet et al., 1995). Gluconic acid, which corresponds to a typical secondary metabolite of *B. cinerea*,

Table 1
Enological parameters determined in Chardonnay base wines

	Healthy wine	Botrytized wine
Global composition		
pH	2.87	3.20
Alcohol content (%, v/v)	10.44	11.23
Total acidity (g/l H_2SO_4)	6.0	4.0
Potassium (mg/l)	140	210
Calcium (mg/l)	92	97
Gluconic acid (g/l)	0.02	0.17
Color		
L^*	83.63	82.65
a^*	−0.97	+7.67
b^*	15.42	32.63

can be used as an indicator of the fungal attack. Its content is correlated with both the percentage of rotten grapes visually estimated and with the laccase activity (Bocquet, Moncomble, & Valade, 1996). The healthy wine contained 8.5 times less gluconic acid than did the wine prepared from botrytized grapes. At the time of the grape harvest, the rate of grapes infected by *B. cinerea* was visually estimated to be about 20%. The low gluconic acid content detected in the healthy wine suggests that a source of *B. cinerea* infection was likely present in the core of some grape bunches. Another change observed was an oxidized colour of the botrytized wine, which was correlated with the values of the parameters measured by the CIEL$^*a^*b^*$ method: an increase of a^* (red: +8 units) and b^* (yellow: +17 units). These results can be explained by the presence of a laccase (phenol-oxidizing extracellular enzyme) produced by *B. cinerea* (Dubernet, Ribéreau-Gayon, Lerner, Harel, & Mayer, 1977).

3.2. Foaming properties and protein content

Significant differences were observed for H_M and H_S, between the healthy wine and the botrytized wine (Table 2). The healthy wine had a better capacity to foam (H_M, +91.0%) than had the botrytized wine. It also presented a better foam stability (H_S, +50.0%). The viscosities of two samples were very close (difference 0.04 unit mm^2/s) in spite of the possible presence of *B. cinerea* polysaccharides and could not account for the differences of foam characteristics observed for these wines. The reduction of the foaming properties of the botrytized wine could be explained partially by its potassium content (+50%, Table 1), indeed known as a foam-destabilizing agent in white wines (Maujean et al., 1990).

The main parameter which must be taken into account to explain the changes in wine foaming properties is the protein content, since proteins are known to play an important role in the stabilization of wine foam (Brissonnet & Maujean, 1991; Malvy et al., 1994), as well as in beers (St. John Coghlan, Woodrow, Bamforth, & Hinchliffe, 1992; Onishi et al., 1999). According to the data presented in Table 2, the protein concentration of the botrytized wine was slightly higher than that of the healthy wine (+13.8%). Our results are in agreement with those of Marchal et al. (2001) which showed no significant differences in the protein content between healthy and botrytized Champagnes, even for 40% infection. The protein content of botrytized Chardonnay Champagnes were even higher (+27% for 20% infection) than that of the control Champagne. In contrast to previous studies, that have shown a relationship between the protein concentration and the foaming properties of wines (Andrés-Lacueva et al., 1996; Malvy et al., 1994; Maujean et al., 1990; Moreno-Arribas, Pueyo, Nieto, Martín-Álvarez, & Polo, 2000; Pueyo et al., 1995), we did not find any relationship in our work. These discrepancies are probably linked to the modification of the grape (and wine) protein biochemical characteristics during and after *Botrytis* infection. To further understand this phenomenon, SDS–PAGE and 2-DE were carried out in order to precisely compare the protein profile of both healthy and botrytized wines.

3.3. Specificity of antigen–antibody recognition

A control experiment using the preimmune antiserum did not show any positive reaction with proteins extracted from either the healthy or the botrytized wines, and similarly with the *B. cinerea–616* secreted proteins (Fig. 1, strip 1). The antibodies raised against *B. cinerea-616* proteins were able to recognize the fungal antigens (Fig. 1, strip 2, spot B) and displayed a high positive reaction against proteins from the botrytized wine, as well (Fig. 1, strip 2, spot D). A low infection level of *B. cinerea* in healthy grape berries was also demonstrated by the low reactivity of *B. cinerea-616* antibodies against healthy wine protein extracts (Fig. 1, strip 2, spot C). Must protein antibodies can differentiate *B. cinerea* proteins and grape proteins, since they displayed a negative response with the *B. cinerea-616* proteins (Fig. 1, strip 3, spot B) and they recognized the grape

Table 2
Effects of *B. cinerea* infection on the foaming properties and the protein content of Chardonnay base wines

	Healthy wine	Botrytized wine	Variation[a] (%)
Foamability, H_M (mm)	128 ± 3	67 ± 3	−47.7
Foam stability, H_S (mm)	15 ± 1	10 ± 1	−33.3
Viscosity (mm^2/s)	1.503 ± 0.004	1.543 ± 0.002	− 2.7
Protein (mg/l BSA)	4.85 ± 0.12	5.52 ± 0.30	+13.8

[a] Variation is calculated as [((botrytized wine value – healthy wine value)/healthy wine value) × 100].

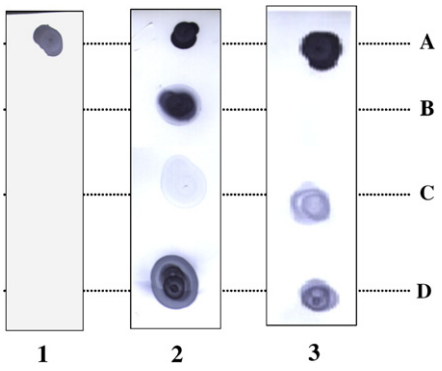

Fig. 1. Dot-blot on nitrocellulose strips with colour developed using 4-chloronaphthol/peroxidase-labelled goat IgG in PBS buffer. Strip 1, rabbit preimmune antiserum; strip 2, rabbit anti-*B. cinerea-616* protein polyclonal antibodies; strip 3, rabbit anti-must protein polyclonal antibodies. Spot A, preimmune serum; spot B, *B. cinerea-616* proteins; spot C, healthy wine proteins; spot D, botrytized wine proteins.

proteins present in the healthy wine, as shown in Fig. 1, strip 3, spot C. Grape proteins were also detected in the botrytized wine (Fig. 1, spot D, strip 3), showing that some grape proteins are not degraded by the fungus.

3.4. SDS–PAGE

3.4.1. Comparison of total proteins from healthy and botrytized wines

Analysis of the protein fraction from both the healthy and the botrytized wines was initially performed by SDS–PAGE, to better evaluate the impact of *B. cinerea* infection (Fig. 2). The electrophoretic profile of healthy wine proteins is shown in Fig. 2, lane 2. Eleven bands with molecular masses ranging from 13.3 to 90 kDa were silver-stained. These bands were distributed as follows: a diffuse band above 90 kDa, a major component around 61 kDa, three bands at 41.8, 32.7 and 29.1 kDa, four large bands between 22.3 and 26.4 kDa and two minor proteins with molecular masses close to 15.4 and 13.3 kDa. These results are relatively similar to previous data obtained from the analysis of total proteins of a Chardonnay wine, after Coomassie brilliant blue staining (Dambrouck et al., 2003). The protein pattern resulting from the botrytized wine (Fig. 2, lane 3) presented some bands with molecular masses similar to those of the healthy wine (Fig. 2, lane 2) but differing significantly in quantitative terms. Indeed, bands at 61 and 15.4 kDa were less intense and the band at 41.8 kDa disappeared. This decrease could be linked to protein degradation caused by fungal proteases secreted by *B. cinerea* (Movahedi & Heale, 1990; Urbanek & Kaczmarek, 1985). Particularly, the band around 61 kDa, which was less abundant in the botrytized wine, corresponds to a glycoprotein (Dambrouck et al., 2003; Marchal et al., 1996) identified as a grape invertase (Ruffner, Hürlimann, & Skrivan, 1995; Ruiz & Ruffner, 2002). This molecule does not seem to be one of the major components of the adsorption layers, which are formed at the air/champagne interface and thus is believed not to significantly contribute to the stabilization of foam (Puff, Marchal, Aguié-Béghin, & Douillard, 2001). However, a recent study, carried out by our group, demonstrated that a decrease in the invertase content of wine is correlated with a decrease in its foaming properties (Dambrouck, Marchal, Cilindre, Parmentier, & Jeandet, 2005). Besides, a greater amount of bands at 90, 32.7, 29.1 and of the diffuse band around 25 kDa was observed in the botytrized wine, as compared to the healthy wine. New protein bands were also detected at 101.8, 76.8, 48.8, 40.7 and 19.7 kDa. These new bands could be either proteins secreted by *B. cinerea*, grape proteins resulting from partial hydrolysis by fungal proteases (Marchal et al., 1998), or proteins secreted by the grape as a response to *B. cinerea* infection. Our results clearly show that the infection of grape berries by *B. cinerea* has an impact on the protein content of the base wine in both qualitative and quantitative terms.

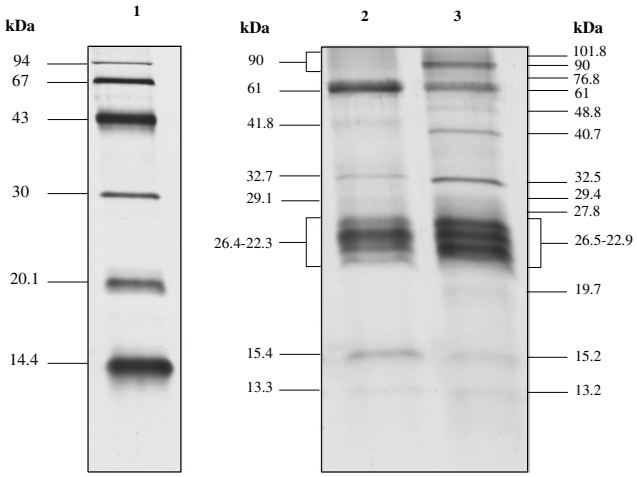

Fig. 2. SDS–PAGE analysis of Chardonnay wine proteins visualized by silver-staining. Lane 1, molecular weight standards; lane 2, healthy wine proteins; lane 3, botrytized wine proteins. The molecular mass (kDa) was assigned to each band using the Quantity One software.

3.4.2. Immunodetection of grape and fungal proteins

Grape proteins were detected in both wines after SDS–PAGE, followed by Western blotting and immunostaining with 4-chloro-1-naphthol (Fig. 3A). The protein patterns obtained after immunostaining (Fig. 3A) and silver-staining (Fig. 2) were similar, except for proteins showing molecular masses below 25 kDa, which were not detected by the antimust antibodies. These proteins of low molecular mass were probably not immunogenic. In the two samples, antibodies were able to recognize proteins with molecular masses ranging from 24.7 to 115.1 kDa, though some bands showed significant differences in intensity. Seven bands at 87.6, 76.4, 54.2, 37.6, 30.5, 28.4 and 24.7 kDa were analogous in both wines. However, the intensity of the bands at 87.6, 54.2, 37.6 and 30.5 kDa was higher in the botrytized wine (Fig. 3A, lane 3), whereas the intensity of the other bands was stable. Moreover, a

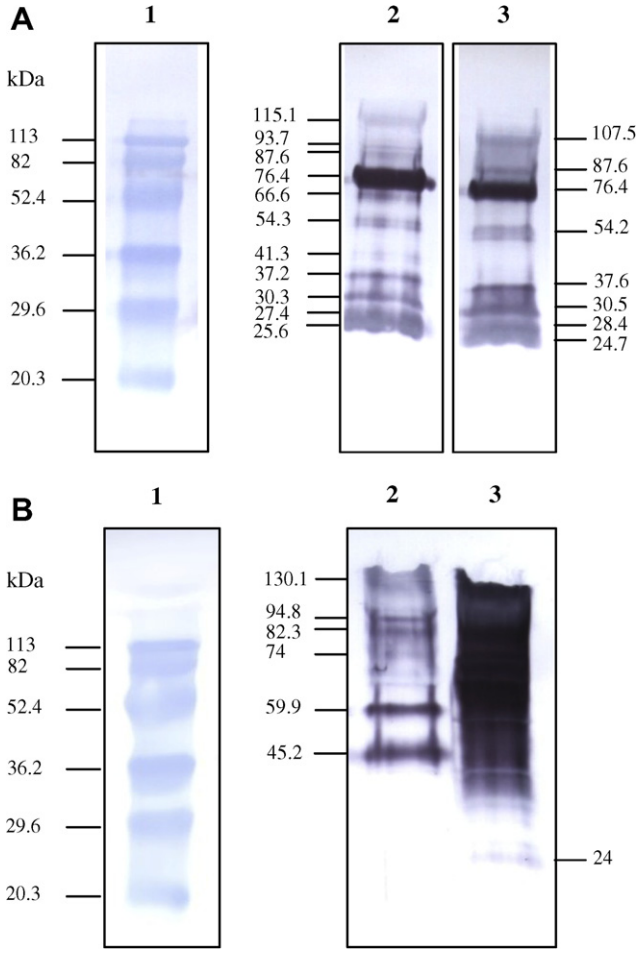

Fig. 3. SDS–PAGE and immunoblotting using rabbit antimust polyclonal antibodies (A) and rabbit anti-*B.c*-616 polyclonal antibodies (B): lane 1, prestained molecular weight standards; lane 2, healthy wine total proteins; lane 3, botrytized wine total proteins. The molecular mass (kDa) was assigned to each band using the Quantity One software.

107.5 kDa band appeared in the botrytized wine. These findings suggest that most of the proteins of the botrytized wine originate from the grape and a few are degraded by *B. cinerea*. Only four protein bands, found in the healthy wine (Fig. 3A, lane 2) at 115.1, 93.7, 66.4 and 41.3 kDa, were absent in the botrytized wine (Fig. 3A, lane 3).

The second step was to detect fungal proteins with anti-*B. cinerea-616* polyclonal antibodies (Fig. 3B). One can observe the presence of six fungal proteins at 130.1, 94.8, 82.3, 74, 59.9 and 45.2 kDa in the healthy wine (Fig. 3B, lane 2). No protein was detected in the low molecular range (molecular mass < 45.2 kDa) in this wine, suggesting that apparently healthy grape berries were probably infected by *B. cinerea* at a very low level, which was impossible to detect by visual scrutiny. This result is also in agreement with the low gluconic acid content observed in this sample (0.02 g/l) and with the results obtained by dot-blot experiments. Reaction of the anti-*B. cinerea* antibodies was much stronger with proteins from the botrytized wine (Fig. 3B, lane 3) than in the healthy wine (Fig. 3B, lane 2). A zone above 29 kDa presented a high coloration intensity with 4CN and a band at 24 kDa was weakly stained. The concentration of fungal proteins was more important in the botrytized wine than in the healthy one.

3.5. 2-DE

3.5.1. 2-DE analysis of wine proteins

In order to study, in more detail, the protein content of both healthy and botrytized wines, 2-DE analyses were carried out, which allow resolving proteins on the basis of their molecular mass as well as their electric charge. Separations were first performed in a wide pH range (pH 3–10, data not shown), revealing a much higher number of proteins than did detection by SDS–PAGE. Protein spots were mainly distributed on the acidic side (pH 3–7) of the gel with molecular masses ranging from 13 to 64 kDa. Although the electrophoretic profiles of both samples presented some similarities, significant differences were observed in the number, the position and the intensity of some spots (data not shown). In order to increase the resolution of the method, further analyses were performed, using narrow pH gradient gels (pH 3–6) (Fig. 4). The protein profiles of the two wine samples were significantly different in terms of numbers of spots and intensity. The number of protein spots detected in the healthy wine was about two times more abundant than in the botrytized one, showing that a large proportion of the proteins was partially or completely degraded because of the *B. cinerea* infection. Indeed, more than 70% of the spots of the healthy wine were not present (Fig. 4A, □) in the pattern of the botrytized wine and 15% of them had a lower intensity (Fig. 4B,▽). A recent paper (ten Have, Dekkers, Kay, Phylip, & van Kan, 2004) reported that *B. cinerea* secretes a group of aspartic proteinases, which has an optimal activity at pH 4. These enzymes, which probably remained active in acid media, such as the grape juice and the wine, would thus be responsible for the degradation of grape proteins. Girbau et al. (2004) studied the pathogenesis-related (PR) protein content of a *Botrytis*-infected free run juice and showed a decrease in the total PR protein concentration of the infected juice compared to the healthy one. These authors also suggested the action of proteolytic enzymes of *B. cinerea*. In our study, only five proteins (Fig. 4A, spots 346, 636, 651, 725, 806) were apparently resistant to the proteolytic activity of *B. cinerea*.

Interestingly, the intensity of some spots (Fig. 4B, spots 162, 196, 198, 261) was stronger in the botrytized wine than the corresponding spots in the healthy wine. Some of these proteins could probably be involved in the grape defence mechanisms, being over-expressed following fungal infection. The most abundant spot displayed a molecular mass around 50 kDa, a p*I* 4.35–4.39 and appeared in both gels (Fig. 4A, spot 139; 4B, spot 113), though it showed a slightly higher intensity in the botrytized wine sample. Twenty spots were also newly synthesized in the botrytized wine (Fig. 4B, ○), representing almost half of total proteins

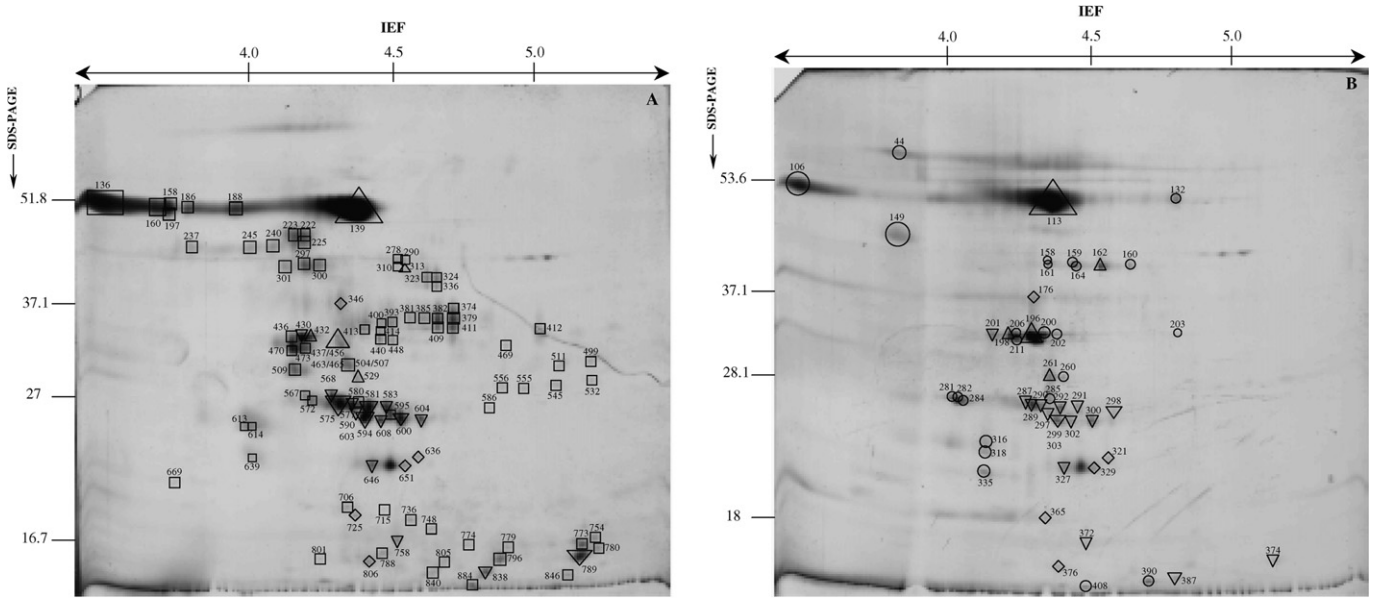

Fig. 4. Differentially expressed proteins in a healthy (A) and botrytized (B) Champagne base wine after IEF/SDS–PAGE and silver-staining. Locations of newly synthesized (circle) and disappeared (square) proteins in the 2-D gel, as well as those spots whose quantity significantly increased (upward pointed triangle), decreased (downward pointed triangle) or was stable (diamond), are indicated. The numbers at the top of the gels denote the pH gradient in the first dimension, while molecular masses of 2-D standards are displayed on the left.

found in this wine. These spots probably correspond to proteins secreted by the fungus or grape protein fragments resulting from fungal proteolysis. Some spots of the botrytized wine had M_r and p*I* very close to those of the healthy wine, suggesting that they were slightly modified by the presence of *B. cinerea* (for example: Fig. 4A, spot 580 and Fig. 4B, spot 285).

3.5.2. Recognition of grape proteins by 2-DE and immunodetection

2-DE was coupled with immunoblotting by using specific polyclonal antibodies raised against must proteins in order to gain more insights about the origin of wine proteins. Most of the proteins recognized by these antibodies in both wines were mainly located in a mass range of about 25 kDa and p*I* of about 4.3 (Fig. 5A and B, area I). In this area, which certainly corresponds to the spots 568 to 608 (Fig. 4A, ▽), and the decline of intensity of which is much more evident with silver-staining, we could even note that some of these spots were less intense or disappeared in the botrytized wine (Fig. 5). A large spot of ca. 50 kDa was detected in both healthy and botrytized wine samples (Fig. 5, spot a), which was also detected with silver-staining (Fig. 4A spot 139; 4B, spot 113). On the other hand, three groups of grape proteins present in the healthy wine extract (Fig. 5A, area II, III and V) were absent in the botrytized wine. The proteins of area II were also observed in the healthy wine after silver-staining (Fig. 4A, spots 222, 223, 225, 297, 300 and 301) as well as for area III (Fig. 4A, spots 379, 382, 409 and 411) and area V (Fig. 4A, spot 188). Another group of proteins, at a mass range of about 50 kDa, was shown to be induced after *B. cinerea* infection (Fig. 5A and B, area IV). A protein of about 46 kDa (Fig. 5B, spot b) also appeared in the botrytized wine. These proteins (area IV and spot b) could be related to the defence mechanisms of the plant following fungal attacks. After comparison with the silver-stained protein patterns (Fig. 4), one can observe that most of the proteins detected in the healthy wine are of plant origin. In addition, proteins of molecular masses below 20 kDa were not detected in 2-D gels, as for the results observed in Fig. 3A.

3.5.3. Detection of fungal proteins by 2-DE and immunoblotting

The Western- blots from 2-DE gels were probed with anti-*B. cinerea-616* polyclonal antibodies and fungal proteins were only detected in the botrytized wine (Fig. 6), contrary to the results obtained by SDS–PAGE (Fig. 3B). It is thus possible that the fungal proteins that are detected in the healthy wine (Fig. 3B, lane 2) have p*I* different from this range of pH. Revelation, with the anti-*B. cinerea-616* antibodies, of the botrytized wine proteins separated by one-dimensional electrophoresis (Fig. 3B, lane 3), showed an absence of proteins below 40 kDa and a very strong reaction between 40 and 120 kDa. In the 2-DE revelation of these same botrytized wine proteins, numerous proteins are seen in the same M_r range. The absence of spots below 40 kDa, as well for 1-DE as for 2-DE, is likely due to the fact that proteins with molecular masses below 40 kDa and used for the preparation of the antibodies are hardly or non immunogenic.

The spots detected in Fig. 6 were, for the most part, spots expected to be of fungal origin after analysis of the silver-stained gels (Fig. 4), even though a precise identifica-

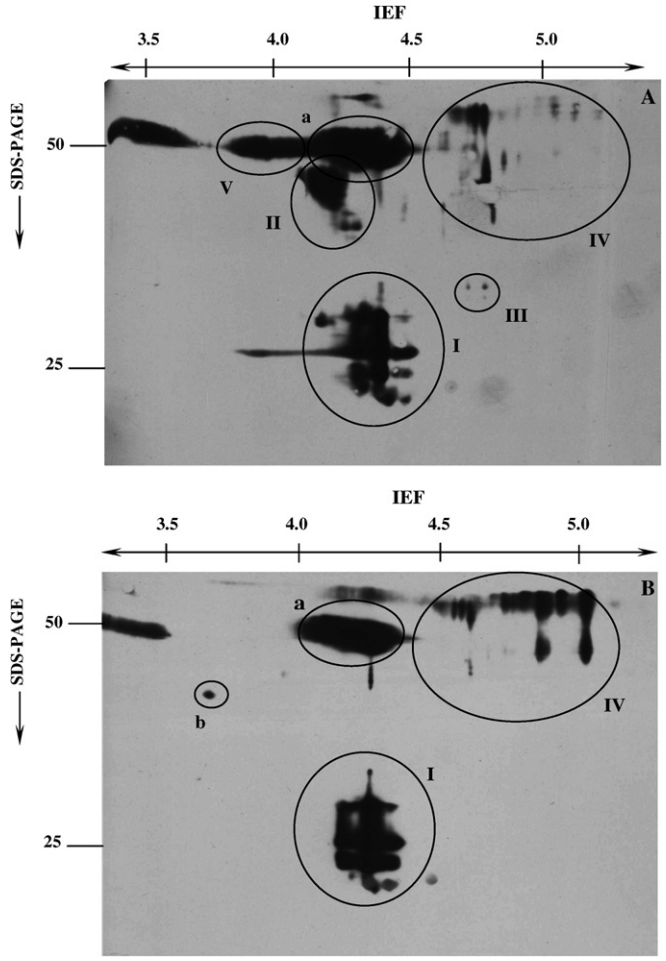

Fig. 5. Detection of plant proteins in healthy (A) and botrytized (B) Champagne base wines after 2-DE combined with immunoblotting using rabbit anti-must polyclonal antibodies. Detection was carried out by chemiluminiscence. Circles denote the main groups of changing proteins.

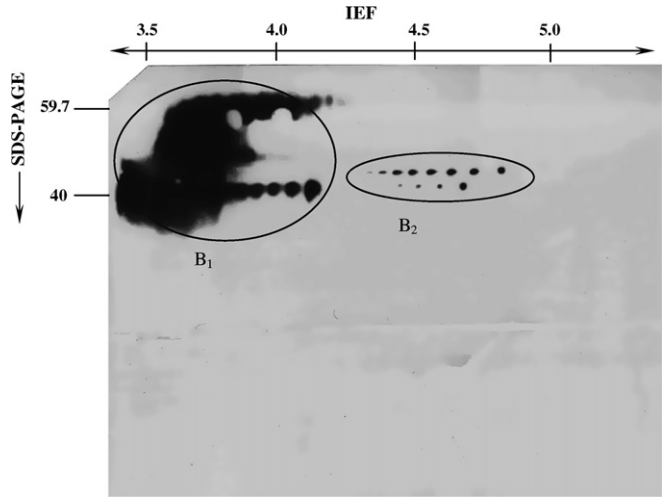

Fig. 6. Detection of *B. cinerea-616* proteins in the botrytized wine after 2-DE combined with immunoblotting using rabbit anti-*B.c*-616 polyclonal antibodies. Detection was carried out by chemiluminescence. Circles denote two main groups of proteins.

tion of these spots is difficult. However, spots 44, 106 and 149 (Fig. 4B) are certainly of fungal origin since their M_r and p*I* are included in area B_1. The group of spots 158, 159, 160, 161 and 164 (Fig. 4B) probably corresponds to some spots of another group of twelve spots (Fig. 6, area B_2). In fact, eight of the nine spots (>40 kDa) that were suspected to be of fungal origin, are most probably secreted by *B. cinerea*.

4. Conclusion

This study has demonstrated that infection of grape berries by *B. cinerea* induces changes in the chemical composition and colour of a Champagne base wine and results in a strong reduction of its foaming properties. Moreover, the protein fraction, which is a "foam-active" agent because of its surface-active properties, was also shown to be altered by the presence of the fungus. The use of one and two-dimensional electrophoretic techniques, in combination with specific antibodies, showed significant changes in the protein content of the Champagne base wine after *B. cinerea* infection. Indeed, many proteins could be partially or completely degraded by fungal proteases, whereas some others were newly synthesized, either by the fungus during the infection process, or by the plant, as a part of the defence mechanisms triggered by *B. cinerea* (Derckel et al., 1999). Current sequencing analysis will allow the identification of proteins whose expression pattern varies in the rotten wine. This may include fungal and plant defence proteins, as well as plant proteins subjected to proteolysis by the action of fungal proteases. These data will be useful for determining whether *B. cinerea* infection results either in the degradation of "foam-active" proteins, or the synthesis of "anti-foam" compounds or both.

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