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Life traits of four Botryosphaeriaceae species and molecular responses of different grapevine cultivars or hybrids

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Botryosphaeriaceae is a fungal family comprising many species involved in botryosphaeria dieback, a worldwide grapevine trunk disease. Currently, the interactions between Botryosphaeriaceae species and various grapevine cultivars are poorly understood and little data is available. This study investigated various life traits of five isolates belonging to four species of Botryosphaeriaceae found in French vineyards (*Diplodia mutila*, *Diplodia seriata*, *Lasiodiplodia viticola* and *Neofusicoccum parvum*). The two species *N. parvum* and *L. viticola* exhibited the highest optimal growth temperature and the best growth rates. They were also responsible for the most extensive necrosis and cankers in three *Vitis vinifera* cultivars (Cabernet Sauvignon, Merlot and Ugni-Blanc) that differed in susceptibility to botryosphaeria dieback, and in two genotypes resistant to downy and powdery mildew (RV4 and RV5). Identification of the extracellular toxins produced by isolates in culture media showed that the *N. parvum* isolate had a different metabolite profile from the others, producing terremutin and salicylic acid derivatives, which are known to be compounds associated with virulence. In a second step, life traits were associated with nondestructive monitoring of gene expression involved in the defence mechanisms of five grapevine cultivars and genotypes after inoculation of wood cuttings with Botryosphaeriaceae. The transcript analyses were carried out at different times and were associated with principal component analysis (PCA). Each cultivar presented a specific transcript signature and several transcripts were correlated either with the size of necrosis/cankers or with symptom reduction, thus offering useful markers for breeding or estimating the defence status of plants.

Keywords: botryosphaeria dieback, defence gene expression, fungal toxin, grapevine trunk diseases, resistant genotypes, wood necrosis

Introduction

Viticulture is one of the most extensive agricultural activities in the world with more than 7554 million ha of cultivated land and 240 million hL of wine produced in 2014 (OIV, 2015). Grapevine trunk diseases affect production and lead to the premature death of the vine. As such, they represent a threat to sustainable viticulture worldwide (Fussler *et al.*, 2008). The economic cost of replacing dead grapevines is estimated to be more than $1.5 billion per year (Hofstetter *et al.*, 2012). Three main fungal diseases are responsible: eutypa dieback, esca and botryosphaeria dieback (Bertsch *et al.*, 2013). All three disease complexes can occur alone or together in the same plant and lead to leaf and trunk symptoms or, in the medium/long-term, to plant death (Larignon *et al.*, 2009). The banning, in the early 2000s, of the only effective treatment (sodium arsenite) coincided with a sharp increase in these symptoms (Fussler *et al.*, 2008; Úrbez-Torres *et al.*, 2009). The incidence of trunk diseases has increased tremendously over recent decades (e.g. 13% of French vineyards affected, Grosman & Doublet, 2012; Bruez *et al.*, 2013) and is highly variable, depending on the region and grapevine cultivar (Fussler *et al.*, 2008; Grosman & Doublet, 2012). For example, Chardonnay is less susceptible to botryosphaeria dieback and esca than Gewürztraminer and Mourvèdre, with variable plant responses (Spagnolo *et al.*, 2014). The influence of region is also important, because cultivar susceptibility varies with the region and climatic conditions (Sosnowski *et al.*, 2007). No cultivar is resistant to grapevine trunk diseases, unlike the case for downy or powdery mildew, but *Vitis vinifera* subsp. *sylvestris* could be a resource for resistance (Guan *et al.*, 2016).

Botryosphaeriaceae, the causal agent of botryosphaeria dieback, is a large family of ascomycetes that are found worldwide as endophytes, parasites or saprophytic fungi in many perennial plants (Slippers & Wingfield, 2007) or forest trees such as grapevine, avocado, apple and pine. More than 22 different species have been associated, to date, with botryosphaeria dieback in grapevine (Úrbez-Torres, 2011) and their distribution depends on

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the country and climate, with the main ones being *Diplodia seriata* (teleomorph: *Botryosphaeria obtusa*), *Neofusicoccum parvum* (teleomorph: *Botryosphaeria parva*), *Diplodia mutila* (teleomorph: *Botryosphaeria stevensii*), *Lasiodiplodia theobromae* (teleomorph: *Botryosphaeria rhodina*), *Botryosphaeria dothidea* (anamorph: *Fusicoccum aesculi*), *Neofusicoccum australe* (teleomorph: *Botryosphaeria australis*) and *Neofusicoccum luteum* (teleomorph: *Botryosphaeria lutea*) (Phillips *et al.*, 2013). *Diplodia seriata* has been isolated in at least 10 countries including France (Úrbez-Torres, 2011), *L. theobromae* and *L. viticola* have been isolated in USA vineyards (Úrbez-Torres *et al.*, 2012), *B. dothidea* and *N. parvum* in Portuguese vineyards (Phillips, 2002), *D. mutila* and *N. luteum* in New Zealand vineyards (Baskarathevan *et al.*, 2012) and *N. australe* in Australian vineyards (Pitt *et al.*, 2010). *Neofusicoccum parvum* is often considered as the most virulent species of the Botryosphaeriaceae, whereas *D. seriata* is ranked among the most moderate (Pitt *et al.*, 2013). In the northern hemisphere, symptoms of botryosphaeria dieback appear earlier in the season (May–June) than those of esca (June–July), and vary according to whether white or red cultivars are attacked. White cultivars present yellowish-orange spots on the leaf margins and the blade, whereas red cultivars show wine-red spots. In both cases, the wood of the infected grapevines exhibits necrosis (Larignon *et al.*, 2001). Botryosphaeria dieback symptoms can lead to wedge-shaped cankers, necrosis, progressive bud-break failure and plant dieback (Carlucci *et al.*, 2015).

Although foliar symptoms are observable, these pathogens are rarely found elsewhere than in the wood. It has been demonstrated that they are able to produce cell wall-degrading enzymes and phytotoxic metabolites, the synergistic action of which plays a role in the development of foliar symptoms (Andolfi *et al.*, 2011). Recent studies have identified four dihydro-isocoumarins (mellein, 4-hydroxymellein, 7-hydroxymellein and 4,7-dihydroxymellein) produced by *D. seriata*, and similar toxins, plus other metabolites derived from terremutin and sphaeropsidone, produced by *N. parvum* (Andolfi *et al.*, 2011; Abou-Mansour *et al.*, 2015). Despite the identification of these metabolites, their exact role remains unclear, but their accumulation in plant tissues suggests a potential effect during the appearance of foliar symptoms.

To date, very few studies on grapevine responses following infection by Botryosphaeriaceae have been conducted. Recently, Spagnolo *et al.* (2014) investigated the transcriptomic and proteomic expression in wood, with and without symptoms, of three cultivars (Chardonnay, Gewürztraminer, Mourvèdre) in French vineyards. Expression levels of genes coding for PR-proteins and enzymes involved in stilbene biosynthesis varied in plants with and without symptoms. Moreover, the genes and proteins involved in primary metabolism were also modified depending on the sanitary status of the plants. RNA sequencing of gene expression in leaves after wood infection showed that certain genes could act as host markers of the latent period of *N. parvum* infection (Czemmel *et al.*, 2015). Overall, these studies have thrown light on the interactions between plants and botryosphaeria dieback fungi. However, the molecular or proteomic markers associated with the resistance of plants to this disease are still unidentified.

The objectives of this study were: (i) to estimate the life traits of five isolates (four species) collected in French vineyards by evaluating their growth rates at different temperatures, their toxin profiles and their aggressiveness *in planta* to assess their pathogenicity; and (ii) to nondestructively examine the responses of three cultivars and two hybrids by monitoring the transcript expression of specific defence genes in the presence or absence of Botryosphaeriaceae in the wood. The pathogenicity of Botryosphaeriaceae species and the value of plant defences and protection markers are discussed in relationship to the various responses of the grapevines.

Materials and methods

Plant material

Three grapevine cultivars (*V. vinifera* 'Cabernet Sauvignon', 'Merlot' and 'Ugni-Blanc') and two genotypes with introgressed QTLs for powdery and downy mildew resistance (RV4, accession 3083-219 and RV5, accession 3179-24-7) were propagated from wood cuttings in a greenhouse. The Merlot and Cabernet Sauvignon wood cuttings were provided by Château Couhins (Gironde, France), clones 182 and 191 respectively, and the Ugni-Blanc wood cuttings came from a Cognac vineyard, clone 459 (Graves Saint-Amand). RV4 and RV5 originated from a grapevine breeding project on downy and powdery mildew resistance conducted by A. Bouquet at INRA Montpellier (Bouquet *et al.*, 2000) by backcrossing between *Muscadinia rotundifolia* and *V. vinifera*; RV4 arose from crosses with the cultivars Cabernet Sauvignon, Grenache, Merlot and Aubun, and RV5 from a supplementary cross with Grenache. After 3 weeks, rooted cuttings were potted in sandy soil and were grown with 16 h light per day. Two-month-old plants with 10–12 leaves were used for the experiments.

Fungal material

Five isolates of four species of Botryosphaeriaceae from the laboratory collection were used: *N. parvum* isolate PER20, *L. viticola* isolate LAG05, *D. mutila* isolate BRA08, and *D. seriata* isolates LAT16 and F98-1 obtained from the vineyard (Table 1). Identification was based on morphological characteristics and PCR analyses of nucleotide sequences of genes for ITS, 28S and β-tubulin; sequences were deposited in GenBank (Table 1). Fungi were grown on malt agar medium (MA, 20 g L^{-1} malt, 15 g L^{-1} agar). Isolates were maintained on medium at 23 °C and subcultured at regular intervals by transferring colonized agar plugs (5 mm diameter) to fresh medium.

Influence of temperature on *in vitro* growth rate of isolates

Cardinal temperatures for growth were determined for each isolate. Mycelial plugs (5 mm diameter) from the margins of actively growing 1-day-old cultures were transferred to MA medium. Petri dishes were closed and incubated at 15, 22, 25,

Table 1 Characteristics of Botryosphaeriaceae isolates from French vineyards

Isolate	Species	Location	Cultivar	Sampling year	Plant state	GenBank accession no.			Mycelium pigmentation
						ITS	β-tubulin	28S	
PER20	*Neofusicoccum parvum*	Champagne	Chardonnay	2008	Plant with symptoms, necrotic wood	KP699097	KP699090	KP699097	Yellow
LAG05	*Lasiodiplodia viticola*	Gironde	Sauvignon	2007	Plant with symptoms, healthy wood	KP699095	KP699091	KP699099	White
BRA08	*Diplodia mutila*	Champagne	Pinot noir	2008	Plant with symptoms, bark	KP699094	KP699092	KP699098	White
LAT16	*Diplodia seriata*	Gironde	Cabernet-Franc	2008	Plant with symptoms, necrotic wood	KP699096	KP699089	KP699100	White
F98-1	*Diplodia seriata*	Pyrénées-Orientales	Syrah	1998	Plant with symptoms	KP699093	KP699088	KP699102	White

28, 33 or 36 °C with a 16/8 h light/dark photoperiod. Two perpendicular diameters of the mycelium were measured at daily intervals for 4 days or until such time as the mycelium covered the entire surface of the agar plate. The growth rate (mm per day) was calculated for each fungal isolate at each growth temperature. The experiment was carried out in triplicate and two independent experiments were performed. Means of the growth rates for each isolate at each temperature were subjected to statistical analyses by a nonparametric test (Kruskal–Wallis) using R x64 v. 3.0.3 software and significant differences were determined by Tukey's test at the 5% significance level.

Toxin identification

The extraction and identification of toxins produced by the different isolates were performed according to Abou-Mansour *et al.* (2015). Briefly, fungal isolates were grown on 10 Petri dishes with potato dextrose agar (PDA) medium for 10 days in the dark at 25 °C. The agar medium was cut into small pieces and extracted with one volume of ethyl acetate and methanol (3:1 v/v). This step was repeated three times. After evaporation of the solvent to dryness, a crude extract was obtained that was dissolved in methanol (1 mg mL^{-1}), then 40 μL were analysed by reverse-phase HPLC with a MNNucleosil100-5 C18 column (250 nm × 4.6; Machery-Nagel). The mobile phase consisted of two solvents: water (0.5%)/formic acid (A) and acetonitrile (B). The linear gradient started at 5% B and increased to 70% in 30 min and 100% in 35 min. The flow rate was 1 mL min^{-1} at 27 °C and the injection volume was 200 μL. LC-DAD analysis was carried out on a Thermo SCIENTIFIC Dionew HPLC with an ultimate 3000 diode array detector. The toxins were detected at 314, 270, 260 and 240 nm with diode array online detection. The spectra were recorded between 200 and 600 nm. Quantification was performed using an external standard calibration method and the data reported as μg mL^{-1} of a solution of 1 mg mL^{-1} of crude extract.

Pathogenicity studies: mean length of necrosis and cankers

Excised 1-year-old 20 cm canes, including two nodes, were used to produce 2-month-old rooted plants with 10–12 leaves. Each plant was perforated with a drill between the two nodes and inoculated by depositing in each wound a malt agar plug (control) or a mycelial plug (5 mm) taken from the actively growing 1-day-old margin. Each inoculation point was covered with wax. Sixty plants were perforated for each cultivar and genotype (Merlot, Ugni-Blanc, Cabernet Sauvignon, RV4 and RV5). Fifty plants were inoculated with five fungal isolates (PER20, LAG05, BRA08, LAT16 or F98-1, 10 plants for each isolate); 10 plants were used as a control and were not inoculated. Plants were placed in the greenhouse with a 16/8 h day/night photoperiod and watered with a dripper. Development of lesions on the plants was observed 11 months after inoculation (10 plants per modality). Lignified stems were cut and the bark was peeled back from the lower to the upper eye. Lengths of canker lesions at the surface of the wood were measured around the inoculation point (hole included). The trunk was cut longitudinally and the necrosis length and lesions in the wood tissue were also measured (hole not included). Means of the necrosis/canker lengths on each cultivar with each isolate were subjected to statistical analyses by a nonparametric test (Kruskal–Wallis) or by parametric tests (ANOVA) followed by pairwise comparisons using Tukey's post hoc test and significant differences were determined at the 5% significance level. The choice of the parametric or nonparametric test depended upon the homogeneity of variances (Levene's test). Statistical analyses were carried out using R x64 v. 3.0.3 software. Reisolation tests (five repetitions) were performed on pieces of wood (5 mm^2) sampled from necrotic zones. After disinfection with calcium hypochlorite (5%) by immersion for 10 s, the wood pieces were put on MA medium in Petri dishes and incubated at 22 °C.

Gene expression analysis by RT-qPCR

Grapevine leaves (the third leaf fully extended from the apex) from each cultivar and genotype were sampled 0, 2, 6 and 17 days after inoculation (or wounding only), frozen in liquid nitrogen and stored at −80 °C. For each modality (perforated-noninoculated or perforated-inoculated with PER20, LAG05, BRA08, LAT16 or F98-1), three batches of three leaves were harvested. Each sample was crushed in liquid nitrogen and total RNA was extracted using the Spectrum Plant Total RNA kit (Sigma), according to the manufacturer's instructions. Samples were incubated for 15 min with the DNase I digestion set (Sigma). RNA concentration was determined with NanoDrop 1000 (Thermo SCIENTIFIC) using spectrophotometric technology. Ten micrograms of total RNA were reverse-transcribed using 2 μM oligo-$d(T)_{15}$, ribonuclease inhibitor and M-MLV reverse transcriptase (Invitrogen), according to the manufacturer's instructions. Expression of 17 genes was monitored by quantitative polymerase chain reaction (qPCR) as described previously (Dufour *et al.*, 2013), including genes involved in PR protein biosynthesis (*VvPR1*, *VvPR2*, *VvPR3*, *VvPR5*, *VvPR6*, *VvPR8* and *VvPR10*), cell-wall strengthening (*VvCALS* and

VvPER), phenylpropanoid pathway (*VvPAL*, *VvSTS* and *VvCHS*) and indole pathway (*VvANTS*, *VvCHORM* and *VvCHORS*) (Table 2). Two housekeeping genes (*VvEF1γ* and *VvGAPDH*) were used as internal standards to normalize the starting template of cDNA (Table 2), as described previously (Dufour *et al.*, 2013). Three biological replicates, each comprising two technical replicates, were performed for each gene. The expression of the selected genes was assessed by using the Stratagene Mx3005P qPCR system (Agilent Technologies) with SYBR Green to detect dsDNA synthesis. Each reaction contained 1 μL of each primer at 1 μM and 7 μL of MESA Blue SYBR qPCR MasterMix (including Hot Start DNA polymerase, dNTP and $MgCl_2$; Eurogentec) and 5 μL of cDNAs, according to the manufacturer's instructions. The cycling conditions were: denaturation cycle (94 °C for 15 min), followed by an amplification and quantification cycle repeated 40 times (94 °C for 10 s, 55 °C for 20 s and 72 °C for 20 s). MxPro software was used to analyse the data. The relative levels of gene expression were determined, as described in Dufour *et al.* (2013), using the formula $2^{-\Delta\Delta Ct}$, where $\Delta\Delta C_t = [C_t^{\text{studied gene}}$ (unknown sample) $- C_t^{\text{reference gene}}$ (unknown sample)] $- [C_t^{\text{studied gene}}$ (reference sample) $- C_t^{\text{reference gene}}$ (reference sample)]. This was performed with each of the two reference genes (*VvEF1γ* and *VvGAPDH*) and the geometric mean was calculated to normalize the results. The relative expressions were finally $\log_2$ transformed. Genes were observed as differentially expressed for $P < 0.05$ in rank-based nonparametric multiple comparisons with the NPARCOMP package in the R statistical software. Principal component analysis was performed to analyse plant defence behaviour after elicitation with the RCMDR package and the plug-in FactoMiner of R statistical software.

Results

Life traits of Botryosphaeriaceae

Influence of temperature on growth rate of the various species and/or isolates

Botryosphaeriaceae isolates grew over a range of temperatures from 15 to 36 °C (Fig. 1). At 15 °C, no significant difference was observed between the five isolates, with rates ranging from 5.67 ± 0.38 to 10.42 ± 0.60 mm per day. Maximum growth rates were obtained between 22 and 25 °C for *D. mutila* (BRA08: 26.1 ± 0.9 mm per day), at 25 °C for *D. seriata* (LAT16: 25.8 ± 0.5 mm per day; F98-1: and 26.7 ± 0.7 mm per day), between 22 and 28 °C for *N. parvum* (PER20: 30.7 ± 2.1 mm per day), and from 22 to 33 °C, with a maximal growth rate of 37.2 ± 1.8 mm per day for *L. viticola* (LAG05). At higher temperatures from 28 to 33 °C, the growth rates of *D. seriata* decreased from 21.0 ± 0.8 to 5.2 ± 0.6 mm per day (LAT 16) and from 19.7 ± 1.1 to 7.1 ± 0.6 mm per day (F98-1), while growth rates of *D. mutila* BRA08 decreased from 20.0 ± 0.4 to 4.4 ± 1.0 mm per day. For *N. parvum* (PER20), a decrease was observed at 33 °C, from 30.2 ± 1.9 to 10.1 ± 0.7 mm per day. At 36 °C, the growth rate of the isolates was nil for PER20 (*N. parvum*) and BRA08 (*D. mutila*), very low for *D. seriata* isolates (0.9 to 1.4 ± 0.4 mm per day) and low for LAG05 (*L. viticola*) (11.7 ± 0.6 mm per day). *Lasiodiplodia viticola* exhibited the highest growth rate over all temperatures.

Identification of Botryosphaeriaceae extracellular metabolites

To assess the potential role of extracellular metabolites in aggressiveness of the five isolates, toxins were extracted from their culture media. Eight major metabolites were identified by HPLC analyses, and four isolates (LAG05, BRA08, LAT16 and F98-1; Table 3) produced three major metabolites belonging to the dihydro-isocoumarin family [(*R*)-(−)-mellein, (3*R*,4*R*)-4-hydroxymellein and (3*R*,4*S*)-4-hydroxymellein)] and one belonging to the dihydrotoluquinone family ((+)-*epi*-sphaeropsidone). One *D. seriata* isolate (LAT16) also produced 4,7-dihydroxymellein. Isolate PER20 (*N. parvum*) produced most of the compounds mentioned above, a large quantity of terremutin, which belongs to the dihydrotoluquinone

Table 2 Selected genes and corresponding primer sets used for analysis of transcript profiles in grapevine leaves

Gene	Forward primer (5′–3′)	Reverse primer (3′–5′)	GenBank accession no.	PCR primer efficiency
VvEF1 γ	GAAGGTTGACCTCTCGGATG	AGAGCCTCTCCCTCAAAAGG	AF176496	0.97
VvGAPDH	GAAATCAACGGCCCAGCGCG	CCGGTGGATACTGGGGCGGA	XM 002263109	0.83
VvPR1	CCCAGAACTCTCCACAGGAC	GCAGCTACAGTGTCGTTCCA	AJ536326	0.88
VvPR2	GGGGAGATGTGAGGGGTTAT	TGCAGTGAACAAAGCGTAGG	AF239617	1.18
VvPR3	TATCCATGTGTCTCCGGTCA	TGAATCCAATGCTGTTTCCA	VVU97521	1.14
VvPR5	CCCCGGCACCACCAATGCTC	TGGGGGAGAACCGTAGCCCTG	XM 002283444	1.18
VvPR6	ACGAAAACGGCATCGTAATC	TCTTACTGGGGCACCATTTC	AY156047	1.23
VvPR8	AATGATGCCCAAAACGTAGC	ATAAGGCTCGAGCAAGGTCA	Z68123	1.05
VvPR10	GCTCAAAGTGGTGGCTTCTC	CTCTACATCGCCCTTGGTGT	AJ291705	1.03
VvPAL	ACAACAATGGACTGCCATCA	CACTTTCGACATGGTTGGTG	X75967	1.08
VvSTS	ATCGAAGATCACCCACCTTG	CTTAGCGGTTCGAAGGACAG	X76892	0.97
VvCHS	CCAACAATGGTGTCAGTTGC	CTCGGTCATGTGCTCACTGT	X75969	1.12
VvCALS	TGGAAATGCAATTCAAACGA	CGAATGCCATGTCTGTATGG	AJ430780.1	0.86
VvPER	TAAGCGCCACAAGAACACTG	GGACCTCCTTGTTGAGTCCA	XM 002274762.1	0.93
VvANTS	AAAAATCCAAGAGGGGTGCT	AAGCTTCTCCGATGCACTGT	XM 002281597	0.84
VvCHORM	TCATTGAGAGGGCCAAATTC	AGGAGGCAGAAAAAGCATCA	FJ604854	1.05
VvCHORS	GCCTTCACATGCAGATGCTA	CTGCAACTCTCCCAATGGTT	FJ604855	1.00

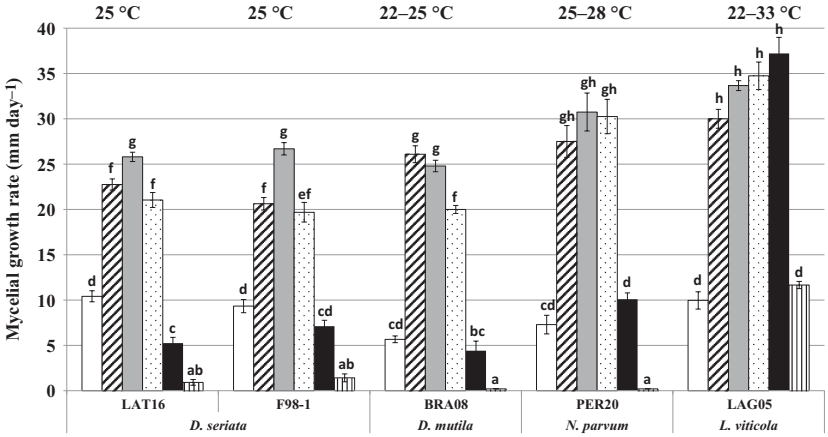

Figure 1 Influence of temperature on growth rate of five Botryosphaeriaceae isolates at six temperatures. Results represent means (± standard deviations) of six experiments. For each isolate, different letters above columns indicate significant differences at $P \leq 0.05$. (□) 15 °C; (▨) 22 °C; (▩) 25 °C; (▩) 28 °C; (■) 33 °C and (▥) 36 °C. Temperatures above the graphic represent the optimal growth temperature for each isolate.

Table 3 Toxins identified by HPLC analysis in the different isolates, expressed as μg per mg of crude extract

	Neofusicoccum parvum PER20	*Lasiodiplodia viticola* LAG05	*Diplodia mutila* BRA08	*Diplodia seriata* LAT16	*Diplodia seriata* F98-1
6-methyl salicylic acid	**0.07 ± 0.00**	nd	nd	nd	nd
6-hydroxypropyl salicylic acid	**0.77 ± 0.00**	nd	nd	nd	nd
(−)-terremutine	**5.87 ± 0.44**	nd	nd	nd	nd
(+)-*epi*-sphaeropsidone	nd	0.24 ± 0.02	**2.72 ± 0.58**	1.06 ± 0.20	**2.50 ± 0.53**
(*R*)-mellein	0.02 ± 0.01	0.04 ± 0.02	**0.10 ± 0.06**	0.01 ± 0.00	0.06 ± 0.03
(3*R*,4*S*)-dihydroxymellein	0.34 ± 0.02	**1.31 ± 0.03**	0.04 ± 0.01	0.08 ± 0.01	0.33 ± 0.03
(3*R*,4*R*)-dihydroxymellein	0.03 ± 0.00	0.03 ± 0.00	0.03 ± 0.00	0.24 ± 0.00	**3.04 ± 0.05**
(3*R*)-hydroxymellein	nd	nd	nd	**0.08 ± 0.02**	nd

nd, not detected.
Figures in bold font indicate higher concentrations found in extracts.

family, and two salicylic acid derivatives (6-methyl salicylic acid and 6-hydroxypropyl salicylic acid) (Table 3). Thus, the metabolic profile of PER20 was different from that of the other isolates. Likewise, amongst the *D. seriata* species, isolate LAT16 also produced an original compound: 4,7-dihydroxymellein.

Necrosis and cankers in planta

Three grapevine cultivars (Merlot, Cabernet Sauvignon and Ugni-Blanc) and two hybrid genotypes (RV4 and RV5) were inoculated to assess the aggressiveness of the isolates *in planta* and the susceptibility of plants by measuring canker (surface lesions) and necrosis (wood lesions) lengths (Figs 2 & 3). In noninoculated plants, the necrosis and canker lengths ranged from 2.8 ± 0.4 mm to 4.7 ± 0.7 mm, and from 0.0 ± 0.0 mm to 2.9 ± 0.3 mm, respectively. In Merlot cuttings inoculated with isolates PER20 (*N. parvum*) and LAG05 (*L. viticola*), significant necrosis was observed, even though this cultivar is considered to be tolerant to grapevine trunk diseases (Grosman & Doublet, 2012; Bruez *et al.*, 2013). Necrosis was fourfold greater in inoculated plants (PER20 = 19.4 ± 3.3 and LAG05 = 19.0 ± 5.1 mm) than in noninoculated ones (4.5 ± 0.5 mm; Fig. 3a). Isolates PER20 (*N. parvum*) and LAG05 (*L. viticola*) always produced the greatest necrosis in all plants, with necrosis length that varied depending on the cultivars or hybrids; the highest necrosis in comparison to the control was in Ugni-Blanc (63.4 ± 7.8 and 49.1 ± 9.5 mm for PER20 and LAG05, respectively) which was 17- to 22-fold greater than in control plants (2.8 ± 0.4 mm), followed by necrosis of 38.8 ± 5.6 mm (PER20) and 15.9 ± 7.0 mm (LAG05) in RV4. The necrosis was less in Cabernet Sauvignon (17.6 ± 1.9 and 10.9 ± 0.7 mm for PER20 and LAG05, respectively) and in RV5 plants (15.8 ± 8.4 mm and 18.4 ± 0.8 mm for PER20 and LAG05, respectively). Thus, PER20 (*N. parvum*) was the most aggressive isolate and LAG05 (*L. viticola*) was the second most aggressive, with significant necrosis in all plants. The *Diplodia* isolates often caused significant necrosis, ranging from 5.6 ± 0.6 to 14.4 ± 8.7 mm (Fig. 3a), depending on the cultivars or hybrids. For example, significant necrosis lengths were observed in Cabernet Sauvignon with isolates BRA08 (11.1 ± 0.4 mm) and LAT16 (11.1 ± 1.2 mm; Fig. 3), but not with isolate F98-1 (*D. seriata*). The latter produced significant necrosis in Ugni-Blanc (5.2 ± 0.8 mm), which is known to be very susceptible to grapevine trunk diseases.

Generally, canker lengths followed the same pattern as necrosis lengths in plants inoculated with *N. parvum* and *L. viticola* isolates. For example, canker lengths

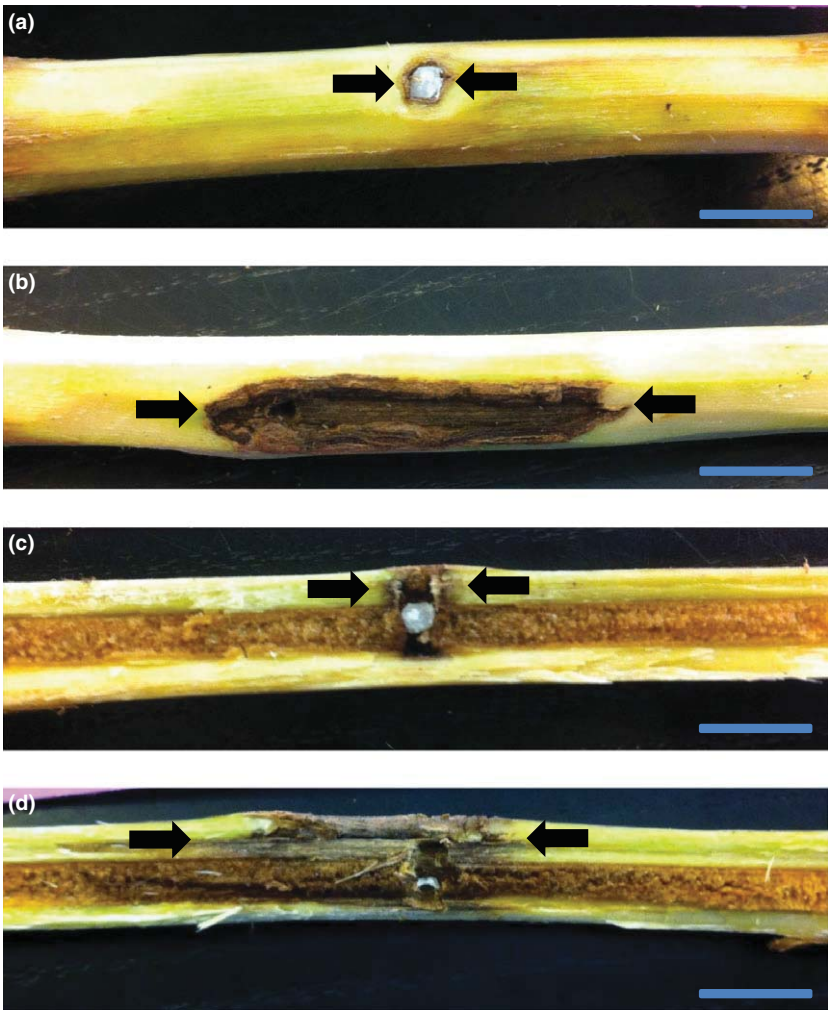

Figure 2 Cankers and necrotic lesions of grapevine wood cuttings, wounded or inoculated with Botryosphaeriaceae isolates. (a, b), canker lesions at surface of wood, and (c, d), necrotic lesions in wood, after wounding (a, c) or wounding and inoculation (b, d). Two arrows indicate the extent of symptoms around wound holes. Scale bars = 1 cm. [Colour figure can be viewed at wileyonlinelibrary.com]

were 10-fold greater (PER20: 3.7 ± 1.7 and LAG05 = 4.1 ± 0.1 mm) than in noninoculated plants (0.4 ± 0.0 mm) in Merlot, and 15- to 20-fold greater than controls in Ugni-Blanc (PER20 = 43.6 ± 5.3 and LAG05 = 33.3 ± 6.3 mm; Fig. 3b). Depending on the cultivar or the hybrid, some exceptions were observed. For example, cankers produced by the *D. seriata* (LAT16) isolate in Cabernet Sauvignon were significantly longer than necrosis. Conversely, in Ugni-Blanc, necrosis caused by isolate BRA08 (*D. mutila*) was significantly longer than cankers. Similarly, in hybrid RV4, only isolates *N. parvum* and *D. mutila* produced significant cankers but not the *L. viticola* isolate (LAG05).

Overall, all species induced significant cankers except F98-1 (*D. seriata*). Major necrosis and canker lesions were caused generally by isolates PER20 (*N. parvum*) and LAG05 (*L. viticola*), regardless of the cultivars or genotypes investigated. Necrosis and cankers were the most extensive in Ugni-Blanc, after inoculation with the most aggressive isolates. In general, cankers were shorter than necrosis but there was a significant correlation between the two symptoms depending on the cultivar (R^2 ranged from 0.458 to 0.871, $P < 0.0001$; Fig. S1): the greater the susceptibility of a cultivar or a hybrid, the stronger the correlation between the necrosis and the canker lengths. In Ugni-Blanc and RV4, i.e. plants with extensive lesions, R^2 were 0.87 and 0.65, respectively. The least susceptible cultivars or genotypes had the lowest R^2 values, e.g. 0.55 (RV5), 0.51 (Cabernet Sauvignon) and 0.46 (Merlot). Necrosis and canker lengths were therefore dependent on the genetic background of the plant and on the aggressiveness of each isolate.

Molecular responses of *V. vinifera*

Wounding affects the transcript profile of leaves from noninoculated plants

To decipher the effect of wounding stress, foliar defence-related transcripts were monitored by RT-qPCR at 2, 6 and 17 days after wounding (without fungal inoculation; Fig. 4). As expected, in the short term after 2 and 6 days, the relative expression of genes coding for PR-proteins (pathogenesis-related proteins), such as *VvPR1*, *VvPR2*, *VvPR3* and *VvPR10*, was significantly more intense, in most conditions. The stilbene synthase gene (*VvSTS*), a gene involved in the phenylpropanoid

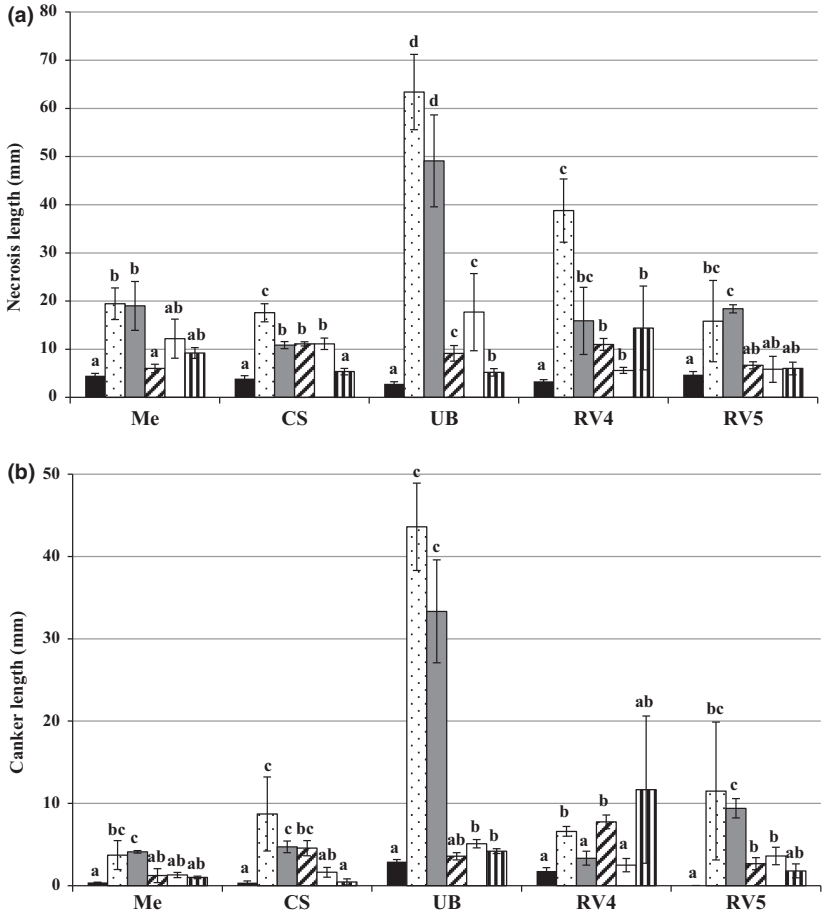

Figure 3 Mean length of internal necrosis (a) and external cankers (b) 11 months after inoculation, in wood of five cultivars and hybrids of *Vitis vinifera* inoculated with five different species of Botryosphaeriaceae. Me = Merlot; CS = Cabernet Sauvignon; UB = Ugni-Blanc; RV4 = downy mildew and powdery mildew resistant hybrid RV4; and RV5 = downy mildew and powdery mildew resistant hybrid RV5. Results represent means (± standard deviations) of 10 wood cuttings. Within each cultivar/hybrid, different letters in columns indicate significant differences between treatments at $P \leq 0.05$. (■) Mock-inoculated; (▒) PER20 - *Neofusicoccum parvum*; (▒) LAG05 - *Lasiodiplodia viticola*; (▨) BRA08 - *Diplodia mutila*; (□) LAT16 - *D. seriata* and (▥) F98-1 - *D. seriata*.

pathway, was also over-expressed at 2 days except in the RV5 hybrid, and the chitinase 3 gene (*VvPR8*) was only over-expressed in Merlot (Fig. 4). Unexpectedly, the genes involved in cell wall reinforcement (*VvPER* and *VvCALS*) and those involved in the anthranilate pathway (*VvCHORM*, *VvCHORS*, *VvANTS*) were slightly suppressed in many cases after wounding and over time. Seventeen days after wounding, only *VvPR2* and/or *VvPR3* were commonly over-expressed in the three cultivars and in the RV5 hybrid.

Defence gene expression in leaves of plants inoculated with different Botryosphaeriaceae isolates

Transcripts in leaves of cultivars and hybrids were measured nondestructively after wounding or fungal inoculation to determine the plant defence responses. Two days post-inoculation (dpi), gene expression patterns indicated a response specific to the plant genetic background, regardless of the isolate inoculated (Fig. 5). Thus, in Merlot leaves, the over-expression of *VvPR2*, *VvPR10*, *VvSTS*, *VvCHORM* and *VvCALS* was characteristic of this cultivar, while *VvPR5*, *VvPR8* and *VvCHORM* gene expression was characteristic of Cabernet Sauvignon. In Ugni-Blanc, the majority of PR-protein genes (*VvPR1*, *VvPR2*, *VvPR3*, *VvPR5* and *VvPR8*) and the *VvSTS* gene were up-regulated at 2 dpi in most cases. Note that genes *VvPR2* and *VvPR5* were up-regulated in the three cultivars. Little down-regulation was observed in the cultivars, except *VvPR1* and *VvANTS* in Cabernet Sauvignon and *VvPER* in Ugni-Blanc leaves. Conversely, few genes in the hybrids (RV4 and RV5) were over-expressed, except the genes involved in the cell wall strengthening (*VvCALS* and *VvPER)* in RV4. Unexpectedly, in RV5 leaves, gene regulation at 2 dpi was different from that observed with hybrid RV4, although the two are genetically very close.

In each of the three cultivars, few differences were noticeable between inoculated isolates. Nevertheless, in plants inoculated with isolates PER20 or LAG05 that induced the most extensive necrosis in all plants, gene *VvPR8* was down-regulated in Merlot and in Ugni-Blanc (Fig. 5). In contrast, in hybrid plants, responses were observed that were more specific to the isolate inoculated. For example, in RV5, many PR-protein genes (*VvPR1*, *VvPR3*, *VvPR5*) were up-regulated and gene *VvCHS* was down-regulated in the presence of isolates BRA08 or LAT16 only (responsible for minor symptoms; Fig. 5), and in RV4, genes *VvPR1*, *VvPR10* and *VvSTS* were down-regulated when inoculated with PER20, F98-1 or LAT16. In RV5, some genes involved in the indole pathways (*VvCHORM* and *VvCHORS*) and cell wall

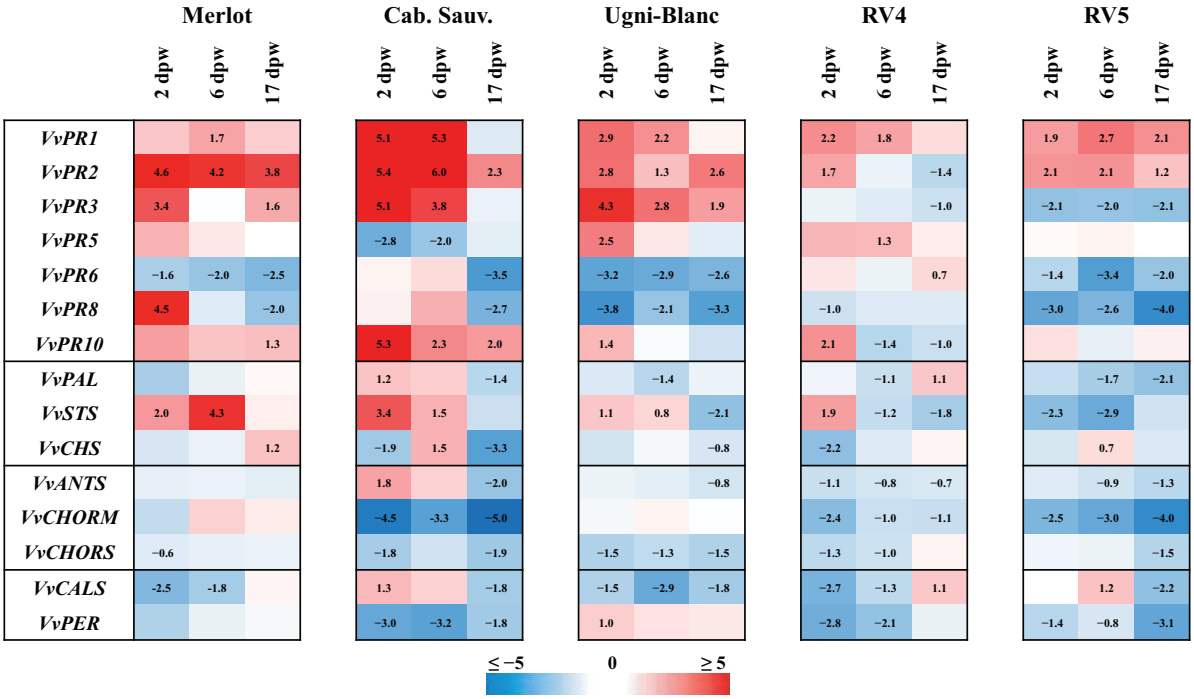

Figure 4 Relative gene expression with $\log_2$ transformation in grapevine leaves sampled after wood wounding without inoculation. Gene expression of unwounded wood was used as a reference to calculate the relative expression. Each column represents one time point after wounding (2, 6 or 17 days post-wounding, dpw) and each line corresponds to one gene represented by a single row of boxes. The colour scale bars represent the ratio values corresponding to the mean of three independent experiments. Over-expressed genes appear in shades of red, with relative expression level higher than five in bright red, while those with reduced expression appear in shades of blue, with relative expression lower than −5 in dark blue. Numbers in boxes represent the significant changes in gene expression ($P \leq 0.05$) compared to unwounded control.

strengthening (*VvCALS* and *VvPER)* were also down-regulated in plants inoculated with BRA08.

At 6 dpi (Fig. S2) and 17 dpi (Fig. S3), the characteristic pattern of cultivars was replaced by profiles that were more specific to the isolate inoculated. For example, in cv. Merlot, the expression of gene *VvPR2* was up-regulated with isolate LAT16, but down-regulated with isolate BRA08. Nevertheless, as at 2 dpi, the systematic up-regulation of genes involved in cell wall strengthening in hybrid RV4 was observed. With each isolate, the expression profile of the host plants could be very different e.g. Merlot or hybrid RV5 inoculated with isolate LAT16 had very different over-expression or down-regulation of genes (Fig. S2). Similar results were obtained with plants inoculated with F98-1 in Ugni-Blanc (up-regulation) or in RV5 (down-regulation). At 17 dpi, down-regulation was more abundant, especially in Cabernet Sauvignon and Ugni-Blanc (Fig. S3). Overall, fewer genes were significantly altered in expression at 6 and 17 dpi than at 2 dpi.

Principal component analysis (PCA) used to summarize gene defence responses and to correlate them with the phenotypes obtained (necrosis and canker lengths) indicated that the first two principal components (Fig. 6) explained 63% of the total data variability. Grapevine responses differed significantly according to the cultivar and hybrid (Fig. 6). Most PR-protein genes and *VvSTS* (stilbene synthase), projected significantly on the first component, represented 33.93% of the total variability, and were strongly correlated with the explanatory quantitative variables (necrosis and canker lengths). Confidence ellipses around the categories of noninoculated genotypes or hybrids revealed significant clusters separated by axis 2 and represented 29.17% of the total variability. These results confirmed the existence of a specific response signature of each plant genetic background, associated with the expression level of genes involved in the salicylic acid and chorismate pathways (*VvCHORM*, *VvCHORS*, *VvANTS* and *VvPAL*), flavonoid pathway (*VvCHS*), cell wall reinforcement (*VvCALS* and *VvPER*) and the serine protease gene *VvPR6*.

Finer examination showed that genes *VvSTS*, *VvPR5*, *VvPR3*, *VvPR10*, *VvPR1* and *VvANTS* were correlated with quantitative variables of canker and necrosis length on axis 1 in the susceptible cultivar Ugni-Blanc and in the tolerant cultivar Merlot (Fig. 7). This was also the case in other cultivars and hybrids. The genes associated with axis 2 (*VvPR8*, *VvPER*, *VvCALS* and *VvCHORM*) in the two cultivars were correlated with defence and reduction of necrosis and canker lesions. In Ugni-Blanc, only significant up-regulation of gene *VvPR8* and a down-regulation of gene *VvPER* were observed, while supplementary gene regulations also involved over-

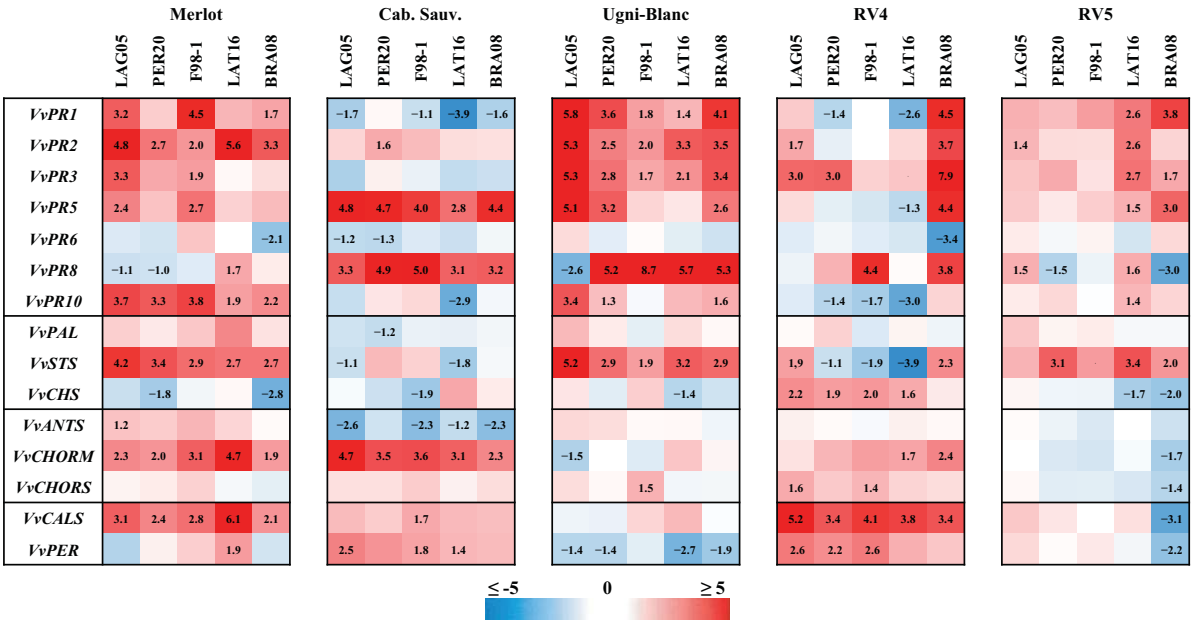

Figure 5 Relative gene expression with $\log_2$ transformation in grapevine cultivar and hybrid leaves 2 days after inoculation of wood with five Botryosphaeriaceae isolates. Gene expression of wounded noninoculated plants was used as a reference to calculate relative expression. Each column represents a combination of one of the five cultivars and genotypes (Merlot, Cabernet Sauvignon, Ugni-Blanc, RV4 and RV5) inoculated with one of the five Botryosphaeriaceae isolates (LAG05, PER20, F98-1, LAT16 and BRA08). Each line corresponds to one gene represented by a single row of boxes. The colour scale bars represent the ratio values corresponding to the mean of three independent experiments. Over-expressed genes appear in shades of red, with relative expression level higher than five in bright red, while those with reduced expression appear in shades of blue, with relative expression lower than −5 in dark blue. Numbers in boxes represent the significant changes in gene expression ($P \leq 0.05$) compared to the wounded noninoculated control.

expression of *VvPAL* or down-regulation of genes *VvCHS* and *VvPR2*.

Discussion

Grapevine trunk diseases are complex and involve not only pathogens but also the defensive capacity of plants in variable environmental conditions. In a simplified model, the capacity of plants to elicit their defences depends on their genetic background, on the time taken to mobilize their defences, and on the aggressiveness of pathogens, which depend respectively on their growth rates, toxin production and ecological requirements. In this study, focusing on the interaction between Botryosphaeriaceae of different species and various cultivars or hybrids of *V. vinifera*, a nondestructive method was used for the early detection of systemic defence signals in leaves of inoculated plants, 11 months before measuring necrosis and canker lengths. To the authors' knowledge, no study has yet investigated the defence dynamics of various genetic backgrounds of *Vitis* plants (cultivars and hybrids) after inoculation of species with different levels of aggressiveness.

The Botryosphaeriaceae species are commonly found in French vineyards and in most vineyards worldwide, and their prevalence and distribution are thought to be greatly influenced by climatic conditions (Larignon *et al.*, 2009; Úrbez-Torres, 2011; Phillips *et al.*, 2013; Pitt *et al.*, 2013). Among the species studied here (*D. seriata*, *D. mutila*, *N. parvum* and *L. viticola*), all were pathogenic in grapevine (Úrbez-Torres *et al.*, 2009, 2012). The isolates belonging to the *Diplodia* genus exhibited optimal temperatures between 22 and 25 °C, which is in agreement with the literature (Úrbez-Torres *et al.*, 2006), but their growth rates were higher than those described by Pitt *et al.* (2013) (26 and 27 mm per day versus 19 and 23 mm per day). This difference may be due to genetic evolution between isolates sampled in different areas or countries, as observed in Spain (Elena *et al.*, 2015). Furthermore, isolate *N. parvum* exhibited a higher growth rate than those reported previously by Pitt *et al.* (2013) in Australia (33 versus 23 mm per day). The *L. viticola* species, which was recently found for the first time in a French vineyard (Comont *et al.*, 2016), grew the quickest at high temperatures (optimal growth temperature up to 33 °C with growth until 36 °C). Its ability to grow at high temperatures is consistent with its preferential geographical distribution in hot and tropical regions (Marques *et al.*, 2013; Phillips *et al.*, 2013). The present study shows that the Botryosphaeriaceae species collected in French vineyards grow over a range of temperatures from 15 to 36 °C and that their growth depends on their respective ecological requirements.

The second life trait (aggressiveness) measured symptoms in the wood (necrosis) and at the surface of the wood (canker/open scar) *in planta*. To the authors'

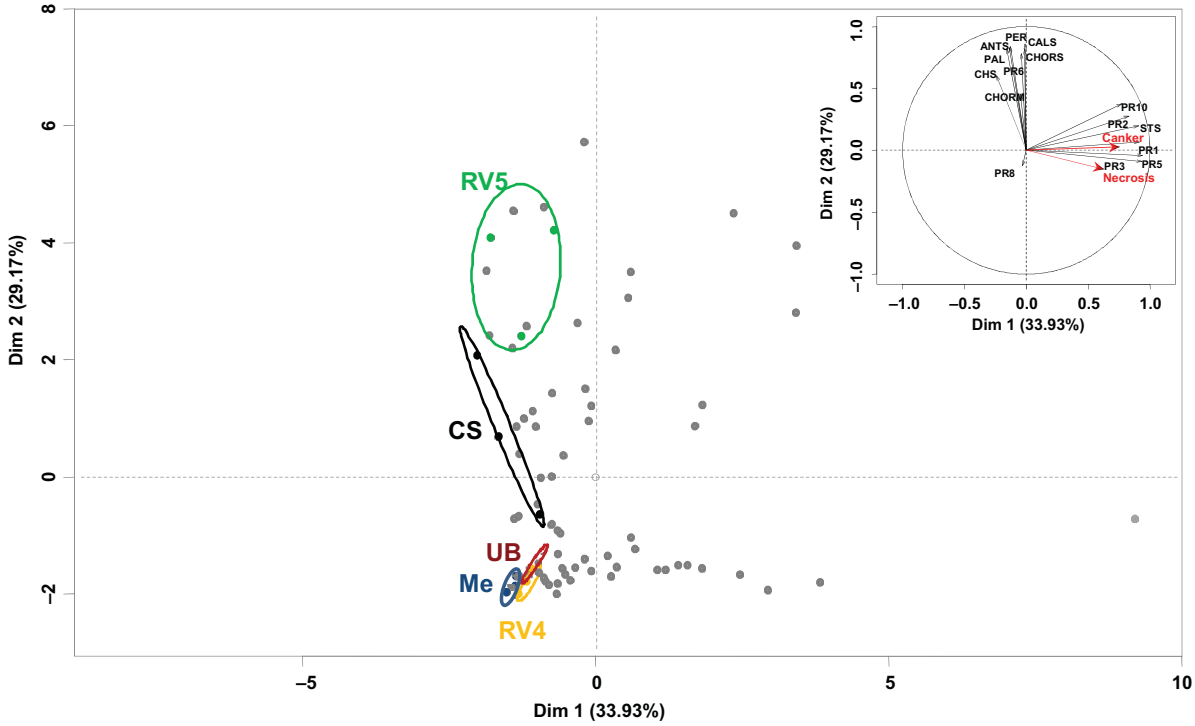

Figure 6 Principal component analysis (PCA) of necrosis/canker length and relative gene expression data obtained from five different grapevine cultivars and hybrids inoculated with five Botryosphaeriaceae isolates. (a) Projections on standard unit circle of quantitative variables (genes) and quantitative illustrative variables (necrosis and canker length). (b) Distribution of plant responses and lesion lengths on principal planes defined by first two axes (Dim 1 and Dim 2) obtained with PCA of gene expression profiles using all inoculated and noninoculated data. The two major principal components explaining ~ 63% of the expression variance are plotted. The points represent the expression patterns obtained in cultivar or hybrid leaves with or without pathogen inoculation. Ellipses represent the 95% confidence intervals calculated for each noninoculated cultivar or hybrid (black, CS, Cabernet Sauvignon; blue, Me, Merlot; green, RV5; yellow, RV4; purple, UB, Ugni-Blanc).

knowledge this is the first report of correlation between necrosis and canker length depending on the cultivar, irrespective of the aggressiveness of the Botryosphaeriaceae isolates. These two criteria (necrosis and canker length) could be useful together to assess the susceptibility of cultivars or the genetic resources of Vitaceae, and could serve as complementary criteria to necrosis area, which is commonly used to assess potential varietal resistance (Travadon *et al.*, 2013; Billones-Baaijens *et al.*, 2014; Guan *et al.*, 2016). As expected, necrosis was less extensive in Merlot than in Ugni-Blanc, in particular for the isolates PER20 (*N. parvum*) and LAG05 (*L. viticola*). Regardless of cultivars or hybrids, isolate PER20 (*N. parvum*) always produced worse symptoms than the *D. seriata* isolates (LAT16 or F98-1), as also described by Guan *et al.* (2016). These results highlight the value of using wood cuttings as a realistic model to measure lesion sizes. The difference in susceptibility observed between Merlot and the other cultivars or genotypes could also partially be due to the dimension of the plant's vessels, which could play a role in tolerance, as suggested by Pouzoulet *et al.* (2014). Indeed, vessel diameter is directly linked to the susceptibility of grapevine to esca (e.g. 90.7 and 99.9 µm for Merlot and Cabernet Sauvignon, respectively). Hybrids RV4 and RV5 were more tolerant to the most virulent isolates and seemed to behave more like Cabernet Sauvignon and Merlot, two cultivars included in their genealogy.

While wood symptoms are known to result from the enzymatic degradation of the wood, foliar symptoms could result from the translocation of phytotoxic compounds, so it is important to analyse the production of extracellular compounds. Indeed, these fungi are responsible not only for trunk symptoms, but also for external symptoms, as reported by Larignon *et al.* (2001) and Reis *et al.* (2016), although they have never been found in the leaves of plants with symptoms. In the last decade, these fungi have been shown to produce many compounds that would result in the onset of foliar symptoms (Ramírez-Suero *et al.*, 2014), but the exact impact of each compound remains unclear (Andolfi *et al.*, 2011). The present study shows that each species and/or isolate produced different secondary metabolites, and this is the first report of these compounds for isolates belonging to the *L. viticola* and *D. mutila* species. Three compounds were produced by all isolates: *epi*-sphaeropsidone, *trans*- and *cis*-4-hydroxymellein, which were previously described by Abou-Mansour *et al.* (2015) as being produced by *N. parvum*. The production of mellein and its derivatives has been previously described for *D. seriata*,

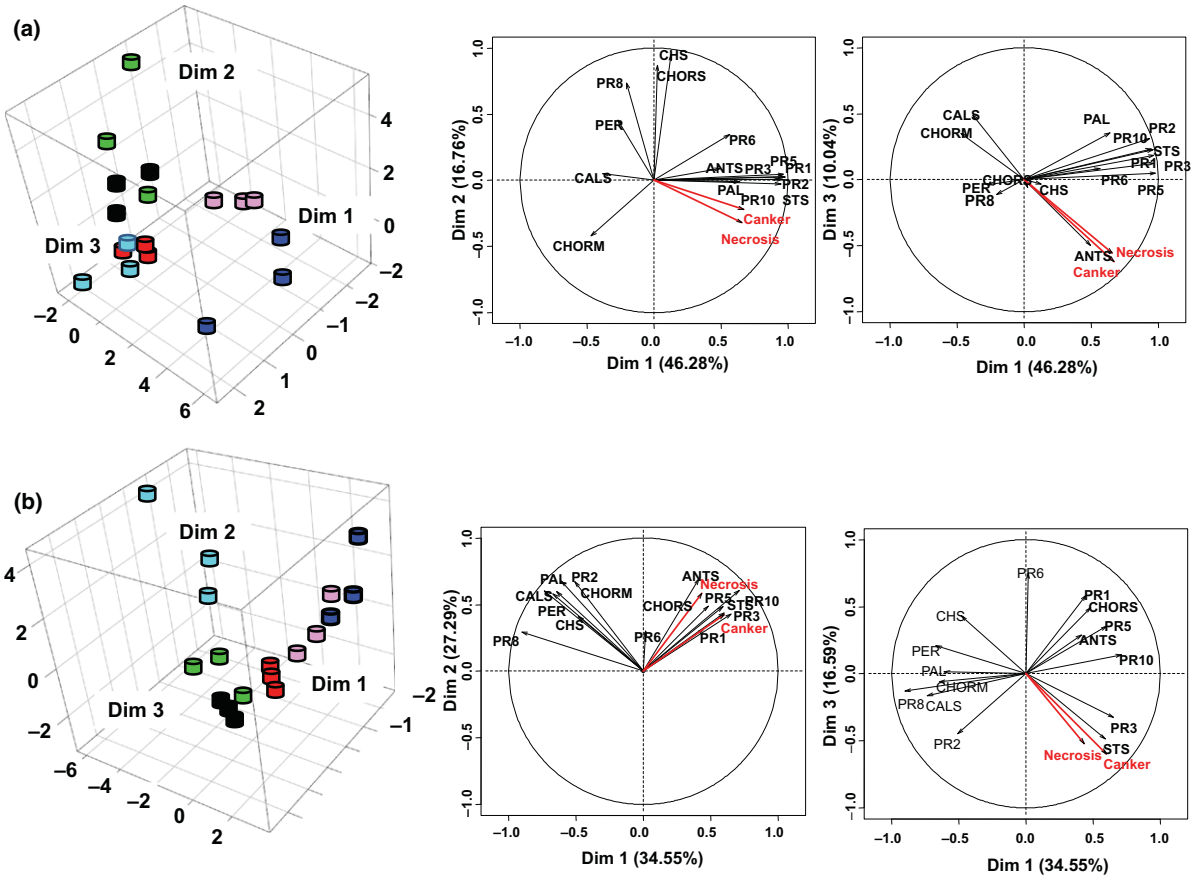

Figure 7 Three-dimensional principal component analyses (3D-PCA) of two cultivar defence responses after inoculation with five isolates compared to noninoculated cultivar and projections on standard unit circle of quantitative variables (genes) and quantitative illustrative variables (necrosis and canker length) in the three major components (Dim1, Dim2 and Dim3). (a) Ugni-Blanc, considered as the most susceptible cultivar and (b) Merlot, the most tolerant. Black circle: noninoculated cultivar; pink circle: inoculated with PER20 (*Neofusicoccum parvum*); dark blue circle: inoculated with LAG05 (*Lasiodiplodia viticola*); red circle: BRA08 (*Diplodia mutila*); sky blue circle: inoculated with LAT16 (*Diplodia seriata*) and green circle: inoculated with F98-1 (*D. seriata*).

but not the production of *epi*-sphaeropsidone. The *N. parvum* isolate (PER20) exhibited a different biochemical profile compared to the other isolates, with the presence of terremutin and two salicylic acid derivatives (6-methyl salicylic acid and 6-hydroxypropyl salicylic acid). This is in agreement with the findings of Abou-Mansour *et al.* (2015), who demonstrated that terremutin and salicylic acid derivatives are highly phytotoxic compounds that are responsible for more extensive necrosis than *cis*-4-hydroxymellein when applied to grapevine leaves. The production of these extracellular compounds may partly explain the wood symptoms obtained after inoculation with this *N. parvum* isolate; the production of salicylic acid derivatives could disrupt the grapevine's salicylic acid-dependent defence responses, which could also partially account for the isolate's pathogenicity on various cultivars and hybrids. However, despite the extensive symptoms observed after inoculation of isolate LAG05 (*L. viticola*), the amount of extracellular compounds that it produced was very similar to that of the less aggressive isolates. *Diplodia seriata* is considered either as a saprophytic species or as a weak secondary pathogen of grapevines in many countries (Taylor *et al.*, 2005; Carlucci *et al.*, 2015), although it is thought to be pathogenic by other authors (Larignon *et al.*, 2001; van Niekerk *et al.*, 2004). The two isolates used in this study were moderately pathogenic, with variability depending on the cultivar. Although LAT16 and F98-1 came from a different background (sampling year, area and cultivar), the life traits of the *D. seriata* species were quite well preserved, with few differences in their extracellular compound production. This finding could be extended to the genus *Diplodia*, because the life traits of the *D. mutila* isolate are very similar to those of the two *D. seriata* isolates. In this work, *L. viticola* and *N. parvum* were the most aggressive species, followed by *D. mutila* and *D. seriata*.

Having sound information on the defence status of plants that can limit the pathogenic attack and lesion extensions is important for understanding the responses

of plants to Botryosphaeriaceae and for designing a new botryosphaeria dieback management method. Using a nondestructive test, this study has shown that transcript expression within a few days of infection is correlated with the extent of necrotic and canker lesions appearing in the same plant 11 months later. In plants that were wounded without fungal inoculation, some gene modulations were observed in cultivars and hybrids. Several PR-protein genes (*VvPR1*, *VvPR2*, *VvPR3* and *VvPR10*) were up-regulated and the gene *VvSTS* was shown to be involved in the stilbene biosynthesis pathway. The common responses of all the cultivars and hybrids were not informative as an efficient defence marker but, rather, reflected an abiotic stress response. The results are in agreement with those of Guan *et al.* (2016) who did not report any correlation between the pattern of PR-protein expression and the level of plant resistance after infection. Nevertheless, genes such as *VvPR8* (chitinase) that were up- or down-regulated depending on the cultivar or hybrid may provide information on specific responses according to the genetic background.

PCA performed with lesion length and gene expression data obtained for the different cultivars and hybrids showed that 34% of the variability observed was explained by the correlation between the modulation of several genes (some PR-protein genes and *VvSTS*) and necrosis and canker sizes. The expression of these genes, mentioned above as being involved in an abiotic stress response, was correlated with increased necrosis and canker sizes and, thus, did not seem to play a major role in the decrease of symptoms. This confirmed the hypothesis that these defence-related genes are involved in the stress response rather than in protection after biotic stress by Botryosphaeriaceae. On the other hand, 29.17% of the variability observed was explained by the modulation of gene expression involved in cell wall reinforcement in the indole and phenylpropanoid pathways and, to a lesser extent, of the PR-proteins (*VvPR6* and *VvPR8*). These genes were correlated with a reduction in necrosis and canker length and could be better markers of defence and protection. Therefore, besides the characteristic gene expression pattern of each cultivar or hybrid to a biotic or abiotic stress, the expression of some genes could be a better marker of plant protection during attack by a pathogen than others that do not seem to have any direct link with necrosis size.

Until now, no genetic resources have been identified in Vitaceae against botryosphaeria dieback. Only a difference in susceptibility or in the expression of symptoms has been reported, depending on the cultivar, but with no complete qualitative resistance (Grosman & Doublet, 2012; Travadon *et al.*, 2013; Billones-Baaijens *et al.*, 2014; Guan *et al.*, 2016). In the present study, two hybrids were used that exhibited resistance to mildew arising from introgression of the resistance cluster of *M. rotundifolia* and backcrosses with various cultivars including Merlot and Cabernet Sauvignon. However, they did not reveal any reduction in the size of necrosis/cankers compared to Cabernet Sauvignon or Merlot. This suggests that the gene clusters involved in resistance to cryptogamic biotrophic pathogens (downy and powdery mildew) are inefficient against Botryosphaeriaceae attack.

To conclude, this study of the pathogenicity of Botryosphaeriaceae shows that they are well adapted to a wide range of temperatures (*L. viticola* and *N. parvum*) and that they are potentially linked with recent mortality of woody species during global climate change (e.g. heat, drought). It has been shown that wood inoculation creates a defence signal in leaves characterized by the modulation of expression of some genes, which is correlated positively or negatively with necrosis and canker length, depending on the susceptibility of the plants. This nondestructive approach in the necrosis/canker bioassay, combined with assessment of gene expression in leaves, could provide better understanding of grapevine defences and their potential for protection. It could also help to assess the health of plants (defence status) in the nursery or in breeding material that is less susceptible or resistant. To evaluate botryosphaeria dieback resistance better and to reinforce plant defences, future studies should focus on plant defence stimulators to combat Botryosphaeriaceae infection in controlled or vineyard conditions. Understanding the mechanisms involved in infection by Botryosphaeriaceae in plants and having markers linked to their development are prerequisites to using resistance inducers and to developing strategies that limit the spread of disease.

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site.

Figure S1. Correlations between Botryosphaeriaceae canker and necrosis length in five grapevine cultivars and genotypes. (a) Correlations with Merlot (black circle and full line) and Cabernet Sauvignon (grey shape and dash line). (b) Correlations with Ugni-Blanc (black circle, black full line) and with the two genotypes RV4 (grey shape and black dotted line) and RV5 (grey triangle and black dotted line). Correlations made independently of inoculated isolate, in each cultivar or genotype.

Figure S2. Relative gene expression with $\log_2$ transformation in grapevine cultivar and hybrid leaves, 6 days after inoculation of wood with five Botryosphaeriaceae isolates. Gene expression of wounded but non-inoculated plants was used as a reference to calculate the relative expression. Each column represents a combination of one of the five cultivars and genotypes (Merlot, Cabernet Sauvignon, Ugni-Blanc, RV4 and RV5) inoculated with one of the five Botryosphaeriaceae isolates (LAG05, PER20, F98-1, LAT16 and BRA08). Each line corresponds to one gene represented by a single row of boxes. The colour scale bars represent the ratio values corresponding to the mean of three independent experiments. Over-expressed genes appear in shades of red, with expression level higher than five in bright red, while those with reduced expression appear in shades of blue, with intensity lower than −5 in dark blue. Numbers in boxes represent significant changes in gene expression ($P \leq 0.05$) compared to the wounded but noninoculated control.

Figure S3. Relative gene expression with $\log_2$ transformation in grapevine cultivar and hybrid leaves, 17 days after inoculation of wood with five Botryosphaeriaceae isolates. Gene expression of wounded

noninoculated plants was used as a reference to calculate relative expression. Each column represents a combination of one of the five cultivars and genotypes (Merlot, Cabernet Sauvignon, Ugni-Blanc, RV4 and RV5) inoculated with one of the five Botryosphaeriaceae isolates (LAG05, PER20, F98-1, LAT16 and BRA08). Each line corresponds to one gene represented by a single row of boxes. The colour scale bars represent the ratio values corresponding to the mean of three independent experiments. Over-expressed genes appear in shades of red, with expression level higher than 5 in bright red, while those with reduced expression appear in shades of blue, with intensity lower than −5 in dark blue. Numbers in boxes represent significant changes in gene expression ($P \leq 0.05$) compared to noninoculated wounded control.