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Mining new resources for grape resistance against Botryosphaeriaceae: a focus on *Vitis vinifera* subsp. *sylvestris*

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Botryosphaeria dieback is an important grapevine trunk disease with global impact. Susceptibility differences between grape varieties manifest as different expression of canopy symptoms in the field. However, the cause of these dieback symptoms and their relation with wood necrosis remain only partially understood. As a first step towards future strategies for resistance breeding, wood necrosis was investigated over a large selection of the Vitaceae family members following artificial inoculation of the Botryosphaeriaceae fungi *Neofusicoccum parvum* and *Diplodia seriata* into woody internodes. Large variation of resistance levels was found, with good performance in several accessions from *V. vinifera* subsp. *sylvestris*, the ancestor of cultivated grapevine. To get insight into the mechanisms of this apparent resistance, expression of defence genes was studied in *V. vinifera* cv. Chardonnay, Gewürztraminer and different *V. vinifera* subsp. *sylvestris* genotypes, in both green and necrotic areas of inoculated woods. Resistance to Botryosphaeriaceae in *V. vinifera* subsp. *sylvestris* correlated with earlier and higher induction of some defence genes, both in green and necrotic wood. Moreover, leaves of several *V. vinifera* subsp. *sylvestris* accessions were also less susceptible to necrosis induced by treatment with a culture filtrate of Botryosphaeriaceae, compared to commercial cultivars of *V. vinifera*. The results show that *V. vinifera* subsp. *sylvestris* provides interesting genetic resources for breeding new varieties with enhanced resistance to botryosphaeria dieback.

Keywords: botryosphaeria dieback, defence responses, grapevine, grapevine trunk diseases, *Vitis vinifera* subsp. *sylvestris*

Introduction

Botryosphaeria dieback, esca and eutypa dieback are three economically important grapevine trunk diseases causing severe yield reduction and affecting the viability of plants in vineyards worldwide. The incidence of trunk diseases has increased considerably over the past few decades and, so far, no efficient curative treatment is available to control these diseases (Grosman & Doublet, 2012; Bruez *et al.*, 2013). Grapevine infection by fungal trunk pathogens results in the formation of cankers and wood discolouration. It is believed that infection takes place primarily via pruning wounds, although contamination of nursery stock does occur (Bertsch *et al.*, 2013).

Botryosphaeria dieback, one of the major trunk diseases, is associated with the development of fungi from the Botryosphaeriaceae family, especially *Neofusicoccum parvum* and *Diplodia seriata*, in the wood of diseased grapevines. Botryosphaeriaceae species are well known pathogens causing dieback in apples, pine trees and grapevines. These fungi are latent pathogens in many woody hosts and are characterized by a quiescent passive life phase, followed by an active pathogenic phase (Slippers & Wingfield, 2007). There are more than 22 different species in the Botryosphaeriaceae family, which have been identified in vineyards from different countries based on morphological and taxonomic studies, as well as on analysis of nucleotide sequences from multiple genes (for reviews see Úrbez-Torres (2011) and Bertsch *et al.* (2013)). Species of Botryosphaeriaceae infecting grapevines can be divided into three different virulence rankings and *Neofusicocum* spp. belong to the highly virulent species, whereas *Diplodia* spp. are moderately virulent (Úrbez-Torres, 2011). The first description of botryosphaeria dieback disease of grapevine was reported in 1974 in Hungary and was associated with *Diplodia mutila* (Lehoczky, 1974). This disease was originally called 'black dead arm' (BDA) and was characterized by diffuse chlorosis of the leaves followed by wilting and black streaking of the wood in the xylem (Lehoczky, 1974). It was shown thereafter that Botryosphaeriaceae species are

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associated with a broad number of different grapevine trunk disease symptoms including bud necrosis, shoot dieback, leaf spots, fruit rots, vascular discolouration of wood and perennial cankers, leading to a loss in economic production, and eventually culminating in the death of the whole plant. Úrbez-Torres (2011) thus proposed the disease name 'botryosphaeria dieback' to include all the symptoms caused by Botryosphaeriaceae species on grapevine. It is known that Botryosphaeriaceae produce cell wall-degrading enzymes and phytotoxic metabolites whose synergistic action may cause the disease symptoms of botryosphaeria dieback (dead spurs, shoot dieback, stunted shoots) (Andolfi *et al.*, 2011). However, even when pathogens do infect the vascular system successfully, this does not necessarily lead to the expression of dieback symptoms in the first season. Moreover, it can happen that in the subsequent year, the same vine shows reduced or even no symptoms at all (Bertsch *et al.*, 2013). In the search for fungal toxins, four dihydroisocoumarins (mellein, 4-hydroxymellein, 7-hydroxymellein and 4,7-dihydroxymellein) were isolated from a culture filtrate of *D. seriata* (Andolfi *et al.*, 2011). In addition, analysis of the metabolites produced by 13 isolates of *N. parvum* revealed that these compounds belong to four different families (dihydrotoluquinones, epoxylactones, dihydroisocoumarins and hydroxybenzoic acids; Abou-Mansour *et al.*, 2015). A recent study also highlighted the role of secreted proteins for the cytotoxicity of culture filtrates from *N. parvum* (Bénard-Gellon *et al.*, 2015).

Studies on botryosphaeria dieback have been conducted at multiple levels: in naturally infected vineyards, under controlled conditions using greenhouse cuttings, and using *in vitro* grapevine models that were artificially infected (Bruez *et al.*, 2013; Travadon *et al.*, 2013; Ramírez-Suero *et al.*, 2014). The physiological and histochemical alterations associated with Botryosphaeriaceae infection include blocked xylem vessels by induction of tyloses or production of gums (such as black goo), as well as by physical impedance by the pathogen itself (Bertsch *et al.*, 2013). Moreover, pathogen-derived macromolecules can plug the pits, reducing translocation of water (Edwards *et al.*, 2007; Bertsch *et al.*, 2013). Not surprisingly, the expression of symptoms caused by Botryosphaeriaceae in grapevines is accentuated by water stress (Niekerk *et al.*, 2011). Grapevines exposed to water stress developed more severe disease symptoms (lesion lengths) in response to inoculation with Botryosphaeriaceae.

Defence responses to trunk diseases have been studied at the leaf level in the case of esca, another major grapevine trunk disease. The main causal agents of esca are the tracheomycotic agents *Phaemoniella chlamydospora* and *Phaeoacremonium aleophilum*, as well as several basidiomycete species, with *Fomitiporia mediterranea* being the most common (Bertsch *et al.*, 2013). Symptoms associated with esca are brown wood streaking and leaf discolouration resulting in a 'tiger stripe' appearance (Surico *et al.*, 2006). The leaves of esca-affected plants are characterized by high tannin contents (Valtaud *et al.*, 2011). Resveratrol and other phenolic compounds could also be detected in leaves and berries before the appearance of esca symptoms (Letousey *et al.*, 2010) and were accompanied by induction of defence genes such as *PAL* and *STS* (encoding phenylpropanoid biosynthesis enzymes), and the accumulation of pathogenesis-related (PR) proteins (Letousey *et al.*, 2010). At the wood level, analysis of the proteome of black-streaked trunks lends further support for the induction of proteins involved in defence (Magnin-Robert *et al.*, 2014). Defence responses to botryosphaeria dieback were less studied. However, PR proteins, members of the enzymatic antioxidant system, total phenolics and stilbenes were found to be more abundant in the brown-striped wood of botryosphaeria dieback-affected grapevines (Spagnolo *et al.*, 2014).

Introgression of resistance factors from wild grape into cultivated varieties has been a successful strategy to contain important grapevine diseases such as downy or powdery mildews (Eibach *et al.*, 2007). However, so far, no genetic resources for a similar strategy have been identified in the case of fungal trunk diseases. In the field, sensitivity of *Vitis vinifera* against fungal trunk diseases seems to vary according to the cultivar (Grosman & Doublet, 2012; Bruez *et al.*, 2013), suggesting that the resistance level might be under genetic control. For instance, Chardonnay infected by the causal agents of trunk diseases presents fewer leaves with symptoms compared to other cultivars. However, so far, no totally resistant cultivar has been reported (Surico *et al.*, 2006). Several studies with artificial inoculation of cultivated grapevines or rootstocks with *N. parvum* and *D. seriata* demonstrated a differential susceptibility of different accessions, but again no complete qualitative resistance was found (Travadon *et al.*, 2013; Billones-Baaijens *et al.*, 2014).

In an attempt to identify novel genetic resources for resistance against trunk diseases, the current study conducted a broad screen to evaluate the susceptibility levels of a large selection of accessions from the Vitaceae family to *N. parvum* and *D. seriata*. The leaf and wood symptoms of *V. vinifera* subsp. *sylvestris*, the ancestor of *V. vinifera*, were also studied. Differential defence gene expression was examined in wood symptoms in a variety of accessions.

Materials and methods

Plant material

The members of the Vitaceae family used in this study are presented in Figure 1.

Inoculation experiments were conducted on detached canes, which consisted of 0·8 × 10 cm sections between two nodes of 1-year-old dormant lignified canes collected from two non-*Vitis* genera (*Parthenocissus tricuspidata* and *Ampelopsis japonica*), one subgenus (*Muscadinia rotundifolia* cv. Regale), four *Vitis* species (*V. rupestris*, *V. amurensis*, *V. riparia*, *V. aestivalis*), 16 *V. vinifera* commercial cultivars (Cabernet sauvignon, Chardonnay, Chasselas, Chenin, Colombard, Danlas, Fantasy seedless, Gamay, Gewürztraminer, Grenache, Muscat d'Alexandrie, Pinot noir, Riesling, Savagnin blanc, Sultanine, Trousseau) and two rootstocks (41B (*V. vinifera* × *V. berlandieri*), 110R

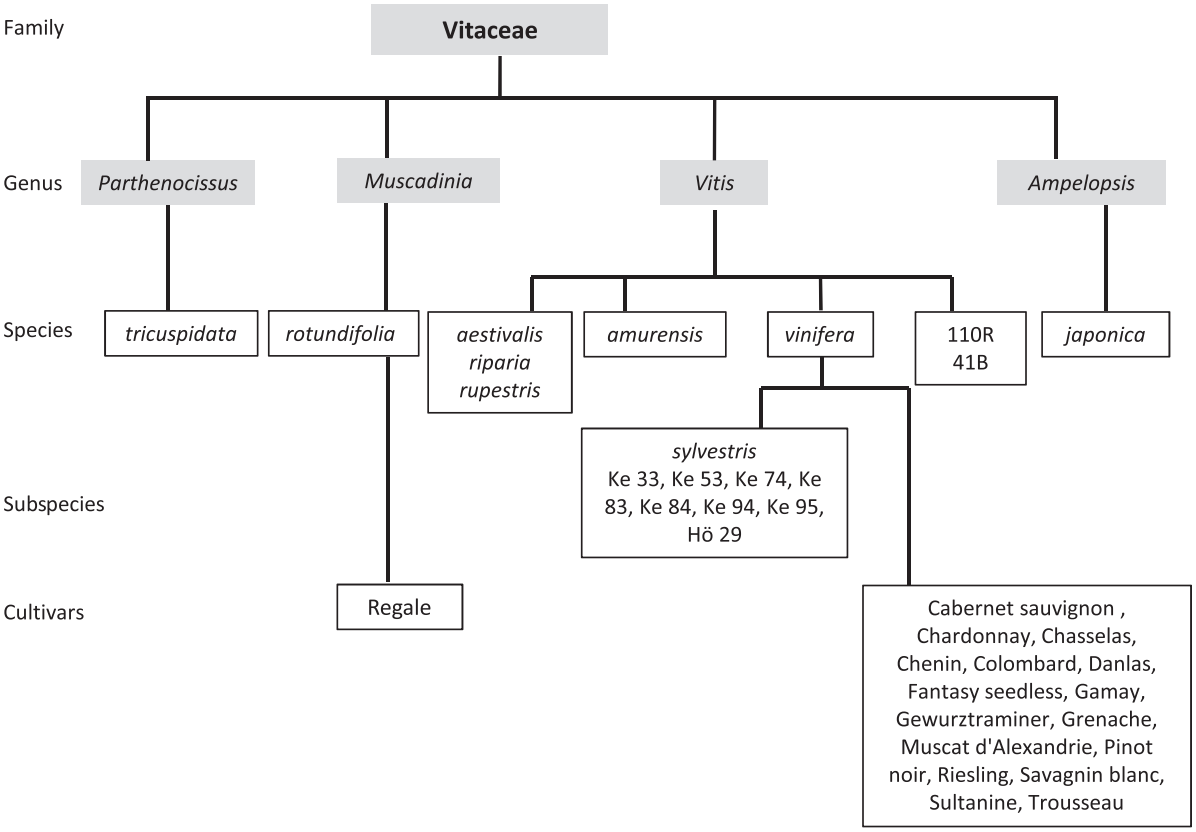

Figure 1 Partial classification of Vitaceae adapted from Galet (1967).

(*V. berlandieri* × *V. rupestris*). All accessions were obtained from a collection from INRA Colmar, France (December 2012 and 2013, February 2014). Inoculation experiments with *V. vinifera* subsp. *sylvestris* were conducted on 1-year-old internodes (December 2012 and 2013) collected from *V. vinifera* subsp. *sylvestris* accessions Ke 33, Ke 53, Ke 74, Ke 83, Ke 84, Ke 94, Ke 95 and Hö 29. The Ke accessions originate from the largest *sylvestris* population in Germany at the Ketsch peninsula, and the Hördt accession from an isolated individual in the Hördt alluvial forest (Upper Rhine valley, Rheinland-Pfalz). These accessions are part of a complete genetic copy for the *sylvestris* genotypes still available in Germany that was established for an *ex situ* conservation project in the Botanical Garden of the Karlsruhe Institute of Technology, and characterized genetically by microsatellite markers (Nick, 2014).

For the study of leaf response to culture filtrates of *N. parvum* and *D. seriata*, the third to sixth fully expanded leaves beneath the apex from the above-cited accessions were collected in May 2013 and May 2014.

Fungal isolates and inoculation experiments

Diplodia seriata strains 99.7 (Rhône-Alpes, France) and 98.1 (Pyrénées Orientales, France) have been described by Larignon *et al.* (2001). *Neofusicoccum parvum* strain Bt 67 and *N. parvum* Bourgogne S-116 were isolated from vineyards in Estremadura (Portugal) and Bourgogne (France), respectively. All strains were grown on solid medium in Petri dishes containing 26·5 g L^{-1} potato dextrose broth (PDB; Laboratorios Conda) and 15 g L^{-1} Bacto agar (Kalys SA Vitro) at 26°C in the dark.

For wood inoculation experiments, internodes were collected from dormant canes. A power drill no. 5 (Bosch PSB 500R) was used to wound the woody stems (5 mm in diameter, 3 mm in depth) at the centre point between nodes. Cuttings were inoculated by filling the wound with a 6 mm diameter plug collected from a 14-day-old fungal culture on potato dextrose agar (PDA), and then sealing this inoculation site with Parafilm. Control internodes were mock inoculated with plugs of non-colonized PDA. After inoculation, detached internodes were incubated in saturating humidity at 28°C in darkness. After 21 days of incubation for *D. seriata* 98.1 and 7–14 days in the case of *N. parvum* Bt 67, the stems were debarked and necrotic areas were quantified from digital images using ImageJ software (http://imagej.nih.gov/ij/). The difference in sampling time is due to a slower growth of *D. seriata* compared to *N. parvum* in the wood.

To validate the inoculation method used in this work, the agar plug inoculation method was compared with the suspension mycelium inoculation developed by Travadon *et al.* (2013). To produce the starter culture, 10 plugs of 2 × 2 cm from a 7-day culture on PDA were inoculated in a 250 mL Erlenmeyer flask containing 100 mL bacteriological malt extract and incubated at 27°C and 150 rpm for 3 days. Agar with mycelia attached was collected into a 50 mL Falcon tube and malt extract medium was added to reach a final volume of 20 mL. Five millilitres of glass beads with a diameter of 3 mm were added into the suspension mycelia and the tube was agitated with a vortexer at full speed to disperse the mycelia. Two millilitres of this homogenized starter culture were then inoculated into a 125 mL Erlenmeyer flask containing 40 mL of bacteriological malt extract. After incubation at 28°C and 150 rpm for

3 days, the entire 40 mL liquid culture was homogenized and the concentration of mycelial fragments, which were primarily < 200 μm in length, was estimated with a haemocytometer. The final concentration of inoculum was adjusted with sterile water to 2×10^5 fragments mL^{-1}.

For the leaf treatment with fungal extracellular compounds, 4 × 2 cm agar plugs with 10-day-old fungi grown on PDA were cultured into 250 mL malt extract (20 g L^{-1}) liquid cultures in 1 L Erlenmeyer flasks at 220 rpm, 28°C in darkness. After 14 days, fungal culture medium was centrifuged for 8 min at 15 000 *g*, 25°C, and the supernatant was collected and filtered through membranes with 0·20 μm pore size (Sartorius Stedim Biotech GmbH) to eliminate spores and sterilize the solution. As it was previously shown that the quantity of extracellular proteins reflects the fungal biomass (M. Bénard-Gellon, Université de Haute-Alsace, Colmar, France, personal communication) and to ensure that the different culture filtrates were comparable, total proteins were quantified in the different experiments by measuring the 280 nm absorbance with a NanoDrop ND-1000 spectrophotometer (Thermoscientific).

Leaf discs of 1 cm diameter were excised using a cork borer and placed on wet filter paper in Petri dishes with the adaxial side up. Before immersion into sterilized fungi culture filtrate solution, the leaf discs were washed with 70% ethanol for 30 s, and rinsed in water. Subsequently, leaf discs were immersed in fungi culture filtrate solution for 24 h, at 25°C in darkness. Necrosis was monitored for three independent experimental series of 5–10 leaf discs for each genotype.

Measurement of necrosis in wood and leaves

For experiments with all the accessions from the Vitaceae family, the necrotic area and the total internode area were quantified using ImageJ software (http://imagej.nih.gov/ij). Specifically, after setting the scale for each image, the 'threshold colour' function was used to highlight the necrotic areas by adjusting 'hue and brightness' parameters. Then the wand (tracing) tool (setup: mode, 8-connected; tolerance, 2·0) and 'ROI manager' were used to precisely select the individual necrotic area from each internode. The percentage of internode necrotic area was calculated by dividing the necrotic area by the total internode area. Data were obtained for three independent experimental series of 7–8 internodes measured for each genotype. For experiments with *V. vinifera* cvs Chardonnay, Gewürztraminer and subsp. *sylvestris*, the necrotic areas were relatively smaller than in the inoculation experiments with the Vitaceae family. The percentage of internode necrotic area in inoculated wood was thus divided by the percentage of internode necrotic area in controls inoculated with PDA. The results are expressed in relative units. For experiments using leaf discs, the percentage of necrotic area was calculated for each leaf disc as described for the woody internodes.

Statistical analysis

The results obtained for wood inoculation were compared for each genotype by using a multifactorial ANOVA followed by a multiple comparison of means using Duncan's test (at $P \leq 0{\cdot}05$, StatgraphicPlus; Manugistics, Inc.).

Gene expression analysis by real-time quantitative RT-PCR

After inoculation, small pieces of internode wood were frozen in liquid nitrogen at 12 h, 3 days and 7 days post-inoculation (pi) for *N. parvum* Bt 67, and 12 h, 3 days and 21 days pi for *D. seriata* 98.1. The green wood section (GW) and the necrotic wood section (NW) immediately surrounding the necrosis were separated. Total RNA was extracted using the RNeasy Plant Mini kit (QIAGEN) following the manufacturer's instructions and RNA concentration was determined with a Qubit fluorometer (Invitrogen). One microgram of total RNA was incubated with one unit of RNase-free DNase I (Euromedex) for 30 min at 37°C according to the manufacturer's instructions. cDNA was synthesized from RNA with the SuperScript II reverse transcriptase (Invitrogen).

Quantitative PCR was performed on the CFX 96 Real Time PCR System with a C1000 thermal cycler (Bio-Rad). Primers described in Ramírez-Suero *et al.* (2014) were used for phenylalanine ammonia lyase (*PAL*), the first enzyme of the phenylpropanoid pathway; stilbene synthase (*STS*) and resveratrol *O*-methyltransferase (*ROMT*), two enzymes involved in the synthesis of stilbene phytoalexins; superoxide dismutase (*SOD*), involved in scavenging of superoxide; lipoxygenase (*LOXC*), the first committed enzyme of the jasmonic acid pathway; the pathogenesis-related proteins *PR1* and *PR6*; and *HSR1*, a cell death marker (Bézier *et al.*, 2002).

Two biological replicates each comprising three technical replicates were conducted for each gene in a total volume of 20 μL containing 10 μL SYBR Green MasterMix (Euromedex), 1 μL each primer at 10 μM and 10 ng cDNA, with the following thermal cycling conditions: 3 min at 95°C; then 40 cycles of 15 s at 95°C, 15 s at 60°C, 20 s at 72°C, 10 s at 77°C. The specificity of the individual PCR amplification was checked using a heat dissociation curve from 55 to 95°C following the final cycle of the PCR and by sequencing the final PCR products. The results obtained for each gene of interest were normalized to the expression of two reference genes (*VvACT1* (AF369524) and *VvEF1α* (CB977561)) and relative expression (fold induction) compared to mock-inoculated controls was calculated as described by Pfaffl (2001). Mean values and standard deviations were obtained from three technical and two biological replicates.

Results

Susceptibility of members of the Vitaceae family to *N. parvum* and *D. seriata*

In preparation for this study, two inoculation methods were compared, i.e. infection using a suspension of mycelia (adapted from Travadon *et al.*, 2013) and infection using a colonized agar plug. Inoculation with the agar plug produced lower variability compared to inoculation with the mycelial suspension, as seen from smaller standard errors between technical replicates (Fig. 2). However, inoculation with the mycelial suspension produced a somewhat higher spread of necrosis. This may be due to the faster spreading rate in the vascular tissues of fungi grown in liquid medium compared to the fungi grown on solid agar plates.

It was therefore decided to use agar plug inoculation to compare susceptibility across different members of the Vitaceae family after inoculation into 1-year-old detached canes. Necrosis was quantified 7–21 days after inoculation with *N. parvum* Bt67 and *D. seriata* 98.1. Although no genotype was completely resistant to either *N. parvum* (Fig. 3a) or *D. seriata* (Fig. 3b), the necrotic

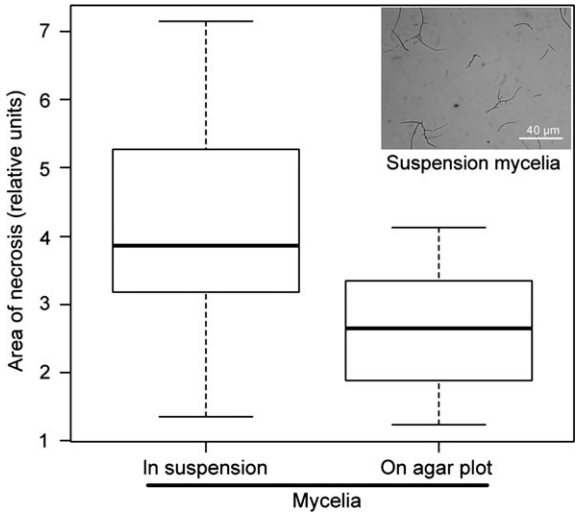

Figure 2 Comparison of the inoculation methods of mycelial suspension versus mycelia on agar tested on *Vitis vinifera* cv. Chardonnay inoculated with *Neofusicoccum parvum*. Necrosis symptoms on *V. vinifera* cv. Chardonnay woody internodes 7 days after *N. parvum* Bt 67 inoculation. Relative units represent the percentage of internode necrotic area in inoculated wood divided by the percentage of internode necrotic area in controls inoculated with potato dextrose agar. Data are expressed as a box-and-whisker plots showing median and interquartile range (IQR). The insert image is the mycelia used for inoculation in suspension medium. Mean ± SE was calculated from three biological replicates each calculated from 7–8 internodes.

area was significantly different ($P < 0{\cdot}05$) and allowed classification of the accessions into four levels of susceptibility.

With respect to inoculation with *N. parvum* (Fig. 3a), *Parthenocissus tricuspidata* was the least susceptible among the tested genotypes. Some genotypes, e.g. *Muscadinia rotundifolia* cv. Regale and the 110R rootstock, known for their resistance to other grapevine pathogens, showed high levels of susceptibility. Grapevine cultivars cultivated in the French Jura, known for a high incidence of canopy symptoms in vineyards (*V. vinifera* cv. Trousseau, *V. vinifera* cv. Savagnin blanc, *V. vinifera* cv. Colombard (Grosman & Doublet, 2012)), also showed a serious level of necrosis after *N. parvum* inoculation. Interestingly, some cultivars like *V. vinifera* cv. Grenache, characterized by a low incidence of esca/BDA canopy symptoms in vineyards, in this experiment showed a high level of susceptibility to *N. parvum*. Finally, *V. vinifera* cv. Chardonnay, Cabernet sauvignon and Pinot noir were among the less susceptible genotypes.

Similarly for inoculation with *D. seriata* 98.1, no completely resistant genotype was identified (Fig. 3b). Again, *P. tricuspidata* showed the smallest necrosis surface, as for *N. parvum* inoculation. Together with *V. vinifera* cv. Fantasy seedless and *Ampelopsis japonica*, this accession fell into the less susceptible group. The accessions 41B and 110R, which have been used as disease-resistant rootstocks, showed a high level of necrosis after *D. seriata* inoculation. Again, the cultivars from the French Jura, which are known for a high incidence of canopy symptoms in vineyards (*V. vinifera* cv. Trousseau, *V. vinifera* cv. Savagnin blanc, *V. vinifera* cv. Chenin, *V. vinifera* cv. Colombard) also exhibited a high level of susceptibility to *D. seriata* inoculation. Interestingly, *A. japonica* that had been highly susceptible to *N. parvum*, showed a low level of susceptibility to *D. seriata*.

Susceptibility of *V. vinifera* subsp. *sylvestris* to *N. parvum* and *D. seriata*

In the next step, the study was extended to *V. vinifera* subsp. *sylvestris*, the ancestor of cultivated grapevine. After inoculation of detached canes with *N. parvum*, all of the tested *V. vinifera* subsp. *sylvestris* accessions were significantly less susceptible than *V. vinifera* cvs Chardonnay and Gewürztraminer (Fig. 4a). Among them, the *sylvestris* genotypes Ke 33 and Ke 95 were ranked as the less susceptible genotypes. In terms of inoculation with *D. seriata*, the less susceptible genotypes were *V. vinifera* subsp. *sylvestris* Hö 29 and *V. vinifera* subsp. *sylvestris* Ke 83 and Ke 84 (Fig. 4b). Surprisingly, *V. vinifera* cv. Gewürztraminer, which had been found to be sensitive to *N. parvum*, exhibited a low level of necrosis upon inoculation with *D. seriata* (similar to Ke 94), whereas Chardonnay was significantly more susceptible. The *sylvestris* Ke 74, which had performed relatively well under challenge by *N. parvum*, exhibited the highest level of susceptibility to *D. seriata*, similar to *V. vinifera* cv. Chardonnay.

Defence gene expression in inoculated internodes

To determine whether the lower susceptibility observed in some *V. vinifera* subsp. *sylvestris* accessions is associated with a differential induction of defence responses, four *sylvestris* accessions with good performance under inoculation of both pathogens were selected (Hö 29, Ke 33, Ke 53 and Ke 83) along with two cultivars (Chardonnay and Gewürztraminer). The expression of several defence genes was evaluated by quantitative RT-PCR in internodes from detached canes 12 h, 3 days and 7 days post-*N. parvum* inoculation (Fig. 5a), and 12 h, 3 days and 21 days post-*D. seriata* inoculation (Fig. 5b), both in necrotic wood and in green wood surrounding the necrotic lesion. Following inoculation with both fungi, defence gene induction began as soon as 12 h pi, and was maximal at 3 days pi (Fig. 5).

The expression of two genes, *SOD* and *HSR1*, correlated with susceptibility to both fungi. The expression of *SOD* was significantly higher both in green and necrotic wood in *V. vinifera* cvs Chardonnay 12 h pi with *D. seriata* and Gewürztraminer 3 days pi with *N. parvum*. The expression of *VvHSR1*, a cell death marker, was also more stimulated in *V. vinifera* cv. Chardonnay 12 h and 3 days pi with *D. seriata* (Fig. 5b).

After *N. parvum* inoculation, the expression of *STS* was activated earlier (from 12 h pi) and to a higher

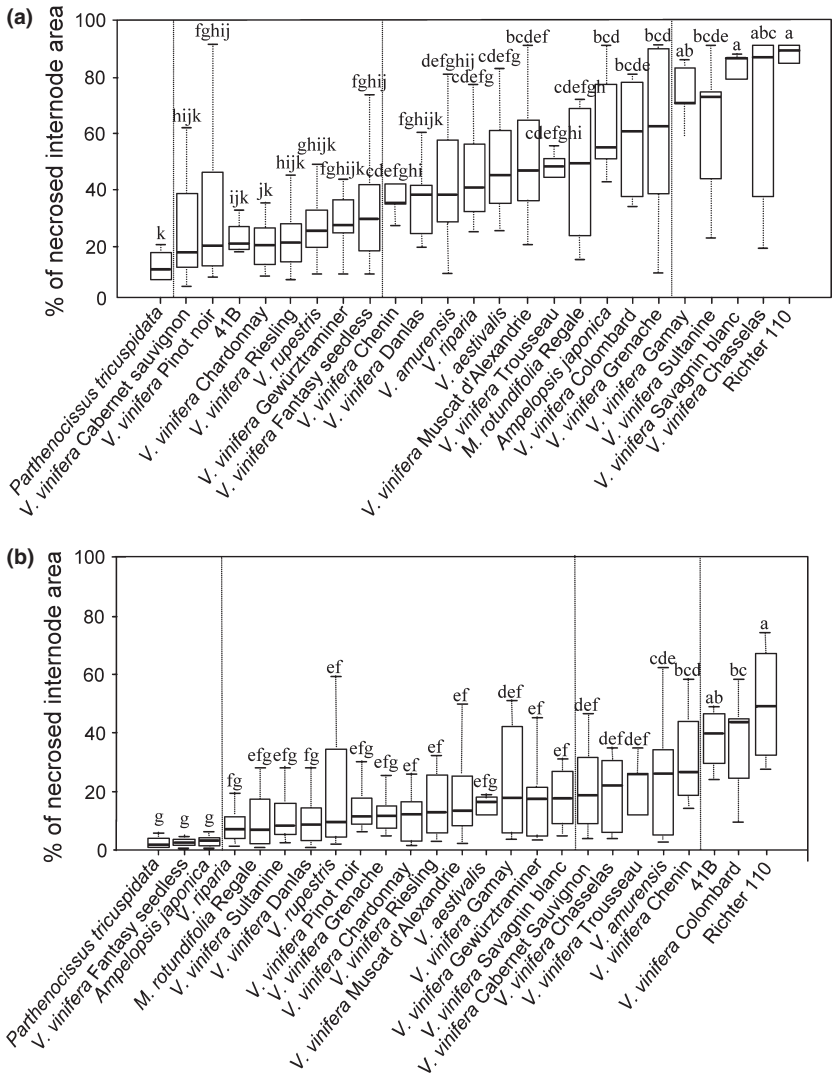

Figure 3 Susceptibility of the Vitaceae family to *Neofusicoccum parvum* Bt 67 (a) and *Diplodia seriata* 98.1 (b). Box plots illustrate the distribution of percentage of internode necrotic area measured within 25 genotypes at day 14 after inoculation with *N. parvum* and at day 21 after inoculation with *D. seriata*. The percentage of internode necrotic area was calculated by dividing the necrotic area by the total internode area. Mean ± SE was calculated from three independent experiments from 2013 to 2014, each calculated from the mean of 7–8 internodes. Data are expressed as a box-and-whisker plots showing median and interquartile range (IQR). The same letter above columns indicates no significant difference at $P < 0{\cdot}05$ (Duncan, 1955). The genotypes are divided into four groups by dotted lines according to their susceptibility.

extent in the resistant genotypes compared to a later activation in the sensitive genotypes (Fig. 5a). However, earlier expression of *STS* was not observed in the resistant genotypes following *D. seriata* inoculation (Fig. 5b). *LOXC* expression was also stimulated earlier (from 12 h pi with *N. parvum*) in the resistant *V. vinifera* subsp. *sylvestris* genotypes compared to *V. vinifera* cv. Chardonnay or Gewürztraminer and again this was not found with *D. seriata*. The overall pattern of PR protein expression did not correlate well with the resistance levels after infection with both fungi, although there was a trend for an earlier expression of *PR6* 12 h after *N. parvum* inoculation in *V. vinifera* subsp. *sylvestris*, except for Ke 53 (Fig. 5a).

Necrosis symptoms on leaf discs treated with culture filtrate of *N. parvum* and *D. seriata*

It is believed that Botryosphaeriaceae produce phytotoxic metabolites that could be translocated via the xylem sap and responsible for the canopy disease symptoms, including wilting and chlorosis of the leaves (Bertsch *et al.*, 2013). The necrotic response to extracellular compounds produced by *N. parvum* and *D. seriata* was therefore examined in leaf discs of the selected four *sylvestris* accessions compared to the cultivars Chardonnay and Gewürztraminer. Foliar discs were incubated with a liquid culture filtrate from two *N. parvum* (Bt 67 and Bourgogne S-116) and two *D. seriata* (98.1 and 99.7) strains. There was no significant difference in leaf necrosis among the different *sylvestris* accessions; however, the necrosis of cv. Gewürztraminer treated with extracellular compounds from *D. seriata* 99.7, and of cv. Chardonnay treated with culture filtrate from *N. parvum* Bourgogne S-116, were significantly higher compared to the other genotypes (Fig. 6). Overall, the two *V. vinifera* subsp. *sylvestris* accessions Ke 33 and Ke 53 developed smaller necrosis after treatment with Botryosphaeriaceae extracellular compounds.

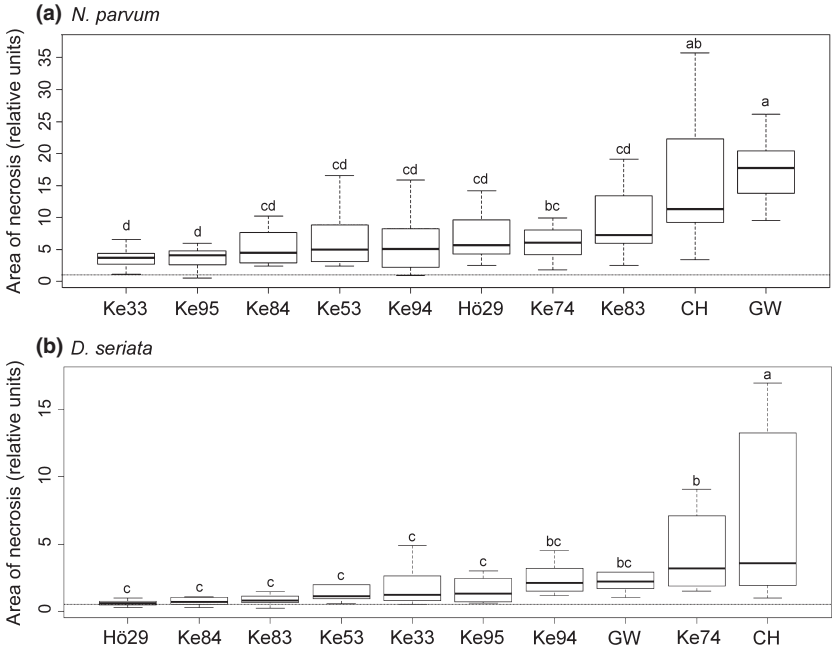

Figure 4 Susceptibility of *Vitis vinifera* and *V. vinifera* subsp. *sylvestris* to *Neofusicoccum parvum* Bt 67 (a) and *Diplodia seriata* 98.1 (b). Box plots illustrating the distribution of necrosis relative units was measured for eight *V. vinifera* subsp. *sylvestris* accessions (Ke 33, 53, 74, 83, 84, 94, 95 and Hö 29) and two *V. vinifera* cultivars (Chardonnay (CH) and Gewürztraminer (GW)) at day 7 after inoculation with *N. parvum*, and at day 21 after inoculation with *D. seriata*. Relative units represent the percentage of internode necrotic area in inoculated wood divided by the percentage of internode necrotic area in controls inoculated with potato dextrose agar (PDA). Mean ± SE was calculated from three independent experiments from 2012 to 2013, each calculated from the mean of 7–8 internodes. Internodes inoculated with PDA were used as control. Data are expressed as a box-and-whisker plots showing median and interquartile range (IQR). The same letter above columns indicates no significant difference at $P < 0{\cdot}05$ (Duncan, 1955).

Discussion

In an attempt to identify potential sources for resistance breeding, this study screened the susceptibility to Botryosphaeriaceae across different members of the Vitaceae, with a focus on *V. vinifera* subsp. *sylvestris*. The results show that genetic variability exists in the response to both *N. parvum* and *D. seriata*, although none of the genotypes showed complete resistance. In general, *N. parvum* was found to be more aggressive than *D. seriata* after artificial inoculation of internodes. This observation is in agreement with the results obtained by Amponsah *et al.* (2011), indicating that *N. parvum* produced longer lesions than *D. seriata*. Also Martos *et al.* (2008) showed a greater phytotoxic activity of culture filtrate from *N. parvum* compared to *D. seriata* on leaves of *V. vinifera* cv. Tempranillo, and Ramírez-Suero *et al.* (2014) further reported that culture filtrate from *N. parvum* Bourgogne S-116 is more aggressive than culture filtrate from *D. seriata* 98.1 on grapevine calli. Although none of the genotypes was completely resistant to any of the pathogens, two *sylvestris* genotypes performed best with respect to reduced necrosis. For *N. parvum*, genotype Ke 33 showed the least necrosis symptoms, whereas genotype Hö 29 was the most resistant to *D. seriata* (Fig. 4). Also, after treatment with the Botryosphaeriaceae extracellular compounds, two *sylvestris* genotypes, Ke 33 and Ke 53, produced the smallest necrotic areas.

Concerning rootstock sensitivity to Botryosphaeriaceae, 110R was more sensitive to both *N. parvum* and *D. seriata*, whereas 41B was relatively less susceptible to *N. parvum* but was highly susceptible to *D. seriata*. The results are in agreement with the study of Billones-Baaijens *et al.* (2014) showing that rootstocks generally produced significantly longer lesions than scions. Rootstocks 110R and 140 Ruggeri were also greatly affected by fungi associated with Petri disease and esca (Gramaje *et al.*, 2010). The relative sensitivity of rootstocks is not surprising because rootstock varieties were not selected for resistance to wood-destroying diseases but for other agronomic traits, such as soil type adaptation, vigour of growth and tolerance or resistance to root parasites. In the present experiments, grapevines originating from North America (*Muscadinia rotundifolia*, *V. riparia* and *V. aestivalis*), which have developed resistance to pathogens such as the biotrophs *Erysiphe necator* and *Plasmopara viticola*, are nevertheless highly susceptible to *N. parvum*. However, *V. riparia* and *M. rotundifolia* exhibited rather low susceptibility to *D. seriata*. In another study susceptibility of genotypes, originating from crosses of North American *Vitis* (*V. berlandieri*, *V. riparia*, *V. rupestris*), to fungi associated with esca

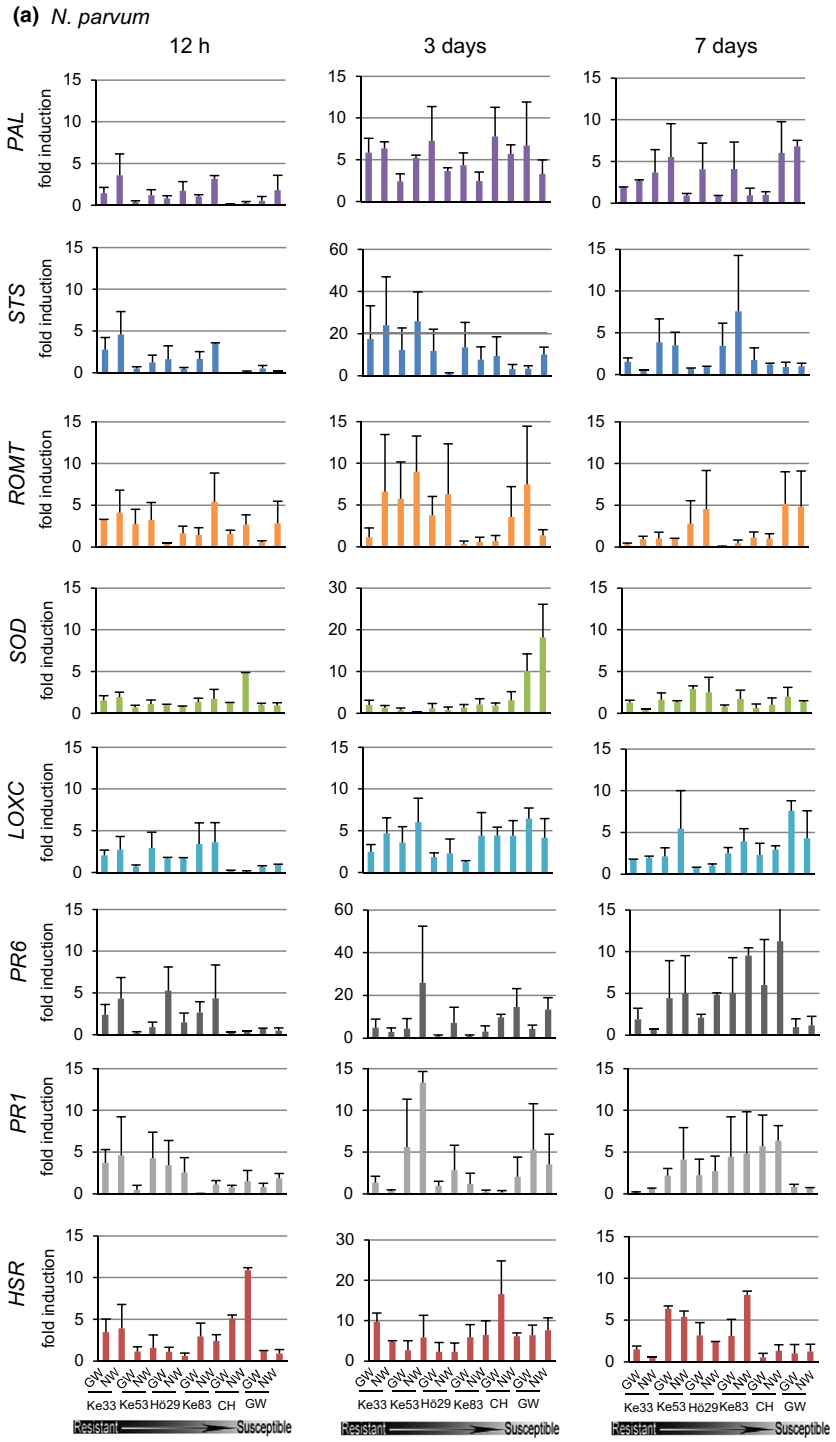

Figure 5 Defence-related gene expression in woody stems of *Vitis vinifera* and *V. vinifera* subsp. *sylvestris* after inoculation of *Neofusicoccum parvum* (a) and *Diplodia seriata* (b). Samples of necrotic wood (NW) and of green wood (GW) were harvested after 12 h, 3 days and 7 days after inoculation with *N. parvum* and 12 h, 3 days and 21 days after inoculation with *D. seriata*. The accessions tested by qRT-PCR were Ke 33, Ke 53, Ke 83 and Hö 29 for *V. vinifera* subsp. *sylvestris* and cv. Chardonnay (CH) and Gewürztraminer (GW) for *V. vinifera*. Relative expression (fold induction) indicates normalized expression levels in inoculated GW or NW compared to normalized expression levels observed in control wood inoculated with potato dextrose agar at the same time point. Mean values and standard error were obtained from three technical and two biological replicates, each consisting of 7–8 internodes per genotype.

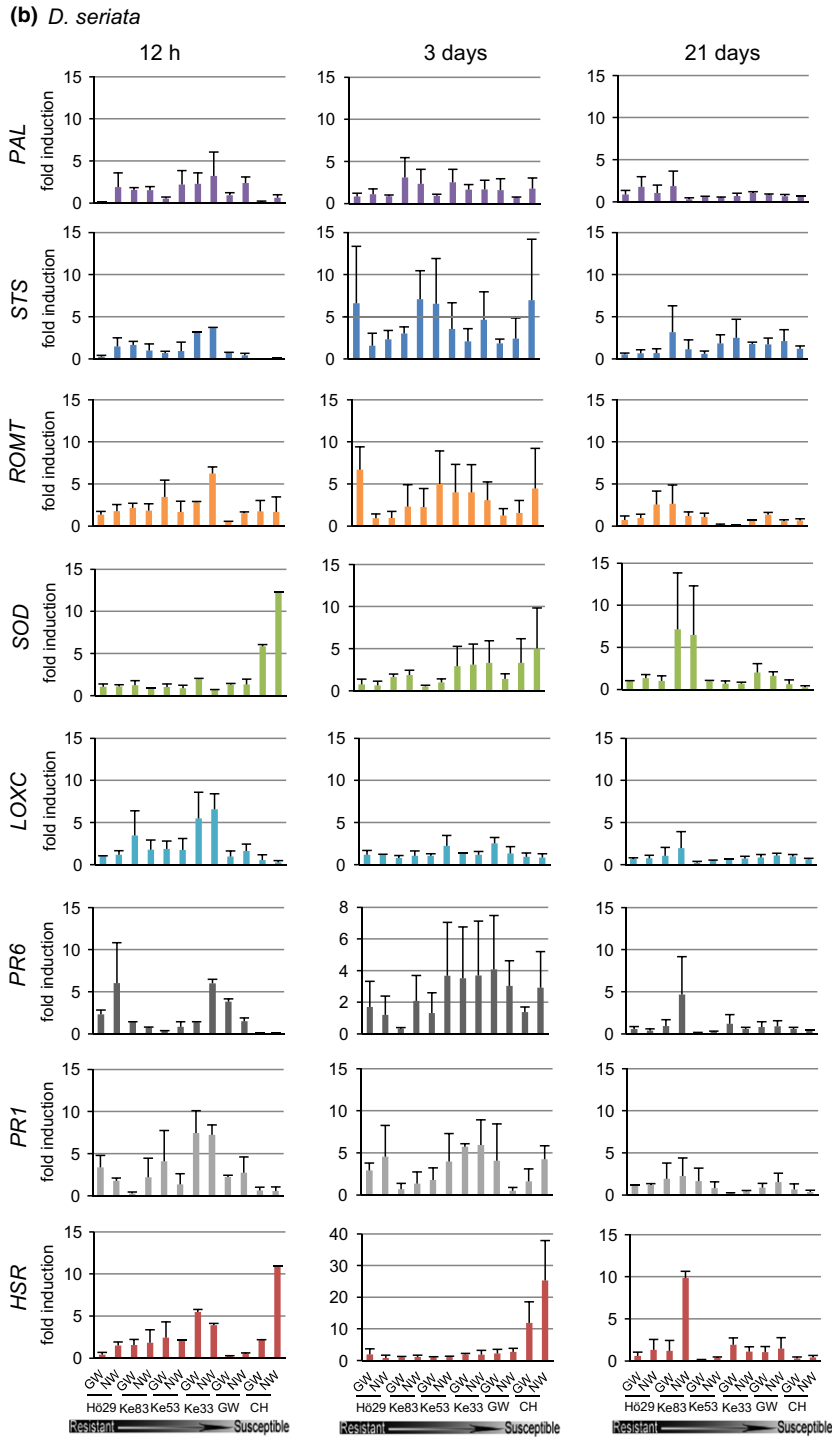

Figure 5 (continued).

has been examined in controlled conditions and in the field. All the genotypes exhibited a wide range of susceptibility to *P. alelophilum* without any resistance (Eskalen *et al.*, 2001; Gramaje *et al.*, 2010).

One of the main questions arising from this work is whether results from the sensitivity of different grapevine cultivars in vineyards (canopy symptoms) can be inferred from results of sensitivity levels after artificial inoculation. This study gives evidence for a correlation: for instance, cultivars characteristic of the French Jura region (Trousseau, Savagnin blanc) are characterized by a high incidence in esca/BDA leaf symptoms (Grosman

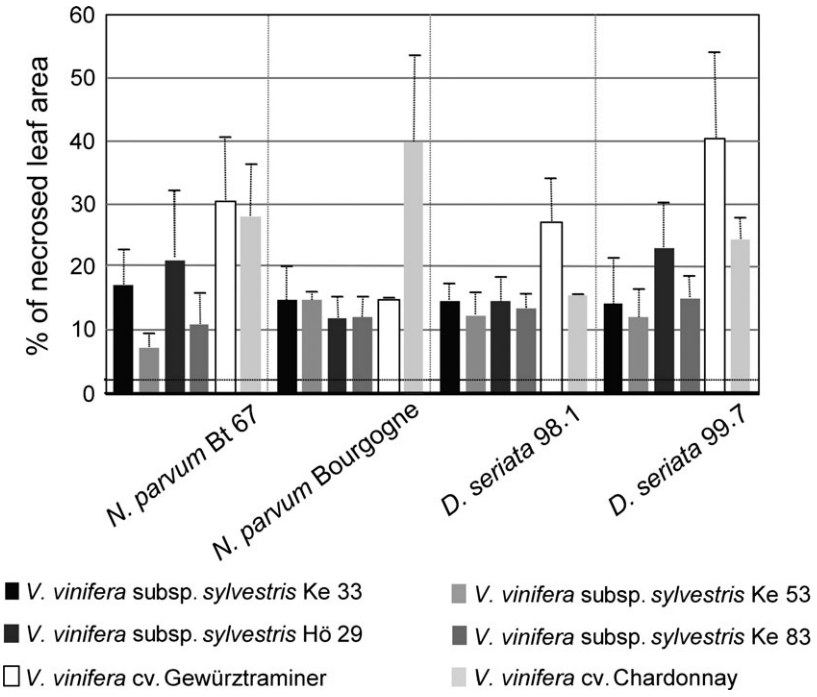

Figure 6 Necrosis symptoms on grapevine leaf discs treated with culture filtrate from *Neofusicoccum parvum* and *Diplodia seriata*. Four *V. vinifera* subsp. *sylvestris* accessions (Ke 33, Ke 53, Ke 83 and Hö 29) and two *V. vinifera* cultivars (Chardonnay and Gewürztraminer) were studied. Percentages of necrosed leaf area at 24 h after inoculation are compared within six genotypes. Mean ± SE was calculated from three biological replicates from year 2012 to 2013 each calculated from 5–10 leaf discs per genotype.

& Doublet, 2012), and these cultivars also range among the most sensitive in the wood inoculation tests for both *N. parvum* and *D. seriata* performed here. Gewürztraminer and Savagnin blanc belong to the same family of 'Traminer', and Gewürztraminer has been selected from an aromatic mutation that occurred in Savagnin rose in the 19th century (Rusjan & Korosec-Korusa, 2008). Similar to Savagnin blanc and Trousseau, Gewürztraminer shows high expression of canopy symptoms in vineyards (Grosman & Doublet, 2012). In contrast, Chardonnay and Pinot noir, which are among the less susceptible genotypes (present study and Travadon *et al.* (2013)), also show a low incidence of dieback symptoms in vineyards. The similar level of susceptibility between these two cultivars could be related to their parental relationship, Chardonnay being the progeny of a single pair of parents, Pinot and Gouais blanc (Bowers *et al.*, 1999). Although the assay system here reflects in many cases the differences observed in vineyards with respect to symptom expression, it is difficult to draw a strict parallel between field observations and inoculation tests, because the esca/BDA canopy symptoms are observed in vineyards and wood necrosis is measured in inoculation tests. For example, Chasselas has a low incidence of leaf symptoms in vineyards but is among the more susceptible cultivars after wood inoculation with both *N. parvum* and *D. seriata* (Fig. 3). Mechanisms of plant defence in wood and canopy may differ, because leaf symptoms are believed to result from the translocation of phytotoxic compounds, whereas wood symptoms result from enzymatic degradation of the wood. Moreover, a complex of fungi is associated with esca and botryosphaeria dieback, which may be one reason why data collected under laboratory conditions are not always correlated with field data (Murolo & Romanazzi, 2014). In addition, field assays are influenced by environmental factors such as climatic and soil conditions. For example, an overview of grapevine trunk diseases in France showed that the same *V. vinifera* cultivar could be significantly more affected in one region than in another (Bruez *et al.*, 2013).

Of course, infection of freshly cut canes represents an artificial system, but at this stage, only experimental reduction will allow mechanisms of infection and defence to be addressed. Two levels of reduction have therefore been administered to screen different genotypes for differences in their response: (i) instead of the naturally occurring variable pathogen communities comprising different fungi, artificial inoculation with single fungal strains has been used; and (ii) instead of working with entire plants that are very difficult to standardize and are limited in number (*V. vinifera* subsp. *sylvestris* is at the verge of extinction), freshly cut canes were used. To what extent the data from this experimental model mirrors the situation in the field will be further tested by future studies.

Overall, this work confirms that the sensitivity of grapevine to botryosphaeria dieback depends on genetic factors. Despite the caveat on the predictability of field performance by laboratory assays, these data on the susceptibility within the Vitaceae family are important readouts for molecular marker-assisted breeding as a strategy for sustainable viticulture. Figure 3 divides the varieties into four groups according to their susceptibility to Botryosphaeriaceae. Based on these data plots, genomic information from the more resistant genotypes could be

used to identify molecular markers for marker-assisted breeding. In addition, defence signalling transduction comparing genotypes with different susceptibilities might identify molecular or cellular events that are relevant for the interaction between host and pathogen. The tested genotypes of *V. vinifera* subsp. *sylvestris* developed significantly smaller lesions to both *N. parvum* and *D. seriata* compared to cv. Chardonnay or Gewürztraminer. As the wild European grapevine is the ancestor of cultivated *V. vinifera*, it is possible to introduce such genetic factors through introgression by simple crosses. In fact, a small crossing population for *V. vinifera* subsp. *sylvestris* Hö 29 × *V. vinifera* cv. Augster weiß (a male-sterile ancient variety) has already been successfully established as a first step to launch a breeding strategy for higher tolerance to botryosphaeria dieback. However, the results from the assay system have to be confirmed by whole plant inoculation and monitoring of necrosis.

To know if the lower susceptibility to Botryosphaeriaceae could be associated with changes in defence gene expression, the expression of several defence markers were monitored after infection. No significant differences in defence gene expression were found between green and necrotic wood, suggesting that signals originating from the necrotic part rapidly migrate to induce defence responses in the green wood surrounding the lesion. The higher expression of *SOD* in susceptible genotypes such as Gewürztraminer might be a consequence of a higher oxidative stress associated with a stronger progression of necrosis in this cultivar.

Several defence genes (*STS*, *LOXC* and *PR6*) were expressed earlier (12 h) after inoculation with *N. parvum* in most of the *V. vinifera* subsp. *sylvestris* accessions compared to *V. vinifera* cv. Chardonnay and Gewürztraminer. At 3 days pi, considering the relatively resistant genotypes *V. vinifera* subsp. *sylvestris* Ke 33 and Ke 53, the expression pattern of *STS* was higher compared to the other genotypes following *N. parvum* inoculation. During a screen for stilbene synthesis in response to stress (using a short UV-C pulse at 254 nm on leaves for induction), the *sylvestris* genotypes Hö 29, Ke 33, Ke 53 and Ke 83 were found to accumulate a much higher level of the antimicrobially active stilbenes *trans*-resveratrol and their oxidative oligomers, the viniferins, than the tested cultivated grape varieties as well as many of the *sylvestris* genotypes (Duan *et al.*, 2015). The same genotypes also showed partial resistance to downy and powdery mildew, indicating that stilbene accumulation might contribute to the performance under Botryosphaeriaceae challenge. Stilbenes, which are associated with higher resistance of grapevine to pathogens (reviewed in Chong *et al.* (2009)), are also toxic to fungi associated with botryosphaeria dieback (Lambert *et al.*, 2012) and a comparative analysis of cultivars with different susceptibility to esca showed that lower susceptibility correlated with more rapid accumulation of *STS* transcripts (Lambert *et al.*, 2013).

However, timely accumulation of stilbenes should not be seen as the only relevant factor for the response to Botryosphaeriaceae. First, it should be noted that the correlation between early induction of *STS* transcripts and partial resistance holds for *N. parvum*, but not for *D. seriata*, which might indicate that this pathogen can silence basal immunity. Secondly, xylem anatomy might modulate the progression of infection. In a recent article, Pouzoulet *et al.* (2014) provide evidence that susceptibility of three *V. vinifera* commercial cultivars to esca disease is correlated to large vessel diameter. Vessel dimensions could play a role in the compartmentalization of the pathogen and at the same time may influence the sensitivity of the plant to drought. Study of the xylem structure of *V. vinifera* subsp. *sylvestris* could help to evaluate whether higher tolerance is related to the diameter of xylem vessels. Thirdly, other secondary metabolites, such as flavonoids, might also contribute to resistance.

In conclusion, this study shows that different accessions from the Vitaceae family are differentially susceptible to wood necrosis caused by Botryosphaeriaceae fungi. It is thus likely that the genotype of the plant plays a role in the resistance to botryosphaeria dieback. Moreover, several accessions of *V. vinifera* subsp. *sylvestris*, the ancestor of *V. vinifera*, are more resistant to artificial inoculation than cultivars such as Chardonnay and Gewürztraminer. These findings suggest that the possibility of creating new grapevine varieties with enhanced resistance to trunk pathogens has realistic potential. Further work will be aimed at elucidating the mechanisms of low susceptibility in some of the *V. vinifera* subsp. *sylvestris* cultivars such as the earlier induction of defence responses linked with basal immunity, secondary metabolite contents, and also wood structure.

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