Reproducing Botryosphaeria dieback foliar symptoms in a simple model system

Reis Pedro[1], Magnin-Robert Maryline[2], Nascimento Teresa[3], Spagnolo Alessandro[2], Abou-Mansour Eliane[4], Cristina Fioretti[2], Christophe Clément[2], Rego Cecilia[1*‡], Fontaine Florence[2*‡]

[1] Centro de Investigação em Agronomia, Alimentos, Ambiente e Paisagem (LEAF), Instituto Superior de Agronomia, Universidade de Lisboa, Tapada da Ajuda, 1349-017 Lisbon, Portugal

[2] SFR Condorcet, Université de Reims Champagne-Ardenne, URVVC EA 4707, Laboratoire Stress Défenses et Reproduction des Plantes, Moulin de la Housse, BP 1039, 51687 Reims Cedex 2, France

[3] Departamento de Ciências e Engenharia de Biossistemas, Instituto Superior de Agronomia, Universidade de Lisboa, Tapada da Ajuda, 1349-017, Lisbon, Portugal

[4] Université de Fribourg, Département biologie végétale, 10 chemin du musée, 1700 Fribourg, Switzerland.

‡ These 2 authors participated equally in this work.

* Corresponding author: Fontaine Florence, E-mail: florence.fontaine@univ-reims.fr

Abstract

Botryosphaeria dieback is a grapevine trunk disease with a worldwide distribution and associated with *Diplodia seriata* and *Neofusicoccum parvum* among several other *Botryosphaeriaceae* species. The aforementioned xylem-inhabiting fungi cause wood lesions, leaf and berry symptoms and eventually lead to the death of the plant. The aim of this work was to develop a simple model system to reproduce the foliar symptoms caused by *D. seriata* and *N. parvum* to better characterize fungal pathogenicity and determine the mechanisms involved in symptom development. Green stems of grafted grapevine cuttings cv. Aragonez were inoculated with three isolates of *N. parvum* and two isolates of *D. seriata* with different degrees of virulence and the experiment was repeated four times from 2011 to 2014. Three months after inoculation, the lesions associated with *N. parvum* were larger than those associated with *D. seriata*. Similarly, eight months after inoculation the percentage of plants showing foliar symptoms was greater in the *N. parvum* treatments than in the *D. seriata* treatments. During the emergence of foliar symptoms, plant stress-related responses were modulated in green stems and leaves, especially a down-regulation of superoxide dismutase (*SOD)* and fasciclin-like arabinogalactan protein (*fascAGP)* and an up-regulation of stilbene synthase (*STS)* with an accumulation of phenolics. In conclusion, the simple model system developed allowed a good characterization of isolate pathogenicity and correlation with foliar symptoms of Botryosphaeria dieback, namely spots on leaf margin and blade.

Several *Botryosphaeriaceae* species are associated worldwide with the grapevine trunk diseases (GTDs) known as Botryosphaeria dieback (Moller and Kasimatis 1978; Larignon and Dubos 1997; Graniti et al. 2000; Fischer 2006; Larignon et al. 2009; Úrbez-Torres 2011; Spagnolo et al. 2014a; Larignon et al. 2015). The most common species isolated from grapevine-growing regions around the world include *Diplodia seriata* De Not. (Cristinzio 1978; Rovesti and Montermini 1987; Larignon et al. 2001; Castillo-Pando et al. 2001; Phillips et al. 2007; Savocchia et al. 2007; Úrbez-Torres et al. 2008) and *Neofusicoccum parvum* (Pennycook & Samuels) Crous, Slippers & A.J.L. Phillips (Crous et al. 2006). These fungi are xylem-inhabiting and attack the framework of grapevines causing perennial cankers in the wood, resulting in leaf and berry symptoms and finally leading to the death of the plant. Symptoms are characterized by yellowish-orange (white cultivars) or wine-red (red cultivars) spots on leaf margins and blade, and in most cases, the emergence of a brown stripe on the wood under the bark (Larignon et al. 2001; Spagnolo et al. 2014a). This symptom is often associated with a grey sector of rotted wood. Shrivelling and drying of inflorescences or fruit clusters are frequently observed.

The incidence of Botryosphaeria dieback, together with two other trunk diseases, esca and Eutypa dieback, has increased over the years. In France it was estimated that 13% of productive vines were affected by GTDs in 2012 (Grosman and Doublet 2012; Bruez et al. 2013). Although GTDs, including Botryosphaeria dieback, appear to be increasingly common, accurate knowledge of host-pathogen interactions poses certain problems, including (i) determining the seasonal influence on field-collected data due to an uncontrolled environment and (ii) distinguishing pathogen effects on grapevines from effects in response to other biotic agents in the field. Research has been developed to gain a better understanding of the mechanisms that are involved in symptom expression by the artificial reproduction of the symptoms through individual or combined inoculations of pathogenic fungi or by the use of simpler grapevine model systems (e.g., cuttings, grapevine *in vitro* plants, or grapevine cultured cells) under controlled conditions. Regarding

Eutypa dieback symptoms, the stunting of new shoots with small cup-shaped, chlorotic and tattered leaves, were reproduced on greenhouse cuttings that were infected with *Eutypa lata* ascospores or mycelial plugs (Petzoldt et al. 1981; Péros and Berger 1994, 1999; Sosnowski et al. 2007). For esca disease, pathogenicity tests were carried out in a greenhouse with vines inoculated with *Phaeomoniella chlamydospora* (Chiarappa 2000). Although a significant reduction of growth was observed, typical foliar symptoms were not reproduced. With *P. chlamydospora,* similar results were obtained with inoculated cuttings as reported by Gerbore (2013) and Pierron et al. (2015). For Botryosphaeria dieback, no studies have been reported on the development of such model systems, both necrosis and foliar symptom development, for the analysis of pathogenicity.

Data crossing of plant-response and fungal-activity in compatible interactions could yield important information about the mechanisms developed by fungi to colonize grapevine and the protective response of the grapevine to limit the development of the fungi. The difficulties of such work could arise from the fact that the grapevine is a perennial plant cultivated worldwide under various environmental conditions. The development of a simple model system for the inoculation of grapevine plants under controlled conditions to optimize and validate disease development is required. With such a tool, knowledge of the interactions between the GTD agents could progress and such a model system could represent a first step towards the development of management solutions against these diseases as the visual presence of symptoms in leaves leads to an early diagnostic. The aim of this work was to develop a simple model system using *D. seriata* and *N. parvum* to better characterize their virulence by measuring the size of lesions and evaluating the percentage of vines developing foliar symptoms, and thereby understand their impact on vine physiology by studying the stress response at a molecular level and by quantification of phenolic compounds.

Materials and Methods

Plant material and fungal inoculation. Each year, during a four-year period (2011–2014), one year old grafted grapevine cuttings cv. Aragonez (= Tempranillo) were potted individually in one litre, free draining bag containing a sandy soil mixture (1/3 sand, 1/3 soil, 1/3 organic matter) and placed using a completely randomized design in a ventilated greenhouse at 24°C under natural light. After one month of growth, plants were inoculated with isolates of *N. parvum* (Np) and *D. seriata* (Ds) with two different origins (Portuguese, isolated by C. Rego and French, isolated by P. Larignon) and different degrees of aggressiveness (Larignon et al. 2001; Rego et al. 2009) (Table 1). A three mm area of the bark was removed with a cork borer from the base of the primary stems (approximately 1.5cm diameter) between the second and third nodes. The wounds were inoculated with three mm mycelial plugs taken from the actively growing margin of 8-day old colonies of Np and Ds growing on potato dextrose agar (PDA, Difco, BD, Sparks, MD, USA) at 24°C in darkness. Each inoculation point was covered with moist cotton wool and sealed with Parafilm. In 2014, the causal agent of black rot of vine *Phyllosticta ampelicida* (Engelm.) Aa (isolates Gb 32 and Gb 17), *Cladosporium* sp. and *Penicillium* sp. isolates (Table 1) were also inoculated in the same manner as positive controls to determine if the expression of foliar symptoms was specific to both Np and Ds. Negative controls were inoculated using the same method but with sterile three mm PDA plugs to confirm that lesions were due to infection by the pathogens and not to the wounding. There were 30 replicates for each treatment and the experiment was kept in the same greenhouse to observe foliar symptom emergence eight months after inoculation.

Determination of lesion size, symptoms appearance and isolation of pathogens. The dimension of lesions was evaluated three months after inoculation on green shoots, before lignification occurred, by measuring the width and the length and therefore calculating the elliptical area of the lesion. All statistical analyses were performed using STATISTICA (StatSoft, Inc. 2007, version 8.0). Homogeneity of variance was tested using Levene's test. Residuals were visually inspected for each experiment, and when necessary the $\log_{10}$ transformation was used to improve

120 homogeneity of variance. One–way analysis of variance (ANOVA) was used to compare
121 differences in mean lesions (width, length and area of discoloration) among fungal isolates and
122 species. Means were separated using Tukey's test at the 5% significance level. Percentage of
123 grapevines in each treatment that showed foliar symptoms was visually inspected. When one or
124 more leaves expressed spots and/or chlorotic areas the plant was considered positive for expression
125 of foliar symptoms. Data collected from each trial were subjected to Chi-Square statistical analysis
126 ($\chi 2$ test) at the 5% significance level. Treatment means were compared using Tukey's test at the 5%
127 significance level. Percentages were transformed to arcsine-square root values before analysis.

128 In order to fulfil Koch's postulates, small pieces of necrotic tissue from the edge of each
129 lesion were cut and placed on PDA medium amended with 250 mg L^{-1} chloramphenicol
130 (BioChemica, AppliChem, Germany) to recover the inoculated fungi.

131 **Plant RNA extraction.** When foliar symptoms first appeared, symptomatic and
132 asymptomatic leaves and stems were collected in 2012 from symptomatic and asymptomatic plants,
133 respectively, immediately wrapped in aluminium foil, frozen in liquid nitrogen and stored at -80°C.
134 Samples were ground in liquid nitrogen to a fine powder. Plant RNA Purification Reagent
135 (Invitrogen, Cergy Pontoise, France) was used to isolate total RNA from 1×50 mg of leaf tissue
136 powder and 2×50 mg of green stem powder. The RNA pellet was resuspended in 20 μL of RNase-
137 free water, treated with RQ1 DNase enzyme (Promega, Mannheim, Germany), and RNA was
138 quantified by measuring the absorbance at 260 nm according to the manufacturer's instructions and
139 stored at -80°C before use.

140 **Real-time RT-PCR analysis of gene expression.** In all, 150 ng of total RNA was reverse-
141 transcribed using the Verso SYBR 2-step QRT ROX enzyme (ABgene, Surrey, UK) according to
142 the manufacturer's protocol. PCR conditions were as described by Bézier et al. (2002). Gene
143 expression was tracked by quantitative Reverse-Transcription Polymerase Chain Reaction using the
144 primers reported in Table 2. The fourteen genes studied were previously selected from a proteomic

study (Spagnolo et al. 2012) focused on grapevine reactions in response to trunk diseases, six genes involved in detoxication and stress tolerance (*Halh*, *epoxHF*, *GST5*, *SOD*, *HSP*, *epoxH2*), one involved in terpenoid synthesis (*DXS1*), 2 genes encoding for PR protein, one involved in wall cell compound synthesis (*fascAGP*), one encoding for an aquaporin (*PIP2.2*) and the three last ones involved in the phenylpropanoid pathway (*STS*, *PPO*, *Lac17*) (Table 2). Two housekeeping genes were used as the internal standard to normalize the starting template of cDNA for each matrix (for leaves: α-chain elongation factor 1 gene *EF1- α* and 39S ribosomal protein L41-A *39SRP*; for green stem *EF1- α* and 60S ribosomal protein L18 *60SRP*). Reactions were carried out in a real-time PCR detector Chromo 4 apparatus (Bio-Rad) using the following thermal profile: 15 s at 95°C (denaturation) and 1 min at 60°C (annealing/extension) for 40 cycles. Melting curve assays were performed from 65 to 95°C at 0.5°C s^{-1}. Melting peaks were visualized to check the specificity of each amplification. Results correspond to the means of the independent experiments that were expressed relative to the control corresponding to a fixed value of 1. Control samples consisted of non-inoculated plant. The genes analysed were considered significantly up- or down-regulated when changes in their expression was >2× or <0.5×, respectively.

Extraction and quantification of phenolic compounds. The protocol used is described in Spagnolo et al. (2014b). Briefly, methanolic extracts were prepared from 50 mg of powdered leaf and green stem tissues mixed with 1 mL of methanol (MeOH) and 25 µL of the internal standard *trans*-4-hydroxystilbene (0.5 mg mL^{-1}). For quantitative analysis of stilbenes, 60 µL of methanolic extract was analysed by high-performance liquid chromatography. Standards such as *trans*-piceid, *trans*-resveratrol and *trans*-pterostilbene (Supplemental Fig. 1) were purchased from Extrasynthèse (Genay - France). The *trans*-ε-viniferin, *trans*-vitisin A and *trans*-vitisin B (Supplemental Fig. 1) were obtained from lignified canes of cv. Syrah as described in Spagnolo et al. (2014). Spectral data for all peaks were accumulated in the range between 220 and 600 nm. The data are reported as µg g^{-1} of fresh weight, with a standard deviation from three independent extractions and analyses. To

170 determine whether concentration of control plants were significantly different from the inoculated
171 plants, a Dunn's Multiple Comparison Test was used. Differences at $P \leq 0.05$ were considered
172 significant.
173
174 **Results**
175 **Observation of lesion and foliar symptom expression on plants inoculated with *N.***
176 ***parvum* and *D. seriata*.** For each year and for each isolate, thirty grafted vines growing under
177 greenhouse conditions were inoculated individually with Np 19, Np 67, Np AR, Ds 98-1 and Ds 99-
178 7 fungal isolates. Control grafted vines were maintained under the same greenhouse conditions.
179 Three months after inoculation, dark brown lesions developed on green stems for all the inoculated
180 isolates (Fig. 1) and their size was evaluated in terms of width, length and surface area (Table 3). V-
181 shaped cankers were also observed when cross sections were made on symptomatic canes. For the
182 four years repetitions, mean lesion widths associated with Ds 98-1, Ds 99-7 and Np AR infection
183 were slightly lower than those associated with Np 19 and Np 67. Mean lesion lengths associated
184 with Np 67 and Np AR were higher than those for Ds 98-1, Ds 99-7 and Np 19. Thus, the mean
185 lesion surface areas associated with Np 67 and Np AR were greater than those associated with Ds
186 98-1, Ds 99-7 and Np 19 (Table 3). Each year, eight months after inoculation, foliar symptoms
187 appeared in some of the thirty infected grapevines per isolate. These were characterized by typical
188 orange/red spots at the margins of the leaf and large chlorotic areas between veins (Fig. 2). Plants
189 were visually evaluated and the percentage of infected plants showing foliar symptoms was greater
190 for Np 67 and Np 19 (for example, 73.3% in 2014) than for Ds 99-7, Ds 98-1 and Np AR with
191 values of 30.0%, 50.0% and 60.0%, respectively. None of the 30 control plants showed symptoms
192 (Table 4). The positive control assay revealed that both Gb 17 and Gb 32 produced lesions on green
193 stems but no foliar symptoms were observed, while *Cladosporium* sp. and *Penicillium* sp. gave rise
194 to small necroses similar to those recorded for the negative controls and foliar symptoms were

absent (Table 5). Reisolation of Ds, Np and Gb isolates was always higher than 70%. No *N. parvum* and *D. seriata* were reisolated neither from the *Cladosporium* sp. and *Penicillium* sp. inoculations nor from the controls.

Stress-related responses in stems and leaves of symptomatic plants inoculated with *N. parvum* and *D. seriata*. The expression of selected stress-related genes was monitored in organs of symptomatic plants to determine if there was a correlation between the severity of symptoms (lesions and foliar symptoms) and the stress induced in plants in response to fungal inoculation. Results of the gene expression analysis in leaves and green stems are summarized in Fig. 3. The genes considered were grouped in different functional categories according to the organ studied.

Concerning the genes involved in detoxification and stress tolerance, no changes in gene expression were observed for *GST5* (glutathione-S-transferase) and both *epoxHF* and *epoxH2* (epoxide hydrolases) in plant tissues under our conditions (Figs. 3 and 4). A slight down-regulation of *Hahl* (haloacid dehalogenase hydrolase) was observed in all inoculated plants irrespective of the fungal species (Fig. 3). The expression of *SOD*, encoding a superoxide dismutase was weakly repressed in asymptomatic (AP) and symptomatic leaves (SP) of plants inoculated with Np 19 and not affected in plants inoculated with other isolates tested (Np 67, Np AR, Ds 98-1 and Ds 99-7) (Fig. 3). The gene expression of an alpha crystalline small heat-shock protein (*HSP*), regarded as a molecular chaperone, was not affected in leaves of inoculated plants. On the contrary, this gene was up-regulated in green stems with AP and SP leaves of plants infected by all 5 isolates of *Botryosphaeriaceae* apart from Ds 99-7 where *HSP* was up-regulated, but only in stems with symptomatic leaves (Fig. 4).

Two genes encoding pathogenesis-related (*PR*) proteins were also investigated, a β-1,3-glucanase (*Gluc*) gene and a serine protease inhibitor (*PR6*) gene. In leaves of plants inoculated with *Botryosphaeriaceae* fungi, *Gluc* was generally weakly repressed in AP, while *PR6* was

generally induced in both AP and SP (Fig. 3). Interestingly, *GLUC* was up-regulated in green stems of plants infected with Np and down-regulated in plants infected with Ds (Fig. 4). The gene *DXS* encodes the enzyme 1-deoxy-D-xylulose 5-phosphate synthase, involved in the first major step of terpenoid synthesis, *DXS* was weakly down-regulated in leaves of SP inoculated with Np or Ds species. Our results also showed that the expression of the gene encoding a fasciclin-like arabinogalactan protein (*fascAGP*) was repressed in green stems of AP and SP infected with Np 67 and Np AR, both these *Botryosphaeriaceae* species produced the biggest necroses. The aquaporin plasma membrane intrinsic protein 2-2 encoding gene (*PIP2.2*) was not affected in green stems, whereas it was repressed in asymptomatic and symptomatic leaves of plants inoculated with both Np and Ds 99-7, with a high repression in SP plants.

For genes involved in the phenylpropanoid pathway, the stilbene synthase (*STS*) gene was weakly up-regulated in green stems of grapevine inoculated with Np67, Np19 and NpAR (Fig. 4). The second gene involved in this pathway, encoding a polyphenoloxidase (*PPO*) was tested. *PPO* was only down-regulated in stems (AP and SP) of plants inoculated with Np 67 and Np 19, such as 7-fold for Np 67 (Fig. 4). Moreover, the laccase 17-like (*Lac17*) gene, encoding the enzyme that belongs to the group of PPO, was also generally repressed in leaves of AP and SP, infected by the 5 isolates of *Botryosphaeriaceae* tested. For plant metabolites monitored, targeted polyphenolic compounds were quantified, especially *trans*-piceid, *trans*-resveratrol, *trans*-ε-viniferin, *trans*-vitisin A and *trans*-vitisin B (leaves - Table 6; green stems – Table 7). Their distribution was different between leaves and green stems. A high level of *trans*-piceid was observed in leaves in comparison to green stems, where *trans*-vitisin B was well detected and quantified in green stems and only weakly accumulated in leaves (Tables 6 and 7). A similar pattern was found for *trans*-ε-viniferin, with a high content in green stems compared to leaves (Tables 6 and 7). This variation may be impacted by the developmental stage of green stems. Thus, the sampling of green stems was carried out when lignification began under greenhouse conditions. Moreover, phenolics were

significantly accumulated in symptomatic organs of plant inoculated with Ds strains. Both *trans*-resveratrol and *trans*-ε-viniferin were detected in symptomatic leaves of plants inoculated with Ds 99-7 (Table 6). A significant accumulation of *trans*-resveratrol and *trans*-vitisin B was also observed in symptomatic green stems of plants inoculated with Np AR (Table 7), which could correlate with an up-regulation of *STS* in the same samples.

Discussion

This study is the first to report the reproduction of foliar symptoms after artificial infection of grapevines with *Botryosphaeriaceae* species as described for *E. lata* and the reproduction of Eutypa dieback (Péros and Berger 1994, Camps et al. 2010). Pathogenicity trial with *Botryosphaeriaceae* species have already been conducted but solely lesions at the initial inoculation point were described and no foliar symptoms were developed (Taylor et al. 2005, Úrbez-Torres and Gubler 2009). In our study, lesion sizes and expression of foliar symptoms differed according to the fungal strains inoculated. Pathogenicity experiments showed that isolates Np 67 and Np AR produced longer lesions with greater surface area than isolates Ds 98-1, Ds 99-7 and Np 19. Furthermore, in plants inoculated with Np species the percentage of infected plants displaying foliar symptoms was more than 50% and could reach 73%.

According to Úrbez-Torres (2011) *Botryosphaeriaceae* species that infect grapevines can be divided into three different groups based on aggressiveness rankings (high, moderate or low) where *Neofusicoccum* spp. belong to the highly aggressive group while *Diplodia* spp. belong to the moderately aggressive group. In pathogenicity tests on grapevine green stems (*cv*. Mourvèdre) inoculated with Np or Ds, Spagnolo et al. (2014a) showed that mean lesion lengths differed significantly between Np and Ds isolates. Lesions associated with Np infection were 39.6 ± 9.1 mm long, while those associated with Ds infection were 14.3 ± 3.9 mm. Moreover, the largest lesions were recorded at the onset of flowering, whereas at separated clusters (G stage) and veraison, no

significant differences between the fungi could be detected. These results reveal that the development of lesions may be influenced by aggressiveness of the fungal strain as well as plant phenological stage (Spagnolo et al. 2014b).

Production of phytotoxic metabolites by *Botryosphaeriaceae* species has been reported by Martos et al. (2008), Andolfi et al. (2011) and Abou-Mansour et al. (2015). Ramirez-Suero et al. (2014) reported necrosis that appeared in calli of cv. Chardonnay sub-cultured on media containing extracellular metabolites produced by different isolates of Np and Ds. The metabolites produced by Np Bourgogne S-116 caused total necrosis of calli whereas metabolites from both isolates Ds 98.1 and Ds 99.7 induced only partial necrosis. These results are also in agreement with those obtained by Martos et al. (2008) who found a greater phytotoxic activity on grapevine leaves cv. Aragonez treated with culture filtrates from Np compared to Ds. These results confirm that Np isolates are more virulent than those of Ds. Moreover, studies from different countries reported differential susceptibility to wood necrosis caused by *Botryosphaeriaceae* fungi (Taylor et al. 2005; Amponsah et al. 2011; Guan et al 2015). Further work needs to be carried out to elucidate the mechanisms of the various susceptibility in cultivars in terms of plant immunity and phytotoxic activity of fungi.

The response of plants to infection and emergence of foliar symptoms were studied in terms of the genes involved in detoxification and stress tolerance. Thus, no modifications of *GST5*, *epoxHF* or *epoxH2* expression were detected under the conditions tested in this paper. In contrast, Spagnolo et al. (2012) showed that these genes were up-regulated in leaves and stems of grapevine affected by GTDs in the vineyard. It has been demonstrated that several toxins produced by GTD agents contain epoxides (Andolfi et al. 2011; Abou-Mansour et al. 2015) and detoxification enzymes have been hypothesized to have a role in the detoxification of these compounds or their active derived-compounds metabolized in grapevines (Spagnolo et al. 2014a; Abou-Mansour et al. 2015). A weak repression of *SOD* was observed in leaves of plants inoculated with Np 19 and Ds 98-1. A similar trend was reported in asymptomatic leaves and green stems of apoplectic plants and

those affected by esca proper (Letousey et al. 2010; Magnin-Robert et al. 2011; Spagnolo et al. 2012). These results suggest that important down-regulation of *SOD* occurs before symptoms appear on the plant. Repression of *SOD* could indicate a lack of oxidative stress control, which could be lethal for plants (Letousey et al. 2010). Similar to previous studies, where an increased abundance of HSPs was reported in green stems of both field-grown plants artificially infected with Np and Ds (Spagnolo et al. 2014b) and grapevine affected by esca proper and apoplexy (Spagnolo et al. 2012), an up-regulation of *HSP* was detected in green stems of plants inoculated with the 5 *Botryosphaeriaceae* isolates. HSPs function by binding partially denatured proteins to prevent irreversible protein inactivation and aggregation (Waters et al. 1995) and could be an indicator of a plant tolerant state.

To compare defence responses of plants inoculated by Np and Ds isolates, we targeted the most frequently observed and the best characterized active defence mechanisms in grapevine, namely the phenylpropanoid pathway. *STS* expression was induced in green stems of vines inoculated with the three Np isolates. These two fungi were previously described as inducing the largest lesion sizes (surface and length). In addition to *STS* up-regulation, only the symptomatic green stems of plants inoculated with Np AR showed significant accumulation of *trans*-resveratrol. Various studies have also described an up-regulation of *STS* in the leaves of field-grown grapevines affected by GTDs (Letousey et al. 2010; Magnin-Robert et al. 2011). Moreover, Ramirez-Suero et al. (2014) showed that extracellular compounds produced by Np and Ds induce the expression of *STS* in cv. Chardonnay calli. All these data suggest that the *STS* gene could be a good marker of stress responses, such as oxidant stress. In this sense, stilbenic polyphenols are also able to scavenge reactive oxygen species (ROS) and so protect the plant cells from oxidative stresses after pathogen attack (Bertsch et al. 2013). Another targeted gene, *PPO*, shown to be involved in plant resistance (Thipyapong et al. 2007) was down-regulated only in stems of plants inoculated with Np isolates, thus inducing larger cankers. Moreover, a repression of *Lac17* expression was observed in leaves of

plants inoculated with Np and Ds strains. These results suggest that GTD fungal agents induce strong perturbations of PPO. Regarding the absence of variation in stilbenoids and the repression of *PPO* expression in plants inoculated with Np strains, another group of phenolics may be impacted such as flavonoids or lignin precursors. Along the same lines, Lima et al. (2010) reported an accumulation of quercetin-3-O-glucoside and caffeic acid in healthy leaves of the asymptomatic cordons of esca-affected plants and a decrease in the same compounds in diseased leaves.

Regarding PR proteins expression, an up-regulation of *PR6* was observed in leaves in response to infection with *Botryosphaeriaceae*. These observations are in accordance with previous studies showing modulation of PR-protein (PR6, chitinase, β-1,3-glucanase) expression in leaves, green stems and wood of vines affected by GTDs (Valtaud et al. 2009; Camps et al. 2010; Letousey et al. 2010; Magnin-Robert et al. 2011; Spagnolo et al. 2012; Spagnolo et al. 2014b, Fontaine et al. 2015). Altogether, these data may indicate that *PR6* plays a defensive role during the response of grapevines to GTD fungal agents. The *fascAGP* genes belong to the large family of hydroxyproline-rich glycoproteins (HRGPs) proteins, which are thought to accumulate in response to elicitor molecules released by fungi and to play a role in plant defence responses (Agrios 2005). Our results showed an alteration of *fascAGP* expression, which suggests its possible role as a marker of stress responses triggered by GTD agents. Meanwhile, expression of *PIP2.2*, encoding a membrane water channel playing a role in controlling the water content of cells, was repressed by fungal infection only in leaves as previously reported in leaves of field-grown vines affected by apoplexy events (Letousey et al. 2010). It seems that GTDs may perceive a water stress signal only in the later steps of the disease and that the appearance of symptoms cannot be simply considered as a water transport-deficit-inducing disease but other physiological mechanisms may be involved (Christen et al. 2007).

To conclude, our study shows that one-year-old vines infected with *Botryosphaeriaceae* species, Ds and Np, induce lesions on the stem and expression of foliar symptoms. This is the first time that the reproduction of foliar symptoms with both species is reported, with a frequency reaching 77% and a value close to those observed in the vineyard. In addition, since the responses of plants artificially infected show similarities to those observed in plants naturally infected in the vineyard, this simple model system could be useful in future studies aimed at determining the relationship between fungi and the appearance of foliar symptoms, especially in a chronic form, and to test eco-friendly strategies to manage Botryosphaeria dieback. Among the gene expressions studied and the phenolics found, some could be selected as markers for the emergence of disease such as *SOD*, *STS* and *fascAGP* and flavonoids. Further work will be aimed at quantifying phytotoxic compounds reported from Ds and Np (Djoukeng et al. 2009; Evidente et al. 2010; Abou-Mansour et al. 2015) such as dihydroisocoumarin and epoxytoluquinol derivatives, on leaves and green stems to better appreciate the molecular dialogue between fungi and plants, since these fungi have never been detected in leaves.

Acknowledgments

This research was financed by the French government CASDAR V903 and V1301 (Compte d'Affectation Spéciale au Développement Agricole et Rural), by Champagne-Ardenne Region, by the national program France Agrimer and was also supported by the European COST Action FA1303 "Sustainable control of grapevine trunk diseases". The authors thank Philippe Larignon for giving *Diplodia seriata* strains; Mariana Mota (PhD) from the University of Lisboa for the support in processing the sequencing data; Phillips Alan, a native speaker and researcher in the University Nova of Lisboa, Portugal, and Buchala Antony, researcher in the University of Fribourg, Switzerland, for revising the English of this manuscript.

Literatured cited

Abou-Mansour, E., Débieux, J., Ramírez-Suero, M., Bénard-Gellon, M., Magnin-Robert, M., Spagnolo, A., Chong, J., Farine, S., Bertsch, C., L'Haridon, F., Serrano, M., Fontaine, F., Rego C., and Larignon, P. 2015. Phytotoxic metabolites from *Neofusicoccum parvum*, a pathogen of Botryosphaeria dieback of grapevine. Phytochemistry 115:207-215.

Agrios, G.N. 2005. Plant Pathology. Academic Press. San Diego, USA.

Amponsah, N.T., Jones, E.E., Ridgway, H.J., and Jaspers, M.V. 2011. Identification, potential inoculum sources and pathogenicity of botryosphaeriaceous species associated with grapevine dieback disease in New Zealand. Eur. J. Plant Pathol. 131:467–482.

Andolfi, L., Mugnai, L., Luque, J., Surico, G., Cimmino, A., and Evidente, A. 2011. Phytotoxins produced by fungi associated with grapevine trunk diseases. Toxins 3:1569–605.

Bézier, A., Lambert, B., and Baillieul, F. 2002. Study of defense-related gene expression in grapevine leaves and berries infected with *Botrytis cinerea.* Eur. J. Plant Pathol. 108**:**111-120.

Bertsch, C., Ramirez-Suero, M., Magnin-Robert, M., Larignon, P., Chong, J., Abou-Mansour, E., Spagnolo, A., Clément, C., Fontaine F. 2013. Trunk diseases of grapevine: complex and still poorly. Plant Pathol. 62:243-265.

Bruez, E., Lecomte, P., Grosman, J., Doublet, B., Bertsch, C., Fontaine, F., Da Costa, J. P., Ugaglia, A., Teissedre, P. L., Guerin-Dubrana, L., and Rey, P. 2013. Overview of grapevine trunk diseases in France in the early 2000s. Phytopathol. Mediterr. 52:262-275.

Camps, C., Kappel, C., Lecomte, P., Léon, C., Gomès, E., Coutos-Thévenot, P. and Delrot, S. 2010. A transcriptomic study of grapevine (*Vitis vinifera* cv. Cabernet-Sauvignon) interaction with the vascular ascomycete fungus *Eutypa lata*. J. Exp. Bot. 61:1719-1737.

Castillo-Pando, M., Sommers, A., Green, C. D., Priest, M., and Sriskanthades, M. 2001. Fungi associated with dieback of Semillon grapevines in the Hunter Valley of New South Wales. Australas. Plant Pathol. 30:59-63.

Chiarappa, L. 2000. Esca (black measles) of grapevine. An overview. Phytopathol. Mediterr. 39:11-15.

Christen, D., Schonmann, S., Jermini, M., Strasser, R. J., and Defago, G. 2007. Characterization and early detection of grapevine (*Vitis vinifera)* stress responses to esca disease *in situ* chlorophyll fluorescence and comparison with drought stress. Env. Exp. Bot. 60:504–514.

Cristinzio, G. 1978. Gravil attachi di *Botryosphaeria obtusa* su vite provincial di Insernia. Inf. Fitopatol. 6:21-23.

Crous, P. W., Slippers, M. J., Rheeder, J., Marasas, W. F. O., Phillips, A. J. L., Alves, A., Burgess, T., Barber, P., and Groenewald, J. Z. 2006. Phylogenetic lineages in the *Botryosphaeriaceae*. Stud. Mycol. 55:235-253.

Djoukeng, J. D., Polli, S., Larignon, P., and Abou-Mansour, E., 2009. Identification of phytotoxins from *Botryosphaeria obtusa*, a pathogen of black dead arm disease of grapevine. Eur. J. Plant Pathol. 124: 303-308.

Evidente, A., Punzo, B., Andolfi, A., Cimmino, A., Melck, D., and Luque, J. 2010. Lipophilic phytotoxins produced by *Neofusicoccum parvum*, a grapevine canker agent. Phytopathol. Mediterr. 49:74-79.

Fontaine, F., Pinto, C., Vallet, J., Clément, C., Gomes, A., and Spagnolo, A. 2015. The effects of Grapevine Trunk Diseases (GTDs) on vine physiology. Eur. J. Plant Pathol. DOI 10.1007/s10658-015-0770-0

Fischer, M. 2006. Biodiversity and geographic distribution of Basidiomycetes causing esca-associates white rot in grapevine: a worlwide perspective. Phytopathol. Mediterr. 45:S30–S42.

Gerbore, J., Benhamou, N., Vallance, J., Le Floch, G., Grizard, D., Regnault-Roger, C., and Rey, P. 2013. Biological control of plant pathogens: advantages and limitations seen through the case study of *Pythium oligandrum*. Environ. Sci.Pollut. Res. 21:4847-4860.

419 Graniti, A., Surico, G., and Mugnai, L. 2000. Esca of grapevine: a disease complex or a complex of
420 diseases? Phytopathol. Mediterr. 39:16–20.
421 Grosman, J., and Doublet, B. 2012. Maladies du bois de la vigne. Synthèse des dispositifs
422 d'observation au vignoble, de l'observatoire 2003–2008 au réseau d'epidémio-surveillance
423 actuel. Phytoma 651:31–35.
424 Guan, X., Essakhi, S., Laloue, H., Nick, P., Bertsch, C., and Chong, J. 2015. Mining new resources
425 for grape resistance against Botryosphaeriaceae: a focus on *Vitis vinifera* ssp. *sylvestris*. Plant
426 Pathol. Doi:10.1111/ppa.12405.
427 Larignon, P., and Dubos, B. 1997. Fungi associated with esca disease in grapevine. Eur. J. Plant
428 Pathol. 103:147–157.
429 Larignon, P., Fulchic, R., Laurent, C., and Dubos, B. 2001. Observation of black dead arm in
430 French vineyards. Phytopathol. Mediterr. 40:S336-S342.
431 Larignon, P., Fontaine, F., Farine, S., Clément, C., and Bertsch, C. 2009. Esca et Black Dead Arm:
432 deux acteurs majeurs des maladies du bois chez la vigne. C. R. Biologies 332:765–783.
433 Larignon, P., Spagnolo, A., Bertsch, C., Fontaine, F. 2015. First report of young grapevine decline
434 caused by *Neofusicoccum parvum* in France. Plant disease, first look, 15 June 2015.
435 Lima, M. R. M., Felgueiras, M. L., Graça, G., Rodrigues, J. E. A., Barros, A., Gil, A. M., and Dias,
436 A. C. P. 2010. NMR metabolomics of esca-disease-affected *Vitis vinifera* cv. Alvarinho leaves.
437 J. Exp. Bot. 14:4033-4042.
438 Letousey, P., Baillieul, F., Perrot, G., Rabenoelina, F., Boulay, M., Valliant-Gaveau, N., Clément,
439 C., and Fontaine, F. 2010. Early events prior to visual symptoms in the apoplectic form of
440 grapevine esca disease. Phytopathology 100:424–431.
441 Magnin-Robert, M., Letousey, P., Spagnolo, A., Rabenoelina, F., Jacquens, L., Mercier, L.,
442 Clément, C., and Fontaine, F. 2011. Leaf stripe form of esca induces alteration of photosynthesis
443 and defence reactions in presymptomatic leaves. Funct. Plant Biol. 38:856-866.

444 Martos, S., Andolfi, A., Luque, J., Mugnai, L., Surico, G., and Evidente, A. 2008. Production of
445 phytotoxic metabolites by five species of Botryosphaeriaceae causing decline on grapevines,
446 with special interest in the species *Neofusicoccum luteum* and *N. parvum*. Eur. J. Plant Pathol.
447 121:451-461.
448 Moller, W. J., and Kasimatis, A. N. 1978. Dieback of grapevines caused by *Eutypa armeniacae*.
449 Plant Dis. Rep. 62:254–258.
450 Péros, J. P., and Berger, G. 1994. A rapid method to assess the aggressiveness of *Eutypa lata*
451 isolates and the susceptibility of grapevine cultivar to eutypa dieback. Agronomie 14:515–523.
452 Péros, J. P., and Berger, G. 1999. Diversity within natural progenies of the grapevine dieback
453 fungus eutypa dieback. Curr. Gen. 36:301–309.
454 Petzoldt, C. H., Moller, W. J., and Sall, M. A. 1981. Eutypa dieback of grapevines: seasonal
455 differences in infection and duration of susceptibility of pruning wounds. Phytopathology
456 71:540-543.
457 Phillips, A. J. L., Crous, P. W., and Alves, A. 2007. *Diplodia seriata*, the anamorph of
458 "*Botryosphaeria*" *obtusa*. Fungal Divers. 25:141-155.
459 Pierron, R. J. G., Pages, M., Couderc, C., Compant, S., Jacques, A. and Violleau, F. 2015. *In vitro*
460 and *in planta* fungicide properties of ozonated water against the esca-associated fungus
461 *Phaeoacremonium aleophilum*. Sci. Hortic. 189:184-191.
462 Ramírez-Suero, M., Bénard-Gellon, M., Chong, J., Laloue, H., Stempien, E., Abou-Mansour, E.,
463 Fontaine, F., Larignon, P., Mazket-Kieffer, F., Farine, S., and Bertsch, C. 2014. Extracellular
464 compounds produced by fungi associated with Botryosphaeria dieback induce differential
465 defense gene expression patterns and necrosis in *Vitis vinifera* cv. Chardonnay cells.
466 Protoplasma. 251:1417-1426.
467 Rego, C., Nascimento, T., Cabral, A., Silva, M. J., and Oliveira, H. 2009. Control of grapevine
468 wood fungi in commercial nurseries. Phytopathol. Mediterr. 48:128-135.

469 Rovesti, L., and Montermini, A. 1987. Un deprimento della vitte causato de *Sphaeropsis malorum*
470 in provincia di Reggio Emilia. Inf. Fitopatol. 1:59–61.
471 Savocchia, S., Steel, C. C., Stodart, B. J., and Somers, A. 2007. Pathogenicity of *Botryosphaeria*
472 species from declining grapevines in sub tropical of Eastern Australia. Vitis 46:27-32.
473 Sosnowski, M. R., Lardner, R., Wicks, T. J., and Scott, E.S. 2007. The influence of grapevine
474 cultivar and isolate of *Eutypa lata* on wood and foliar symptoms. Plant Dis. 91:924–931.
475 Spagnolo A., Magnin-Robert, M., Alayi, T. D., Cilindre, C., Mercier, L., Schaeffer-Reiss, C., Van
476 Dorsselaer, A., Clément, C., and Fontaine F. 2012. Physiological changes in green stems of *Vitis*
477 *vinifera* L. cv. Chardonnay in response to esca proper and apoplexy revealed by proteomic and
478 transcriptomic analyses. J. Proteome Res. 11:461–475.
479 Spagnolo, A., Larignon, P., Magnin-Robert, M., Hovasse, A., Cilindre, C., Van Dorsselaer, A.,
480 Clément, C., Schaffer-Reiss, C. and Fontaine, F. 2014a. Flowering as the most highly sensitive
481 period of grapevine (*Vitis vinifera* cv. Mourvèdre) to the botryosphaeria dieback agents of
482 *Neofusicoccum parvum* and *Diplodia seriata* infection. Int. J. Mol. Sci. 15:9644-9669.
483 Spagnolo, A., Magnin-Robert, M., Alayi,T. D., Cilindre, C., Schaeffer-Reiss, C., Van Dorsselaer,
484 A., Clément, C., Larignon, P., Ramirez-Suero, M., Chong, J., Bertsch, C., Abou-Mansour, E.,
485 and Fontaine, F. 2014b. Differential responses of three grapevine cultivars to Botryosphaeria
486 dieback. Phytopathology 104:1021–1035.
487 Taylor, A., Hardy, G. E. St J., Wood, P., and Burgess, T. 2005. Identification and pathogenicity of
488 Botryosphaeria species associated with grapevine decline in Western Australia. Australasian
489 Plant Pathology 34: 187–95.
490 Thipyapong, P., Stout, M. J., and Attajarusit, J. 2007. Functional analysis of polyphenol oxidases by
491 antisense/sense technology. Molecules 12:1569-1595.
492 Úrbez-Torres, J. R. and Gubler, W. D. 2019. Pathogenicity of Botryosphaeria species isolated from
493 grapevine cankers in California. Plant Dis. 93:584-92.

494 Úrbez-Torres, J. R., Leavitt, G. M., Guerrero, J. C., Guevara, J., and Gubler, W. D. 2008.
495 Identification and pathogenicity of *Lasiodiplodia theobromae* and *Diplodia seriata*, the causal
496 agents of bot canker disease of grapevines in Mexico. Plant Dis. 92:519-529.
497 Úrbez-Torres, J. R. 2011. The status of Botryophaeriaceae species infecting grapevines.
498 Phytopathol. Mediterr. 50:S5–S45.
499 Valtaud, C., Larignon, P., Roblin, G., and Fleurat-Lessard, P. 2009. Development and
500 ultrastructural features of *Phaeomoniella chlamydospora* and *Phaeoacremonium aleophilum* in
501 relation to xylem degradation in esca disease of the grapevine. J. Plant Pathol. 91:37-51.
502 Waters, E. R. 1995. The molecular evolution of the small heat-shock proteins in plants. Genetics
503 141:785-795.
504

505 Table 1. Isolates of *Neofusicoccum parvum*, *Diplodia seriata, Phyllosticta ampelicida, Penicillium*
506 sp. and *Cladosporium* sp. used for inoculation.
507

Strain	Species	Aggressiveness	Origin
Np 19	*Neofusicoccum parvum*	High	Portugal
Np 67	*Neofusicoccum parvum*	Low	Portugal
Np AR	*Neofusicoccum parvum*	nd	France
Ds 98-1	*Diplodia seriata*	High	France
Ds 99-7	*Diplodia seriata*	Low	France
Gb 17	*Phyllosticta ampelicida*	Medium	Portugal
Gb 32	*Phyllosticta ampelicida*	High	Portugal
-	*Penicillium* sp.	Not pathogenic	France
-	*Cladosporium* sp.	Not pathogenic	France

508 [nd] Not determined.
509

510 Table 2. Primers of genes analyzed by real-time reverse-transcription polymerase chain reaction.
511
512

Genes	Primer sequences	Genbank or TC TIGR accession number	Matrix
EF1-α (elongation factor 1-α, housekeeping gene)	5'-GAACTGGGTGCTTGATAGGC-3' 5'-AACCAAAATATCCGGAGTAAAAGA-3'	GU585871	leaf green stem
39SRP (39S ribosomal protein L41-A, housekeepoing gene*)*	5'- GACTGACTTCAAGCTTAAACC-3' 5'-GATATAACAGGGAATACAGCAC-3'	XM_002285709	leaf
60SRP (60S ribosomal protein L18, housekeeping gene*)*	5'- ATCTACCTCAAGCTCCTAGTC-3' 5'-CAATCTTGTCCTCCTTTCCT-3'	XM_002270599	green stem
Hahl (haloacid dehalogenase hydrolase)	5'-CCCTCAGGATAGCCAACATCA-3' 5'-AGGTGCCAACCAGAACTGTGT-3'	XM_002267523	leaf green stem
epoxH2 (epoxide hydrolase 2)	5'-TCTGGATTCCGAACTGCATTG-3' 5'-ACCCATGATTAGCAGCATTGG-3'	XM_002270484	green stem
epoxHF (epoxide hydrolase)	5'-TGCTCGTCTTGGCACTGAGA-3' 5'-TGAGCGCACCACTGTACCAT-3'	XM_003632333	leaf
GST5 (glutathione-S-transferase 5)	5'-GCAGAAGCTGCCAGTGAAATT-3' 5'-GGCAAGCCATGAAAGTGACA-3'	XM_002277883	leaf green stem
SOD (superoxide dismutase)	5'- GTGGACCTAATGCAGTGATTGGA-3' 5'- TGCCAGTGGTAAGGCTAAGTTCA-3'	AF056622	leaf green stem
HSP (alpha crystalline heat shock protein)	5'-TCGGTGGAGGATGACTTGCT-3' 5'-CGTGTGCTGTACGAGCTGAAG-3'	XM_002272382	leaf green stem
DXS1 (1-deoxy-d-xylulose-5-phosphate)	5'-GCAGAAGCTGCCAGTGAAATT-3' 5'-GGCAAGCCATGAAAGTGACA-3'	XM_002277883	leaf
PR6 (serine proteinase inhibitor)	5'- AGGGAACAATCGTTACCCAAG-3' 5'- CCGATGGTAGGGACACTGAT-3'	AY156047	leaf
Gluc (glucanase)	5'- TCAATGGCTGCAATGGTGC-3' 5'- CGGTCGATGTTGCGAGATTTA	AF 239617	leaf green stem
fascAGP (fasciclin-like arabinogalactan protein)	5'- CGAAACCCCAAAGCCTAAGAA-3' 5'- GAAAACACAAAGGGGTTGCA-3'	XM_002280793	green stem
PIP2.2 (aquaporin plasma membrane intrinsic protein 2-2)	5'-GGTTCAGTCTCCATTGCACATG-3' 5'-TTGGCAGCACAGCAGATGTAT-3'	XM_002271336	leaf green stem
Lac17 (laccase like 17)	5'-GGACCCAATGGGACAAAGTTT-3' 5'-CCATTTGATTGCCCAGAGAAG-3'	XM_002284437	leaf
STS (stilbene synthase)	5'- AGGAAGCAGCATTGAAGGCTC-3' 5'- TGCACCAGGCATTTCTACACC-3'	X76892	green stem
PPO (polyphenol oxidase)	5'- TGGTCTTGCTGATAAGCCTAGTGA-3' 5'- TCCACATCCGATCGACATTG-3'	XM_002727606	green stem

513

Table 3. Dimensions of lesions produced by isolates of *Neofusicoccum parvum* (Np) *and Diplodia seriata* (Ds) in green stems, three months after inoculation: width (mm), length (mm), area (mm^2).

Isolates	Width (mm) ± SE[X]				Length (mm) ± SE[X]				Area (mm^2) ± SE[X]			
	2011	2012	2013	2014	2011	2012	2013	2014	2011	2012	2013	2014
Control	4.2 ± 0.5 a	5.6 ± 0.8 a	5.7 ± 0.7 a	5.5 ± 0.7 a	8.7 ± 0.5 a	5.7 ± 1.0 a	5.8 ± 0.9 a	5.4 ± 0.8 a	28.8 ± 4.2 a	25.0 ± 4.6 a	25.7 ± 5.0 a	23.4 ± 4.9 a
Ds 98-1	7.1 ± 1.0 ab	9.1 ± 1.5 ab	10.0 ± 1.6 ab	9.7 ± 1.6 b	12.8 ± 2.3a	17.5 ± 5.4 b	18.3 ± 3.3 b	19.1 ± 4.9 b	71.5±17.6 a	126.3 ± 50.3 ab	143.9 ± 34.5 b	145.9 ± 48.3 b
Ds 99-7	nd	12.8 ±1.1 ab	12.7 ± 1.0 b	13.1 ± 1.1 cd	nd	20.4 ± 2.9 bc	19.5 ± 2.2 b	20.1 ± 2.6 b	nd	205.9 ± 34.6 ab	194.3 ± 24.6 b	206.5 ± 31.2 c
Np AR	9.9 ± 1.1 ab	13.8 ± 1.7 ab	13.9 ± 1.6 bc	12.7 ± 1.7 c	41.3 ± 1.9 c	37.8 ± 2.3 d	38.2 ± 2.6 d	39.7 ± 3.1 d	320.8 ± 42.4 b	410.6 ± 54.3 c	416.4 ± 59.9 d	394.9 ± 68.1 e
Np 19	12.8 ± 1.5 b	14.7 ± 1.7 b	15.1 ± 1.3 c	14.1 ± 1.8 d	20.4 ± 3.5 b	24.6 ± 3.2 c	25.3 ± 2.0 c	24.9 ± 2.6 c	203.9 ± 41.3 ab	282.7 ± 48.4 bc	297.4 ± 34.2 c	274.1 ± 40.0 d
Np 67	28.2 ± 3.6 c	24.6 ± 2.7 c	24.2 ± 2.5 d	23.4 ± 2.6 e	81.3 ± 5.9 d	78.8 ± 3.1 e	79.5 ± 3.2 e	83.4 ± 3.8 e	1793.9 ± 240.5 c	1523.1 ± 165.2 d	1508.3 ±160.1 e	1531.7 ± 168.3 f

[X] Data are means (n=30) and columns with the same letter are not significantly different according to Tukey's test ($P<0.05$). All log values are back transformed to the original scale (millimetres).
[nd] Not determined.

Table 4. Foliar symptoms produced in grapevines of cv. Aragonez (= Tempranillo) by isolates of *Neofusicoccum parvum* (Np) *and Diplodia seriata* (Ds) eight months after inoculation.

	Grapevines with foliar symptoms (%)[x]			
Isolates	2011	2012	2013	2014
Control	0.0 a	0.0 a	0.0 a	0.0 a
Ds 99-7	nd	33.3 b	36.7 b	30.0 ab
Ds 98-1	36.7 b	50.0 bc	53.3 bc	50.0 bc
Np AR	6.7 a	60.0 bc	56.7 bc	60.0 bc
Np 19	63.3 bc	66.7 c	76.7 c	73.3 c
Np 67	76.7 c	73.3 c	76.7 c	73.3 c

[x] Data are percentages (n=30) and columns with the same letter are not significantly different according to Tukey's test ($P<0.05$). All values are back transformed to percentages.
[nd] Not determined.

Table 5. Dimensions of lesions produced by isolates of *Phyllosctita ampelicida* (Gb), *Cladosporium* and *Penicillium* three months after inoculation in green stems: width (mm), length (mm), area (mm^2).

Isolates	Width ± SE[X] (mm)	Length ± SE[X] (mm)	Area ± SE[X] (mm^2)
	2014	2014	2014
Control	5.5 ± 0.7 a	5.4 ± 0.8 a	23.4 ± 4.9
Cladosporium	5.1 ± 0.9 a	5.2 ± 0.8 a	20.9 ± 5.1 a
Penicillium	5.5 ± 0.8 a	5.4 ± 1.0 a	23.3 ± 5.3 a
Phyllosctita ampelicida (Gb17)	11.0 ± 2.2 b	16.0 ± 2.3 b	137.4 ± 33.0 b
Phyllosctita ampelicida (Gb32)	11.0 ± 1.7 b	28.0 ± 2.7 c	243.6 ± 48.2 c

[X] Data are means (n=30) and columns with the same letter are not significantly different according to Tukey's test($P<0.05$).
All log values are back transformed to the original scale (millimetres).

Table 6. Stilbenic compound contents in leaves of control plants, in asymptomatic (AP) and symptomatic (SP) leaves of plants inoculated with three *N. parvum* (Np 19, Np 67 and Np AR) and with two *D. seriata* (Ds 99-7 and Ds 98-1) isolates.

Stilbenes (µg g^{-1} FW)	Control	Np 19		Np 67		Np AR		Ds 99-7		Ds 98-1	
		AP	SP	AP	SP	AP	SP	AP	SP	AP	SP
trans-piceid	202.0 ± 46.0	200.0 ± 5.0	253.0 ± 53.0	233.0 ± 51.0	240.0 ± 41.0	205.0 ± 19.0	265.0 ± 24.0	347.0 ± 68.0	291.0 ± 57.0	228.0 ± 75.0	201.0 ± 45.0
trans-resveratrol	3.0 ± 0.0	12.0 ± 5.0	12.0 ± 6.0	3.0 ± 2.0	11.0 ± 2.0	13.0 ± 11.0	7.0 ± 2.0	9.0 ± 2.0	30.0 ± 17.0*	4.0 ± 1.0	11.0 ± 4.0
trans-ε-viniferin	7.0 ± 1.0	7.0 ± 4.0	18.0 ± 9.0	16.0 ± 3.0	15.0 ± 1.0	7.0 ± 1.0	17.0 ± 12.0	29.0 ± 17.0	33.0 ± 11.0*	11.0 ± 2.0	17.0 ± 5.0
trans-vitisin A	5.0 ± 1.0	nd	6.0 ± 3.0	5.0 ± 2.0	7.0 ± 0.0	3.0 ± 1.0	6.0 ± 2.0	5.0 ± 1.0	6.0 ± 3.0	1.0 ± 1.0	6.0 ± 4.0
trans-vitisin B	5.0 ± 1.0	nd	nd	nd	3.0 ± 3.0	nd	nd	2.0 ± 2.0	nd	1.0 ± 1.0	nd

nd, indicates compounds not detected. Values followed by an asterisk are significantly different to the control value (Dunn's Multiple Comparison Test, $P \leq 0.05$).

Table 7: Stilbenic compound contents in green stems of control plant, in asymptomatic (AP) and symptomatic (SP) green stems of plants inoculated with three *N. parvum* (Np 19, Np 67 and Np AR) and with two *D. seriata* (Ds 99-7 and Ds 98-1) isolates.

Stilbenes (µg g^{-1} FW)	Control	Np 19		Np 67		Np AR		Ds 99-7		Ds 98-1	
		AP	SP	AP	SP	AP	SP	AP	SP	AP	SP
trans-piceid	nd	nd	11.0 ± 10.0	0.021 ± 0.003	nd	39.0 ± 6.0	nd	nd	nd	1.0 ± 1.0	26.0 ± 7.0
trans-resveratrol	nd	31.0 ± 12.0	30.0 ± 5.0	51.0 ± 2.0	22.0 ± 6.0	nd	87.0 ± 19.0*	nd	nd	28.0 ± 10.0	67.0 ± 18.0
trans-ε-viniferin	13.0 ± 2.0	840.0 ± 386.0	907.0 ± 159.0	1615.0 ± 265.0	863.0 ± 267.0	2847.0 ± 503.0	1387.0 ± 404.0	510.0 ± 36.0	146.0 ± 24.0	785.0 ± 251.0	1188.0 ± 341.0
trans-vitisin A	nd	nd	12.0 ± 11.0	166.0 ± 37.0	32.0 ± 9.0	195.0 ± 61.0	nd	nd	nd	34.0 ± 19.0	41.0 ± 13.0
trans-vitisin B	nd	120.0 ± 82.0	174.0 ± 58.0	258 ± 35.0	150.0 ± 31.0	919.0 ± 14.0	263.0 ± 6.0*	78.0 ± 21.0	nd	115.0 ± 65.0	383.0 ± 112.0

nd, indicates compounds not detected. Values followed by an asterisk are significantly different to the control value (Dunn's Multiple Comparison Test, $P \leq 0.05$)

Caption for figures

Figure 1. Lesions observed in the green stems of grafted cuttings cv. Aragonez inoculated with control (**A**), three *N. parvum* (Np 19 (**B**), Np 67 (**C**) and Np AR (**D**)) and two *D. seriata* (Ds 98-1 (**E**) and Ds 99-7 (**F**)) isolates.

Figure 2. Foliar symptoms in leaves of grafted cuttings cv. Aragonez (= Tempranillo) inoculated in green stems of control (**A**) and with three *N. parvum* (Np19 (**B**), Np67 (**C**) and Np AR (**D**)), two *D. seriata* (Ds98-1 (**E**), Ds99-7 (**F**)) isolates.

Figure 3. Relative expression of 10 selected genes in the leaves of asymptomatic (AP) and symptomatic plants (SP) inoculated with three *N. parvum* (Np 67, Np 19 and Np AR) and with two *D. seriata* (Ds 98-1 and Ds 99-7) isolates. The colour scale represents the ratio values corresponding to the mean of two or three independent experiments. Genes over-expressed appear in shades of red, with an expression level higher than 30 in bright red, while those repressed appear in shades of blue, with an intensity lower than 0.1 in dark blue (white: no change in gene expression compared to the control). Gene changes in relative expression were > 2x or <0.5x, respectively.

Figure 4. Relative expression of 10 selected genes in the green stems of asymptomatic (AP) and symptomatic plants (SP) inoculated with three *N. parvum* (Np 67, Np 19 and Np AR) and with two *D. seriata* (Ds 98-1 and Ds 99-7) isolates. The colour scale represents the ratio values corresponding to the mean of two or three independent experiments. Genes over-expressed appear in shades of red, with an expression level higher than 30 in bright red, while those repressed appear in shades of blue, with an intensity lower than 0.1 in dark blue (white: no change in gene expression compared to the control). Gene changes in relative expression were > 2x or <0.5x, respectively.

Supplemental Material

Supplemental Fig. 1 shows structures of stilbenic compounds studied.

Figure 1.

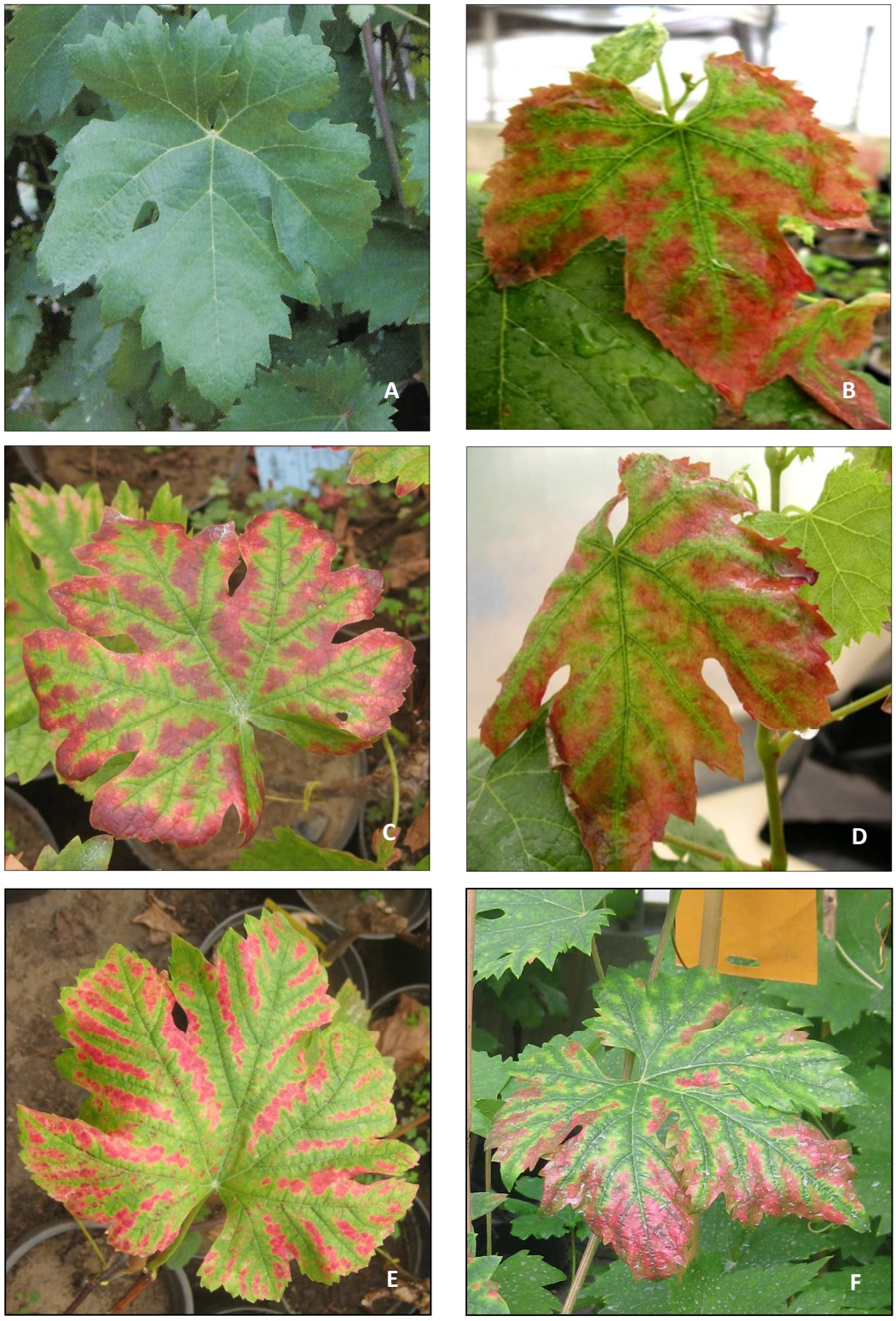


Figure 2.

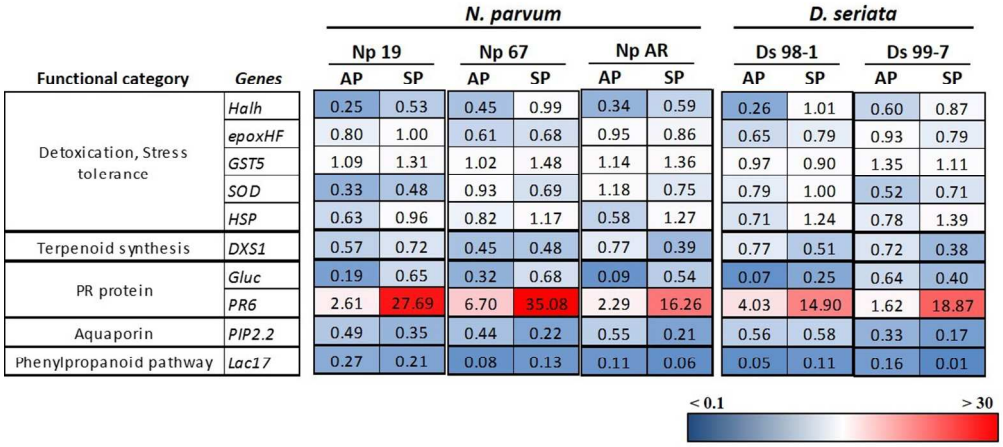
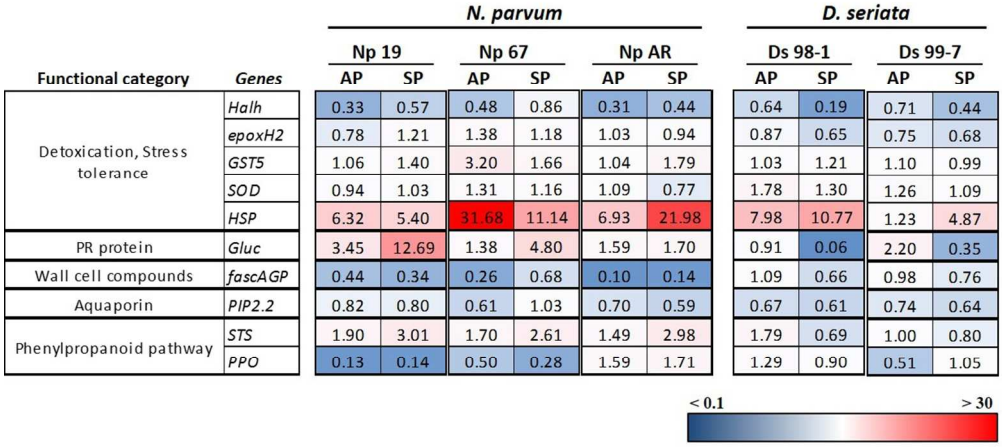


Figure 3.

Fig 3
208x114mm (150 x 150 DPI)



Functional category	Genes	*N. parvum* Np 19 AP	Np 19 SP	Np 67 AP	Np 67 SP	Np AR AP	Np AR SP	*D. seriata* Ds 98-1 AP	Ds 98-1 SP	Ds 99-7 AP	Ds 99-7 SP
Detoxication, Stress tolerance	*Halh*	0.33	0.57	0.48	0.86	0.31	0.44	0.64	0.19	0.71	0.44
	epoxH2	0.78	1.21	1.38	1.18	1.03	0.94	0.87	0.65	0.75	0.68
	GST5	1.06	1.40	3.20	1.66	1.04	1.79	1.03	1.21	1.10	0.99
	SOD	0.94	1.03	1.31	1.16	1.09	0.77	1.78	1.30	1.26	1.09
	HSP	6.32	5.40	31.68	11.14	6.93	21.98	7.98	10.77	1.23	4.87
PR protein	*Gluc*	3.45	12.69	1.38	4.80	1.59	1.70	0.91	0.06	2.20	0.35
Wall cell compounds	*fascAGP*	0.44	0.34	0.26	0.68	0.10	0.14	1.09	0.66	0.98	0.76
Aquaporin	*PIP2.2*	0.82	0.80	0.61	1.03	0.70	0.59	0.67	0.61	0.74	0.64
Phenylpropanoid pathway	*STS*	1.90	3.01	1.70	2.61	1.49	2.98	1.79	0.69	1.00	0.80
	PPO	0.13	0.14	0.50	0.28	1.59	1.71	1.29	0.90	0.51	1.05

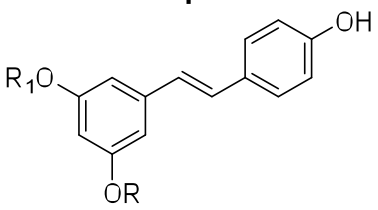
< 0.1 > 30

Figure 4.

Fig 4
208x114mm (150 x 150 DPI)

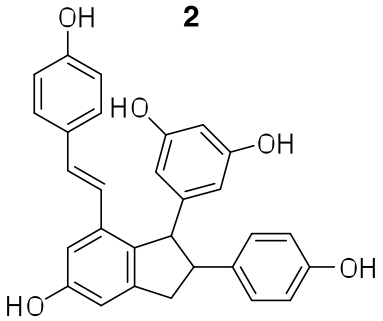
Supplemental Fig. 1: Structures of followed stilbenic compounds: **1**: *tr*-piceid and *tr*-resveratrol, **2**: *tr*-ε-viniferin, **3**: *tr*-vitisin A, **4**: *tr*-vitisin B.

1



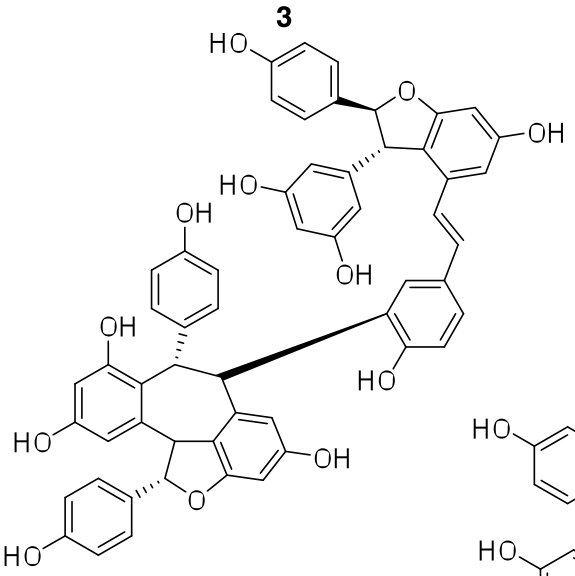
	R1	*R2*
tr-resveratrol	H	H
tr-pterostilben	CH3	CH3
tr-piceid	Glu	H

2



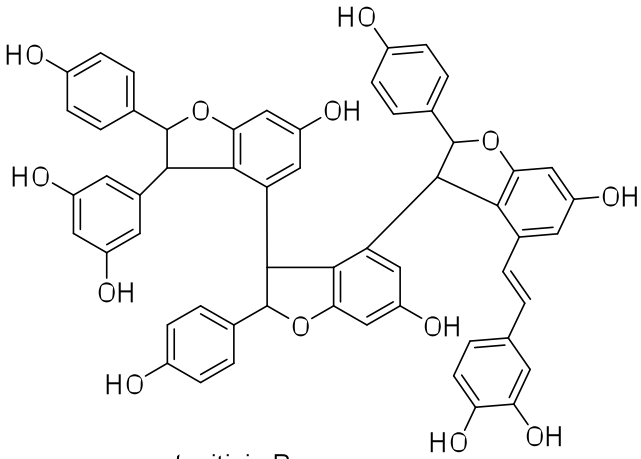
tr-ε-viniferin

3



tr-vitisin A

4



tr-vitisin B