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Grape berry skin features related to ontogenic resistance to *Botrytis cinerea*

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Abstract This work investigated the structural and biochemical changes during grape berry development which account potentially for the onset and increase in susceptibility to *Botrytis cinerea*. Using the cv. Sauvignon blanc, we quantified at seven developmental growth stages from herbaceous to over-mature berries: (1) fruit ontogenic resistance using three strains (II-*transposa*), (2) the morphological and maturity fruit characteristics and (3) preformed biochemical compounds located in the berry skin. From the mid-colour change stage onwards, susceptibility of unwounded fruit increased sigmoidally in both rot and sporulation severities at the berry surface. A principal component analysis identified a very close connection between fruit susceptibility and the level of fruit maturity. Berry susceptibility was significantly and positively correlated with the phenolic compounds in the skin cell walls and negatively correlated with the total tannin content in the skin and with water activity (Aw) at the fruit surface. On the berry, Aw decreased from 0.94 at bunch closure to 0.89 at berry maturity, with a relatively low value (0.90) at the stage of mid-colour change. Using artificial media, different Aw levels led to significant differences in mycelial growth (Aw ≤0.95 resulted in the lowest growth rate ≤0.34 mm day^{-1}). Thus, besides the level of fruit maturity, both water activity on the fruit and the total tannin content in the skin may affect fungal growth and berry colonisation. The potential of these variables for use as indicators of grape berry susceptibility as well as associated mechanisms for the development of disease are discussed.

Keywords Grey mold · Group-II isolates · Ripening · Susceptibility · Tannins · *Vitis vinifera*

Abbreviations

AIM	Alcohol-insoluble material
AIM-TC	Tannin content in skin AIM
AIM-TP	Total phenolics from AIM
Aw	Water activity
BFM	Berry fresh mass
CFM	Cluster fresh mass
MRS	Mean rot severity
MSI	Mean sporulation intensity
MSS	Mean sporulation severity
S	Soluble solids
SFM	Skin fresh mass

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S/TA	Maturity index
STC	Skin tannin content
TA	Titratable acidity
W%	Water content
WSP	Water-soluble pectic compounds

Introduction

Botrytis cinerea (teleomorph: *Botryotinia fuckeliana* is a cosmopolitan filamentous fungus and a necrotrophic pathogen which causes one of the most serious diseases in grapevine (*Vitis vinifera*), *i.e. Botrytis* bunch rot or grey mold. The disease can reduce drastically both yield at harvest and wine quality, for all types of white or red wines (Ribérau-Gayon et al. 1998). However, *B. cinerea* infestation can also be desirable as 'noble rot' when present on grape varieties where sweet, late harvest wines are produced (Ribérau-Gayon et al. 1998).

In vineyards, epidemic development is initiated by primary infections of young vegetative parts by an airborne conidial inoculum following winter conservation by sclerotia of the pathogen (Elmer and Michailides 2004). In late spring, a major epidemiological step in the spread of the pathogen, based on secondary infections leading to the disease of the berries, is the infection of floral tissues and/or fruit pedicel, followed by a period of latency until veraison (Pezet et al. 2004). Later in the growing season, conidial infections of ripening berries are considered to be as important as the development of latent infections of green tissues in the spread of disease (Elmer and Michailides 2004). The rate and extent of epidemic development depends on dynamical changes in the pathogen, the host and their environment. For the pathogen, this depends largely on the genetic structure of *B. cinerea* populations. These comprise two different main genetic groups, *i.e.* clades, Group I and Group II (Fournier et al. 2005). Within the Group-II isolates, classification is according to four transposable element types including 'II-*transposa*' which is the dominant and most aggressive type on grape berries (Martinez et al. 2005, 2008). Epidemic progress is also very dependent on environmental conditions, particularly climate and microclimate (English et al. 1989; Fermaud et al. 2001). In addition, the presence of wounds from an exogeneous origin has also been shown to promote infection by the pathogen, e.g. injuries caused by insects which can also act as vectors of conidia, (Elmer and Michailides 2004). However, disease development has been shown to be driven by various interconnected host-related factors, such as the vegetative growth and development of the host plant (Valdés-Gómez et al. 2008), cluster architecture (Vail and Marois 1991) and intrinsic berry susceptibility or ontogenic resistance (Mlikota-Gabler et al. 2003).

Whilst the characteristics of the grape berry are a critical factor in the epidemiology and severity of the disease, relatively little has been done to relate such ontogenic changes, associated with the grape berry skin, to changes in grape berry susceptibility to *B. cinerea*. Grape berries have a number of characteristics that allow them to resist fungal infection. Among these are the physical, morphological and/or anatomical barriers formed by the skin tissue, between the inner grape and its external environment. On the one hand, a higher frequency of cracks and pores at the berry surface increases fruit susceptibility by creating entry points for penetration by conidial germ tubes of the pathogen (Commeli et al. 1997; Mlikota-Gabler et al. 2003). On the other hand, the number and thickness of epidermal and hypodermal cell layers have been positively correlated with resistance to *B. cinerea* (Mlikota-Gabler et al. 2003). Physical resistance to infection also depends on the cuticle and wax content as well as the cell wall structure and composition in the berry skin (Commeli et al. 1997; Mlikota-Gabler et al. 2003; Vorwerk et al. 2004). Furthermore, cluster compactness has been shown to increase berry susceptibility to *B. cinerea* by affecting both skin morphology and fruit microclimate (Fermaud et al. 2001; Percival et al. 1993; Vail and Marois 1991). An indirect way for assessing physical resistance and hardness (force at fracture) of fruit tissues has been developed recently in apple, showing that such features increased logarithmically as water activity (Aw) decreased in accordance to microstructural changes (Acevedo et al. 2008). Aw is defined as the ratio of the water vapour pressure of a solution to that of pure water at a given temperature and it has long been demonstrated to be a critical factor affecting the growth and metabolism of fungi (Magan and Lacey 1984). Generally, yeasts are more

resistant than bacteria and fungi, *i.e* they can grow at low Aw values (Rousseau and Donèche 2001). For *B. cinerea* as for *Penicillium* spp., it has been shown *in vitro* that a high free water level is necessary to induce spore germination and fungal growth (Lahlali et al. 2007; Magan and Lacey 1984).

Furthermore, various biochemical features of grape berries may also affect fungal pathogenesis, particularly the presence of diverse constituents in the fruit skin. These skin compounds are constitutive or induced factors following stress or infection. Tannins and phenolic compounds, preformed in the skin, are known to be important for grey mold resistance (Goetz et al. 1999; Sarig et al. 1998). Similarly, water-soluble pectins and phenolics linked to the skin parietal structures have been identified as indicators of susceptibility and resistance to the pathogen, respectively (Dubos and Roudet 2003). In response to pathogen attacks, grape berries deploy different mechanisms including (1) the synthesis of small secondary stress metabolites, such as phytoalexins (Bavaresco et al. 1997), and (2) the accumulation of tannins and pathogenesis-related (PR) proteins, notably chitinases and β-1,3-glucanases which impede directly fungal invasion. However, it has been found recently that in healthy grapes, the skins also accumulate both of these PR proteins during fruit ripening (Deytieux et al. 2007).

Botrytis infection is dependent on the evolution of berry resistance during fruit development, *i.e*. ontogenic resistance, in particular at the skin level. Although after contact between a conidium and the host tissue, a number of factors influence germination such as temperature, relative humidity (RH) and the presence of nutrients on the skin surface (Lahlali et al. 2007), an understanding of the mechanisms involved in disease development is far from being comprehensive. Most researchers agree that *B. cinerea* resistance results from a combination of factors, but few studies describe clearly the structural and biochemical changes in the berry associated with the onset of its susceptibility during the ripening process. Thus, the objective of this work was to examine certain skin-related factors associated with grape berry development (mass, Aw, tannins, pectins), and relate these to the ontogenic resistance of the grape berry and, in turn, to the risk of infection by *B. cinerea.*

Materials and methods

Fungal material and routine culture conditions

Three virulent isolates of *B. cinerea* (213, 234 and 344), used throughout the study, were selected from the collection of UMR Santé Végétale INRA-ENITA, Bordeaux. These single-spore isolates have been characterised as belonging to the II-*transposa* type, in the Group-II clade, and their phenotypic features have been described previously (Martinez et al. 2003, 2008). They were obtained originally in 1998, between veraison and harvest, from the cvs Semillon blanc (213 and 234) and Merlot noir (344) in vineyards near Bordeaux. Stock cultures were maintained on solid 1.5% malt-agar medium. All isolates are available from authors upon request.

Fruit sampling

Grapes (*V. vinifera*, cv. Sauvignon blanc) were collected from an INRA experimental vineyard located near Bordeaux during the 2006 growing season. The vineyard was planted in 1991 on a gravely soil and was grafted onto '101–14' rootstock. The planting density was approximately 5,350 vines ha^{-1} with a row by vine spacing of 1.70 m×1.10 m and a north–south row orientation. The experimental vine plot was not treated with anti-*Botrytis* fungicides during the growing season. Grape clusters were sampled at seven different stages of development (Table 1): (1) the end of the first period of active growth (berry touch) or stage 33 according to Eichhorn and Lorenz (1977); (2) beginning of colour change (veraison: phenological stage 35); (3) mid-colour change (stage 36); (4) end of colour change (stage 37); (5) intermediate stage during fruit maturation; (6) full maturity (stage 38) and (7) over-maturity. These stages corresponded to 36, 50, 59, 66, 78, 87 and 97 days after anthesis (DAA), respectively. At each stage, on three consecutive rows, a total of 36 grape clusters were randomly sampled from identified vines, at the rate of one cluster per vine. For the analyses which required fresh grapes, approximately 30 berries per cluster were cut carefully (pedicel attached) from the top of the clusters, then pooled and used. On the other hand, for phenolic analyses and alcohol-insoluble material (AIM) preparation, five berries per cluster were carefully cut from another

Table 1 Dates of sampling and associated developmental stages

DAA	Stage (Eichhorn and Lorenz 1977)	Development stage
36	33	Berry touch
50	35	Beginning of colour change (veraison)
59	36	Mid-colour change
66	37	End-colour change
78	37–38	During maturation
87	38	Full maturity
97	40	Over maturity

56 randomly selected clusters from the same identified vines. These berries were immediately frozen in liquid nitrogen and stored at −80°C until used.

Media preparation and *B. cinerea* growth rate according to Aw

PDA was used as the basal PDA medium (Aw=0.997) and the Aw of the medium was also modified by replacing some of the water by an equal weight of NaCl to obtain Aw levels of 0.990, 0.9815, 0.966, 0.946 and 0.930 at 25°C (Rousseau and Donèche 2001). An Aw-meter, Aw-Sprint (Novasina), was used to check the Aw level which was found to be within a range of ±0.003 of the expected Aw level. The final media were autoclaved at 120°C for 20 min. The growth rate of each of the *B. cinerea* strains was assessed by inoculating the centre of a Petri dish with a 5 mm diam PDA disc of mycelium cut from a 4 day-old culture (three plates per Aw-value). The plates were sealed with Parafilm to avoid loss of water and then incubated at 20°C in the dark. Fungal growth was assessed by measuring mycelium development along four rectangular radii. The experiment was repeated twice.

Pathogenesis tests

Berry susceptibility was determined at each phenological stage on approximately 150 berries with pedicels attached to avoid a risk of wounding. To remove floral and/or other water-soluble organic residues that might be used as a nutrient source by *B. cinerea*, berries were first rinsed in sterile deionised water, dried, and then placed, pedicel downward, on a grid within a moist incubation chamber (*i.e.* a plastic box containing absorbent paper in the base with sterile water). For every *B. cinerea* isolate, 34 berries at the same stage were inoculated by placing one mycelial plug (5 mm diam) with mycelium in contact with the berry surface. This way of inoculating was selected because it was comparable to the method used to assess the mycelial growth rate in Petri dishes. Moreover, this allowed us to test and confirm unambiguously the ontogenic resistance of the grape berry, notably at the early stages of development, under conditions of high inoculum pressure, *i.e.* mycelium more aggressive than conidia, nutrient source added and virulent strains used. The plugs had been cut from the edge of 5 day-old colonies of *B. cinerea* grown on MA plates at 20°C in the dark. Uninoculated control treatments were included by using, at each phenological stage, three incubation chambers, each containing 17 uninoculated and unwounded berries. All boxes were placed in an incubator (TABAI ESPEC CORP EX-111, Osaka, Japan) at a constant temperature of 15°C and with a RH of 100%. Symptom development was measured regularly by assessing visually: (1) the total percentage of the berry surface expressing a typical brown rotted area (sporulating + non-sporulating) and (2) the percentage of the berry surface showing *B. cinerea* sporulation. A semi-quantitative scale was used: 0 = healthy, 5%, 10%, 20%, 33%, 50%, 66%, 80%, 90%, 95% and 100% = totally rotten and/or sporulating. Furthermore, at 27 days post-inoculation (dpi), the number of spores at the surface of every sporulating berry was assessed by haemocytometry after the berry was immersed into 10 ml of sterile distilled water containing 0.001% Tween 80. Three variables of disease intensity were calculated for each *B. cinerea* strain and maturity stage. Considering all the berries tested, the mean rot severity (MRS) was calculated at 24 dpi, as the mean percentage of the berry surface expressing a rot symptom and the mean sporulation severity (MSS) as the mean percentage of the berry

surface showing sporulation at 27 dpi. Considering diseased berries only, the mean sporulation intensity (MSI) corresponded to the mean number of spores (in millions) per sporulating berry at 27 dpi.

Fruit morphological and maturity characteristics

All the clusters sampled at each stage were first weighed individually (CFM) to assess cluster architecture because Vail and Marois (1991) have shown that cluster weight made the largest contribution to cluster tightness among various cluster measurements (number of berries cm^{-1} of rachis, ratio of interior to exterior berries…). Afterward, three samples of 100 cut fresh berries were randomly selected from all the 36 clusters and weighed to assess the mean berry mass (BFM). All berries from each sample were then wrapped in a double-layer of cheesecloth and crushed with a small hand press. The resulting juice was centrifuged (3,000 *g*, 2 min) to remove debris. Aliquots of the supernatant were retained for immediate analysis of titratable acidity (TA) by titration with NaOH, pH and soluble solids (S) by refractometry.

Skin characteristics

At each developmental stage, three samples of 10 randomly selected berries were peeled to isolate fresh skins. The skin and berry fresh masses (SFM and BFM) were evaluated to calculate the skin mass proportion per berry. Finally, skins were desiccated at 100°C (XM60, Precisa) and weighed to assess their water content as a percentage (W%). Moreover, another random sample of 10 berries was used to measure Aw on each berry. Beforehand, the pedicel was surrounded with paraffin to avoid exchanges from this zone and to consider only those from the skin surface. The berry was placed in the chamber of an Aw-meter Aw-Sprint (Novasina), thermo-regulated at 30°C. The stability factor was adjusted to 6 min.

Cell wall material preparation

Cell walls were isolated as alcohol-insoluble material (AIM) by the methods described in Valdés-Gómez et al. (2008) Skins (50 g) were ground in a fine powder in liquid nitrogen and boiled for 10 min in ethanol 95%. They were then cooled at room temperature and centrifuged (10,000 *g*) for 20 min. On a minimum of three occasions, the pellet was then resuspended in cold ethanol 95% and centrifuged similarly until the pellet was decolourised. The resulting solids were dried overnight at 40°C, ground to a fine powder (<100 μm) and stored in the dark until analysis.

Phenolic compound extraction and assays

Phenolic compounds and tannin contents were determined in skins (from three samples of 10 berries) ground in liquid nitrogen and in the AIM fraction. On one hand, for both materials, we performed an extraction process based on two successive steps in 40 ml of methanol containing 0.1% of 12N HCl for 3 h, according to Gagne et al. (2006). On the other hand, using the AIM fraction only, phenolic compounds linked to the parietal structures were extracted using NaOH (1 M) at 70°C (Starck et al. 1988). The amount of total phenolics from the AIM fraction (AIM-TP) was measured by the Folin-Ciocalteu method (Singleton and Rossi 1965) and expressed as mg g^{-1} AIM. Tannin contents were determined by spectrophotometry (Gagne et al. 2006) and expressed as mg g^{-1} skin, namely STC when issued from the skin and AIM-TC from the AIM fraction.

Pectin soluble in water

Water-soluble pectic compounds (WSP) were extracted from the AIM fraction by centrifugation (10,000 *g*) for 30 min of 0.5 g of AIM diluted in 25 ml distilled water. Concentration in uronic acids, expressed as mg g^{-1} AIM, was measured in three replicates by the colorimetric method using metahydroxyphenylphenol (Robertson 1979).

Statistical methods

In order to identify the main relationships among the fruit variables tested and to choose the ones that could better account for grey mould development in our conditions, eighteen variables were submitted to principal component analysis (PCA). In the PCA, 13 possible explanatory variables characterising the fruit were analysed as active variables, whereas the three grey mould variables (MRS, MSS, MSI) were considered as supplementary variables (not used to calculate relative contributions to the axes). Data of

Botrytis rot development at the berry surface were analysed by an analysis of variance (ANOVA) procedure using a randomised factorial design with two main effects: the strain (213, 234 and 344) and the berry developmental stage (four stages: end of veraison, intermediate, maturity and over-maturity). Data analysis was conducted with StatBox software (Version 6.23, Grimmer Logiciels, Paris) and with GraphPad Prism software (Version 4.00 for Windows, 2003).

Results

Characterisation of the fruit maturity parameters

The grapes collected were characterised by a classical evolution profile during their development showing an increase in berry weight and soluble content and a decrease in titratable acidity (Table 2). In the skin, the total tannin content decreased progressively, except for a marked increase, limited in time, at mid-colour change, where the contents peaked at 74 mg g^{-1} skin.

Interrelations among fruit morphology, berry maturity, skin composition and susceptibility to *B. cinerea*

Interrelations were investigated by PCA among various variables characterising the fruit (fruit morphology, maturity components and skin biochemical features) and the response variables pertaining to berry susceptibility to *B. cinerea* (Fig. 1). The first two main factorial axes accounted for 79% of the total variance. PCA showed strong correlations between the variables characterising fruit morphology and maturity during berry development. The first composite axis was principally representative of the berry maturity level by comprising the following variables (relative contribution to the axis in parenthesis): titratable acidity (10.0%) as opposed to sugar content (9.9%), mean mass of 100 berries (9.8%), the maturity index (S/TA) (9.5%), pH (9.3%) and, to a lesser extent, skin water content (8.6%). The second main axis represented mostly the tannin content in the skin cell-walls (AIM-TC) expressed g^{-1} skin (40.4%). Water activity at the skin surface contributed in part (8.7%) to this second axis.

On the PCA diagram, individuals were clearly differentiated and clustered according to four main groups: (1) the immature berries at bunch closure and veraison (stages 36 and 50); (2) berries mid-coloured (stage 59); (3) maturing berries from the end of berry colouration to maturity (stages 66, 78 and 87 mostly with positive coordinate values on both axes); (4) over-mature berries (stage 97). Berries at the 'mid-colour change' stage and at over-maturity were characterised by a lower content in cell wall tannins (AIM-TC) and low Aw values (negative axis-2 coordinates). Furthermore, the mid-colour change stage showed the highest content in total tannins in skins (STC). Besides the previous active variables, the *B. cinerea* variables (supplementary variables not contributing to the factorial axes) were clearly and positively associated with an increased berry maturity (positive coordinates on the first axis). The location of both rot and sporulation severities was very close to the maturity index resulting from highly significant positive correlations ($P<0.001$, Pearson's $r=0.94$ and 0.95, respectively). Furthermore, three other explanatory variables, related partly to the second axis, were

Table 2 Maturity characterisation of berries

DAA	39	50[a)]	59	66	78	87	97
Berry weight (g $berry^{-1}$)	0.93±0.03	1.10±0.02	1.39±0.05	1.65±0.03	1.92±0.07	2.06±0.03	2.04±0.06
Soluble content (g l^{-1})	43.84±0.4	72.59±2.5	105.59±2.2	163.47±10.1	188.03±9.0	184.57±10.7	222.03±4.2
Titrable acidity (g l^{-1})[b)]	20.58±0.1	20.16±0.2	13.62±0.5	9.02±1.8	6.27±0.2	4.80±0.5	3.66±0.2
Total tannins (mg g^{-1} skin)[c)]	58.30±2.5	47.54±0.8	73.74±8.6	39.46±3.2	33.63±0.5	30.32±0.2	23.70±0.7

Each value represents the mean of three measurements based on 100-berry samples (10-berry samples for total tannins) ± standard deviation

[a)] Onset of ripening

[b)] Expressed in g l^{-1} sulphuric acid

[c)] Measurements conduced on isolated skins

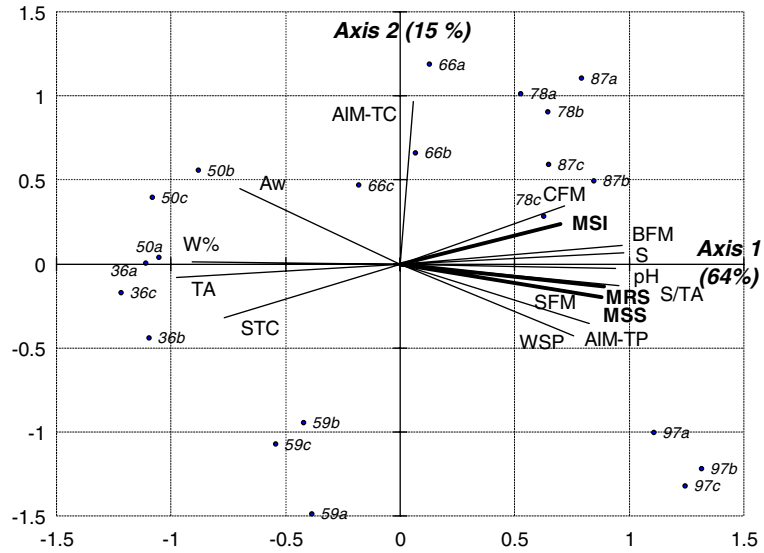

Fig. 1 Relative location on the first two axes of a principal component analysis (PCA) of active variables characterising fruit maturity (BFM, CFM, S, TA, S/TA, pH) and berry skin biochemical and morphological characteristics (SFM, Aw, W%, STC, AIM-TC, AIM-TP, WSP) and of supplementary variables (marked in bold) related to berry susceptibility to grey mould (MSS, MRS and MSI). An explanation of abbreviations is given in the Materials and methods. Individuals, corresponding to berries sampled at seven different development stages, are indicated in italics as DAA numbers (days after anthesis) followed by a replicate letter (*a*,*b* or *c*)

of interest because of a highly significant ($P<0.001$) correlation with the mean rot severity: (1) phenolic compounds in the skin cell walls (AIM-TP), $r=0.80$; (2) total tannin content in the skin (STC), $r=-0.75$; (3) water activity (Aw) located diametrically opposed to both rot severities, $r = -0.69$.

Evolution of berry susceptibility during maturation

The uninoculated control berries showed the following mean sporulation severities: 0% until the mid-colour change stage, 5.9% at the end of colour change, 2.1% during maturation (78 DAA), 7.4% at maturity and 22.9% at over maturity. As for inoculated berries, the development of Botrytis rot symptoms at the berry surface, as indicated by both mean percentages of rot (MRS) and sporulation (MSS) severities, increased significantly ($P<0.001$) as the berry developed (Table 3 and Fig. 2). A similar pattern was observed for each strain of *Botrytis* with no significant difference in virulence detected between strains (Table 3); thus, the mean results from the three strains are presented in Fig. 2. Before and at the onset of veraison (50 DAA), the immature berries were not susceptible to the pathogen. The response of these variables of disease severity to the developmental stage was represented by a sigmoid curve that increased regularly during berry maturation, *i.e.* from mid-veraison (59 DAA) onwards (Fig. 2). A similar pattern was observed for each strain of *Botrytis* with no significant difference in virulence detected between strains (Table 3). Lastly, the mean number of spores per sporulating berry (MSI) was not significantly influenced ($P=0.47$) by the berry developmental stage or by the *B. cinerea* strain ($P=0.07$). Consequently, one sporulating berry produced a similar number of spores irrespective of the fruit developmental stage from the mid-colour change stage to over-maturity. The overall mean number of spores per sporulating berry reached 6.009 million at 27 dpi.

Effect of water availability on mycelial radial growth rate

The effects of the isolate, Aw and their interaction were investigated on the radial growth rate of *B. cinerea*. The ANOVA showed both highly significant effects of Aw ($P<0.0001$) and the isolate ($P=0.0006$), without any interaction effect ($P=0.083$). Aw values ≥ 0.98 resulted in the highest mean growth rates (not significantly different at $P=0.05$), *i.e.* 5.9, 5.4 and

Table 3 Analysis of variance (ANOVA) of rot symptom development at the berry surface according to the berry phenological stage and the *B. cinerea* strain

[a] Five developmental stages were tested, *i.e.* from the mid-colour change stage to overmaturity (Table 1), because the first two stages did not show any rot symptom

Source of variation	df	Mean square	*F* value (*P* value)
ANOVA of mean rot severity (MRS)			
Berry developmental stage[a]	4	5,337	130.5 ($P<0.0001$)
B. cinerea strain	2	73.5	1.8 ($P=0.23$)
ANOVA of mean sporulation intensity (MSS)			
Berry developmental stage[a]	4	4,832	101.2 ($P<0.0001$)
B. cinerea strain	2	49	1.0 ($P=0.40$)
ANOVA of mean number of spores (in million) per sporulating berry (MSI)			
Berry developmental stage[a]	4	3.3	0.9 ($P=0.47$)
B. cinerea strain	2	15.1	4.3 ($P=0.07$)

5.0 mm day^{-1} for Aw values of 0.99, 0.997 and 0.98, respectively. They were significantly different from the two lowest growth rates: 0.34 and 0.31 mm day^{-1} for Aw values of 0.95 and 0.93, respectively. As for strain effect, the two strains 213 and 234 showed the greatest mean growth rates which were not significantly different (3.7 and 4.2 mm day^{-1}, respectively). The strain 344 showed the least mean growth rate (2.3 mm day^{-1}). Furthermore, both the Aw and isolate effects on the growth rate are exemplified clearly based on the data from one of the two experiments for six water activities (Fig. 3). *Botrytis cinerea* was unable to grow at Aw ≤0.946 in NaCl—modified PDA medium. The minimum Aw value allowing mycelial extension was 0.966. From this Aw threshold value upwards, the radial growth rate increased with increasing Aw to 0.99, corresponding to a maximum linear growth. Thereafter, a decline in radial growth with increasing Aw from 0.99 to 0.997 was noticeable for the three isolates.

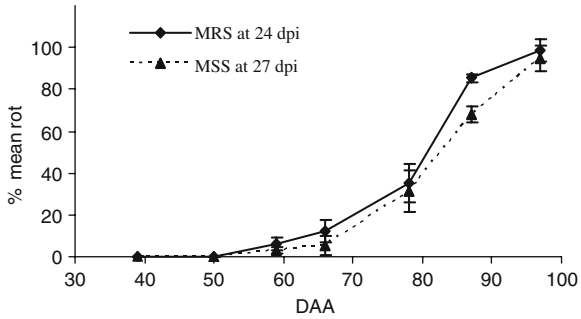

Fig. 2 Evolution of berry susceptibility (ontogenic resistance) to grey mould. The rotted (MRS) or sporulating (MSS) surface of infected berries were scored (%) regularly for 27 days in a controlled-condition experiment using approx. 100 berries per developmental stage inoculated individually with one *Botrytis* mycelial plug

Evolution of water activity (Aw) at the berry surface

At the berry skin surface, Aw decreased progressively during grape development from 0.937 to 0.887 and the effect of the development stage was significant at $P=0.00001$ (ANOVA) (Fig. 4). The maximal Aw value was reached at the bunch closure stage, *i.e.* 36 DAA. Then, Aw decreased significantly to a low level (0.902) at mid-colour change and then increased showing a plateau at intermediate stages during ripening. Lastly, just before harvest, Aw values decreased markedly to the minimal level (approximately 0.89) at over-maturity.

Relationships between possible explanatory fruit features and *Botrytis* rot severity

Figure 5. shows the relationships between susceptibility to *B. cinerea*, measured by MRS, and skin parameters, particularly skin phenolic composition and Aw values. Botrytis rot susceptibility was positively correlated with total phenolics in the skin cell walls, measured by the Folin-Ciocalteu method from the AIM fraction (AIM-TP) ($r^2=0.71$), and negatively correlated with the total tannin contents in the skin (STC), determined by spectrophotometry ($r^2=0.59$), and the Aw measured at the berry surface ($r^2=0.68$).

Discussion

In this study, we have investigated the temporal, developmental changes in various morphological and biochemical features of the grape berry and related these to changes in berry susceptibility to infection by

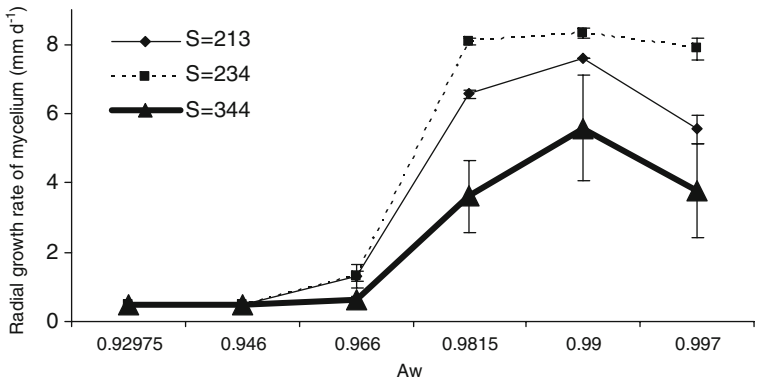

Fig. 3 Effect of water availability on the mycelial radial growth rate in *B. cinerea*. Each mark represents the mean of three measurements (three plates per Aw-value and per strain) ± standard deviation (error bars). S = the *B. cinerea* strain used

B. cinerea. In 2006, the year of this study, the parameters characterising grape berry development were typical for grapevines grown in the Bordeaux region of France. During the season, characterised by rapid vegetative growth, the first grapes to show a change of colour (veraison) were detected on the 23rd July. Berry development was particularly influenced by the weather conditions: alternating warm (end of July and September) and cool temperatures (August). At the beginning of September, good ripening conditions resulted from high temperatures which greatly favoured the accumulation of sugar and anthocyanins. A week before harvest, the grape sugar content was well-balanced by sufficient acidity and a remarkable richness in phenolic compounds was noticeable. Thus, in 2006, Bordeaux wines were of a particularly high quality and classified as 'classic': rich in sugar but with a sufficient acidity, very equilibrated, expressing powerful and complex fruitiness (Geny et al. 2007).

Under controlled conditions, the key stage at which the berry becomes susceptible to the pathogen was clearly identified as the 'mid-colour change' stage, during the veraison period. Afterwards, the susceptibility of inoculated, unwounded berries increased significantly following a sigmoid curve during maturation. A PCA showed that different maturity variables were correlated with changes in berry susceptibility. The increasing susceptibility corresponded to (1) a higher skin tissue colonisation by *B. cinerea* (rot expansion) and (2) a greater sporulating overall surface following the increase in incidence of rotted berries. The maturity variables were autocorrelated and comprised notably: (1) titratable acidity, (2) sugar content, (3) maturity index, (4) mean mass of 100 berries and (5) pH. This positive and close relationship between grey mould susceptibility and grape berry ripening confirmed recent work involving artificial conidial inoculations of non-wounded berries (Kretschmer et al. 2007). Similarly, Mundy and Beresford (2007) have established a significant linear regression between berry sugar concentration and the percentage of rotted berries. In immature grapes, common organic acids may be toxic for the pathogen. This has been demonstrated for glycolic acid (Pezet et al. 2004) and for tartaric and malic acids which inhibited fungal growth at high concentrations on solid media (Vercesi et al. 1997). From fruit set to the period of veraison, tartaric acid is the most abundant organic acid in the fruit, representing a possible carbon source for *B. cinerea*. Yet, at a concentration higher than 7 g l^{-1}, tartaric acid has been shown to reduce the rate of conidial germination and mycelial growth and to inhibit sporulation (Donèche 1986).

In vineyards, this maturity effect may contribute to the well-known epidemiological progression of grey mould after veraison following secondary infections

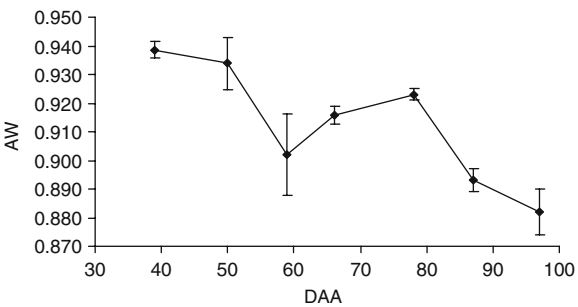

Fig. 4 Evolution of water availability at the berry surface. Each mark represents the overall mean from 10 measurements on a 10-berry sample (± standard deviation: error bars)

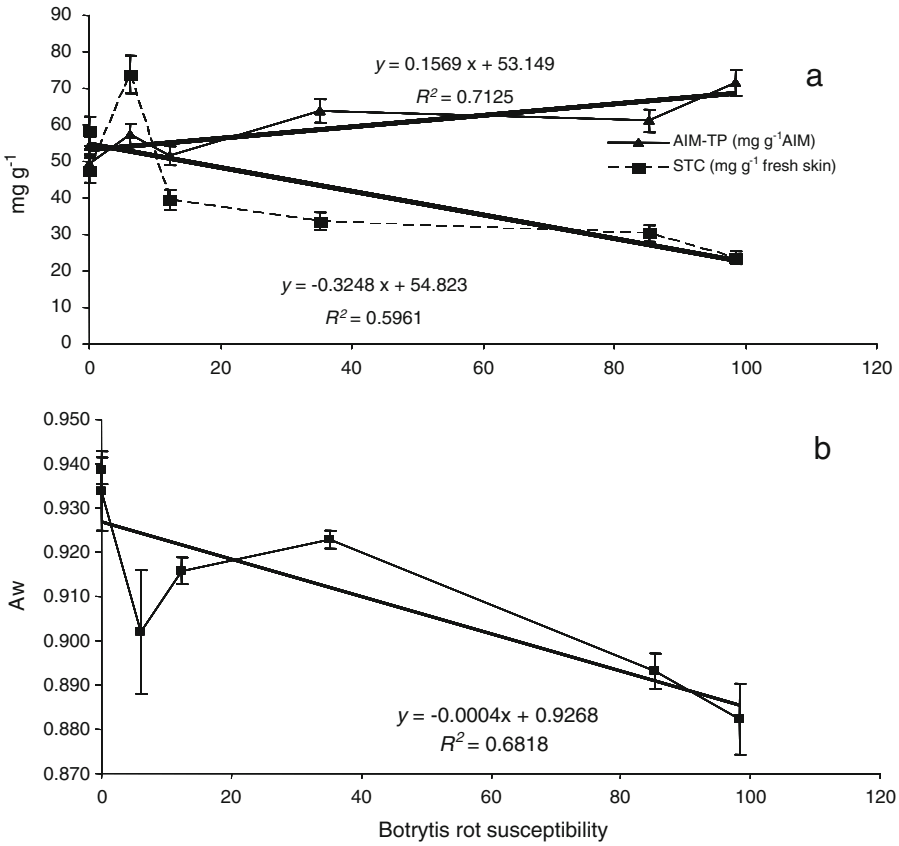

Fig. 5 Linear relationships between Botrytis rot susceptibility, indicated by the mean rot severity (MRS), and either skin phenolic composition (**a**) or water availability Aw at the berry surface (**b**). Each mark represents the mean of three measurements, each based on a 10 or 100-berry sample (± standard deviation: error bars)

caused by air-borne conidia ('pathway V' according to Elmer and Michailides 2004). However, under field conditions, this maturity effect on disease incidence may also be counterbalanced under conditions of high vegetative growth of the grapevine. These conditions are characterised by a relatively low maturity level associated with a fruit microclimate within the canopy very conducive to the disease (Valdés-Gómez et al. 2008). Furthermore, in a recent study of mature berries removed from different grapevine cultivars, significant correlations have been demonstrated between susceptibility to the pathogen and (1) the soluble solids (sugar) content, (2) the number and thickness of cell layers in the epidermis and external hypodermis and (3) the amount of cuticle and wax and the number of pores in the berry surface (Mlikota-Gabler et al. 2003). The first two factors, which were significantly auto-correlated, are connected with the maturation process during which several skin cell layers, located in the hypodermis, are converted into the pulp tissue (Kretschmer et al. 2007).

The importance of Aw in the susceptibility of grape-berries to infection by *B. cinerea*, measured directly at the surface of fresh berries, was highlighted in this work. On the one hand, the significant effect of Aw was confirmed clearly on mycelial growth on PDA medium and, on the other hand, significant changes in Aw have been shown at the berry surface during fruit development. The general trend was a decrease in Aw during development with a transient low value at 59 DAA (mid-colour change) corresponding to an important cell wall rearrangement in the berry skin. At this point, Deytieux-Belleau et al. (2008) have shown an increase in activity and/or gene expression for two cell-wall modifying enzymes, *i.e.* pectin-methyl esterase and polygalacturonase. So, the onset of berry ripening is characterised by large modifications of the skin cell wall, which could cause a transient increase in skin permeability, and thus account for a low Aw value.

On the PCA diagram, Aw was located diametrically opposed to rot development resulting from a significant negative correlation between Aw and berry susceptibility. Aw may, thus, be considered as a key skin feature associated with the fruit ontogenic resistance to the pathogen. According to Maltini et

al. (2003) Aw is clearly related to most physical degradation reactions. The structural alteration of the grape berry skin, with less cell layers, contribute to an exosmosis increase during ripening, thereby providing substantial amounts of nutrients to the epiphytic microorganisms, including *B. cinerea* (Donèche 1986; Padgett and Morrison 1990). Concurrently, the presence of these soluble compounds on the skin must increase the solute concentration in free water at the berry surface, thus leading to a decrease in Aw (as shown in this study). In this context, the Aw value determined for the berry surface can be considered as an indirect assessment of physical and structural alterations of the berry skin. At maturity and overmaturity, the low Aw values corresponded presumably to a high level of skin alteration which is highly favourable to the fungus by facilitating penetration and colonisation by developing hyphae through associated pores or micro-cracks. On the other hand, the *in vitro* growth study on artificial medium did not allow us to test the effect of Aw in the presence of a pellicle at the medium surface. It follows from this that we found such a discrepancy between the relationships between Aw and *B. cinerea* development established either on fruit or on artificial medium. From these results, it can be hypothesised that the presence of both nutrients and micro-cracks at the surface of maturing berries may have a greater effect to favour the pathogen than the expected negative effect following the decrease in Aw. In this connection, the relationships between Aw and (1) nutrient availability at the berry surface (2) the cuticle and wax structure and (3) the pore number in the berry skin, should be further investigated.

According to Pezet et al. (2004), the ontogenic resistance of immature grape berries to *B. cinerea* is certainly the consequence of the presence of constitutive antifungal compounds in active concentrations. These authors considered phenolic compounds and polymeric proanthocyanidins (PPRAs) or tannins from the berry skin as toxic to *B. cinerea*, inhibiting both the polygalacturonase and the laccase-like stilbene oxidase activities. Furthermore, although Mlikota-Gabler et al. (2003) did not find any association, other studies showed a significant correlation between a low grapevine susceptibility and a high phenolic content (Sarig et al. 1998), in particular, phenolic compounds linked to the parietal structures in the berry skin (Dubos and Roudet 2003) and those detected in exudates from the grape berry (Padgett and Morrison 1990). Our results concerning the chemical composition of the berry skin corroborate this. The variables of susceptibility to the disease were located in the PCA close to the total phenolic content from skin cell walls (AIM-TP) and, interestingly, a significant negative correlation was established with the tannin content in the skin (STC).

In accordance with a previous paper (Gagne et al. 2009), it was noticeable that the total tannin content increased at the mid-colour change stage in connection with peaks of activity and expression of LAR and ANR (leucoanthocyanidin reductase, anthocyanidin reductase). These authors hypothesised that this increase activity resulted from the stimulation of the proanthocyanidin pathway (corresponding to the beginning of anthocyanin accumulation in black cultivars). The inhibitory activity of tannins (PPRAs) has been suggested to be specific to some enzymes of *B. cinerea*, in particular the stilbene oxidase (Goetz et al. 1999). Furthermore, the PPRAs from the berry skin could play an important role in berry ontogenic resistance because the inhibitory activity of the PPRAs from mature berries to stilbene oxidase is lower than that from unripe berries (Goetz et al. 1999; Pezet et al. 2004).

Lastly, different structural and biochemical changes that occur during berry ripening may act simultaneously to affect the susceptibility of berries to *B. cinerea*. Classically, our results showed that maturity, measured as sugar content, was positively correlated with berry susceptibility to the pathogen. Furthermore, both water availability at the berry surface and the total tannin content in the skin may also be considered as potential major determinants affecting both growth of the fungus and berry colonisation. The results of this multi-factorial approach suggest that, besides fruit maturity, Aw and tannin contents might be used to estimate the potential susceptibility of grape berries to infection by *B. cinerea*. However, predictions based on extrapolation of these results alone to natural situations and beyond the tested range for each of these indicators should be made with caution since other factors such as nutrient availability, microclimatic conditions and interactions with insects and/or micro-organisms on the fruit surface may also affect the development of *B. cinerea*.

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