

Plant Disease "First Look" paper • <http://dx.doi.org/10.1094/PDIS-10-15-1194-RE> • posted 02/10/2016
This paper has been peer reviewed and accepted for publication but has not yet been copyedited or proofread. The final published version may differ.

1 **Reproducing Botryosphaeria dieback foliar symptoms in a simple model system**

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Abstract

26

Botryosphaeria dieback is a grapevine trunk disease with a worldwide distribution and associated with *Diplodia seriata* and *Neofusicoccum parvum* among several other *Botryosphaeriaceae* species.

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The aforementioned xylem-inhabiting fungi cause wood lesions, leaf and berry symptoms and

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eventually lead to the death of the plant. The aim of this work was to develop a simple model

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system to reproduce the foliar symptoms caused by *D. seriata* and *N. parvum* to better characterize

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fungus pathogenicity and determine the mechanisms involved in symptom development. Green

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stems of grafted grapevine cuttings cv. Aragonéz were inoculated with three isolates of *N. parvum*

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and two isolates of *D. seriata* with different degrees of virulence and the experiment was repeated

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four times from 2011 to 2014. Three months after inoculation, the lesions associated with *N.*

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parvum were larger than those associated with *D. seriata*. Similarly, eight months after inoculation

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the percentage of plants showing foliar symptoms was greater in the *N. parvum* treatments than in

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the *D. seriata* treatments. During the emergence of foliar symptoms, plant stress-related responses

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were modulated in green stems and leaves, especially a down-regulation of superoxide dismutase

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(*SOD*) and fasciclin-like arabinogalactan protein (*fascAGP*) and an up-regulation of stilbene

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synthase (*STS*) with an accumulation of phenolics. In conclusion, the simple model system

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developed allowed a good characterization of isolate pathogenicity and correlation with foliar

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symptoms of *Botryosphaeria dieback*, namely spots on leaf margin and blade.

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

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

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

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

93

94 **Materials and Methods**

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

114 **Determination of lesion size, symptoms appearance and isolation of pathogens.**
115 The dimension of lesions was evaluated three months after inoculation on green shoots, before
116 lignification occurred, by measuring the width and the length and therefore calculating the elliptical
117 area of the lesion. All statistical analyses were performed using STATISTICA (StatSoft, Inc. 2007,
118 version 8.0). Homogeneity of variance was tested using Levene's test. Residuals were visually
119 inspected for each experiment, and when necessary the log₁₀ 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 (χ^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 μ L 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

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

160 **Extraction and quantification of phenolic compounds.** The protocol used is described in
161 Spagnolo et al. (2014b). Briefly, methanolic extracts were prepared from 50 mg of powdered leaf
162 and green stem tissues mixed with 1 mL of methanol (MeOH) and 25 μ L of the internal standard
163 *trans*-4-hydroxystilbene (0.5 mg mL⁻¹). For quantitative analysis of stilbenes, 60 μ L of methanolic
164 extract was analysed by high-performance liquid chromatography. Standards such as *trans*-piceid,
165 *trans*-resveratrol and *trans*-pterostilbene (Supplemental Fig. 1) were purchased from Extrasynthèse
166 (Genay - France). The *trans*- ϵ -viniferin, *trans*-vitisin A and *trans*-vitisin B (Supplemental Fig. 1)
167 were obtained from lignified canes of cv. Syrah as described in Spagnolo et al. (2014). Spectral data
168 for all peaks were accumulated in the range between 220 and 600 nm. The data are reported as μ g g⁻¹
169 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

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

198

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

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

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

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

230 For genes involved in the phenylpropanoid pathway, the stilbene synthase (*STS*) gene was
231 weakly up-regulated in green stems of grapevine inoculated with Np67, Np19 and NpAR (Fig. 4).
232 The second gene involved in this pathway, encoding a polyphenoloxidase (*PPO*) was tested. *PPO*
233 was only down-regulated in stems (AP and SP) of plants inoculated with Np 67 and Np 19, such as
234 7-fold for Np 67 (Fig. 4). Moreover, the laccase 17-like (*Lac17*) gene, encoding the enzyme that
235 belongs to the group of PPO, was also generally repressed in leaves of AP and SP, infected by the 5
236 isolates of *Botryosphaeriaceae* tested. For plant metabolites monitored, targeted polyphenolic
237 compounds were quantified, especially *trans*-piceid, *trans*-resveratrol, *trans*- ϵ -viniferin, *trans*-
238 vitisin A and *trans*-vitisin B (leaves - Table 6; green stems – Table 7). Their distribution was
239 different between leaves and green stems. A high level of *trans*-piceid was observed in leaves in
240 comparison to green stems, where *trans*-vitisin B was well detected and quantified in green stems
241 and only weakly accumulated in leaves (Tables 6 and 7). A similar pattern was found for *trans*- ϵ -
242 viniferin, with a high content in green stems compared to leaves (Tables 6 and 7). This variation
243 may be impacted by the developmental stage of green stems. Thus, the sampling of green stems was
244 carried out when lignification began under greenhouse conditions. Moreover, phenolics were

245 significantly accumulated in symptomatic organs of plant inoculated with Ds strains. Both *trans*-
246 resveratrol and *trans*- ϵ -viniferin were detected in symptomatic leaves of plants inoculated with Ds
247 99-7 (Table 6). A significant accumulation of *trans*-resveratrol and *trans*-vitisin B was also
248 observed in symptomatic green stems of plants inoculated with Np AR (Table 7), which could
249 correlate with an up-regulation of *STS* in the same samples.

250

251 Discussion

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

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

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

273 Production of phytotoxic metabolites by *Botryosphaeriaceae* species has been reported by
274 Martos et al. (2008), Andolfi et al. (2011) and Abou-Mansour et al. (2015). Ramirez-Suero et al.
275 (2014) reported necrosis that appeared in calli of cv. Chardonnay sub-cultured on media containing
276 extracellular metabolites produced by different isolates of Np and Ds. The metabolites produced by
277 Np Bourgogne S-116 caused total necrosis of calli whereas metabolites from both isolates Ds 98.1
278 and Ds 99.7 induced only partial necrosis. These results are also in agreement with those obtained
279 by Martos et al. (2008) who found a greater phytotoxic activity on grapevine leaves cv. Aragonéz
280 treated with culture filtrates from Np compared to Ds. These results confirm that Np isolates are
281 more virulent than those of Ds. Moreover, studies from different countries reported differential
282 susceptibility to wood necrosis caused by *Botryosphaeriaceae* fungi (Taylor et al. 2005; Amponsah
283 et al. 2011; Guan et al 2015). Further work needs to be carried out to elucidate the mechanisms of
284 the various susceptibility in cultivars in terms of plant immunity and phytotoxic activity of fungi.

285 The response of plants to infection and emergence of foliar symptoms were studied in terms
286 of the genes involved in detoxification and stress tolerance. Thus, no modifications of *GST5*,
287 *epoxHF* or *epoxH2* expression were detected under the conditions tested in this paper. In contrast,
288 Spagnolo et al. (2012) showed that these genes were up-regulated in leaves and stems of grapevine
289 affected by GTDs in the vineyard. It has been demonstrated that several toxins produced by GTD
290 agents contain epoxides (Andolfi et al. 2011; Abou-Mansour et al. 2015) and detoxification
291 enzymes have been hypothesized to have a role in the detoxification of these compounds or their
292 active derived-compounds metabolized in grapevines (Spagnolo et al. 2014a; Abou-Mansour et al.
293 2015). A weak repression of *SOD* was observed in leaves of plants inoculated with Np 19 and Ds
294 98-1. A similar trend was reported in asymptomatic leaves and green stems of apoplectic plants and

295 those affected by esca proper (Letousey et al. 2010; Magnin-Robert et al. 2011; Spagnolo et al.
296 2012). These results suggest that important down-regulation of *SOD* occurs before symptoms
297 appear on the plant. Repression of *SOD* could indicate a lack of oxidative stress control, which
298 could be lethal for plants (Letousey et al. 2010). Similar to previous studies, where an increased
299 abundance of HSPs was reported in green stems of both field-grown plants artificially infected with
300 Np and Ds (Spagnolo et al. 2014b) and grapevine affected by esca proper and apoplexy (Spagnolo
301 et al. 2012), an up-regulation of *HSP* was detected in green stems of plants inoculated with the 5
302 *Botryosphaeriaceae* isolates. HSPs function by binding partially denatured proteins to prevent
303 irreversible protein inactivation and aggregation (Waters et al. 1995) and could be an indicator of a
304 plant tolerant state.

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

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

326 Regarding PR proteins expression, an up-regulation of *PR6* was observed in leaves in
327 response to infection with *Botryosphaeriaceae*. These observations are in accordance with previous
328 studies showing modulation of PR-protein (*PR6*, chitinase, β -1,3-glucanase) expression in leaves,
329 green stems and wood of vines affected by GTDs (Valtaud et al. 2009; Camps et al. 2010; Letousey
330 et al. 2010; Magnin-Robert et al. 2011; Spagnolo et al. 2012; Spagnolo et al. 2014b, Fontaine et al.
331 2015). Altogether, these data may indicate that *PR6* plays a defensive role during the response of
332 grapevines to GTD fungal agents. The *fascAGP* genes belong to the large family of hydroxyproline-
333 rich glycoproteins (HRGPs) proteins, which are thought to accumulate in response to elicitor
334 molecules released by fungi and to play a role in plant defence responses (Agrios 2005). Our results
335 showed an alteration of *fascAGP* expression, which suggests its possible role as a marker of stress
336 responses triggered by GTD agents. Meanwhile, expression of *PIP2.2*, encoding a membrane water
337 channel playing a role in controlling the water content of cells, was repressed by fungal infection
338 only in leaves as previously reported in leaves of field-grown vines affected by apoplexy events
339 (Letousey et al. 2010). It seems that GTDs may perceive a water stress signal only in the later steps
340 of the disease and that the appearance of symptoms cannot be simply considered as a water
341 transport-deficit-inducing disease but other physiological mechanisms may be involved (Christen et
342 al. 2007).

343

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

358

359 **Acknowledgments**

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

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- 504

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

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

508 nd Not determined.
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Table 2. Primers of genes analyzed by real-time reverse-transcription polymerase chain reaction.

Genes	Primer sequences	Genbank or TC TIGR accession number	Matrix
<i>EF1-α</i> (elongation factor 1- α , housekeeping gene)	5'-GAACTGGGTGCTTGATAGGC-3' 5'-AACCAAAATATCCGGAGTAAAAGA-3'	GU585871	leaf green stem
<i>39SRP</i> (39S ribosomal protein L41-A, housekeeping gene)	5'-GACTGACTTCAAGCTTAAACC-3' 5'-GATATAACAGGGAATACAGCAC-3'	XM_002285709	leaf
<i>60SRP</i> (60S ribosomal protein L18, housekeeping gene)	5'-ATCTACCTCAAGCTCCTAGTC-3' 5'-CAATCTTGCTCCTTTCCT-3'	XM_002270599	green stem
<i>Hah1</i> (haloacid dehalogenase hydrolase)	5'-CCCTCAGGATAGCCAACATCA-3' 5'-AGGTGCCAACCAGAACTGTGT-3'	XM_002267523	leaf green stem
<i>epoxH2</i> (epoxide hydrolase 2)	5'-TCTGGATTCCGAACTGCATTG-3' 5'-ACCCATGATTAGCAGCATTGG-3'	XM_002270484	green stem
<i>epoxHF</i> (epoxide hydrolase)	5'-TGCTCGTCTGGCACTGAGA-3' 5'-TGAGCGCACCCTGTACCAT-3'	XM_003632333	leaf
<i>GST5</i> (glutathione-S-transferase 5)	5'-GCAGAAGCTCGAAGTAAAATT-3' 5'-GGCAAGCCATGAAAGTGACA-3'	XM_002277883	leaf green stem
<i>SOD</i> (superoxide dismutase)	5'-GTGGACCTAATGCAGTGATTGGA-3' 5'-TGCCAGTGGTAAGGCTAAGTCA-3'	AF056622	leaf green stem
<i>HSP</i> (alpha crystalline heat shock protein)	5'-TCGGTGGAGGATGACTTGCT-3' 5'-CGTGTGCTGTACGAGCTGAAG-3'	XM_002272382	leaf green stem
<i>DXS1</i> (1-deoxy-d-xylulose-5-phosphate)	5'-GCAGAAGCTGCCAGTAAAATT-3' 5'-GGCAAGCCATGAAAGTGACA-3'	XM_002277883	leaf
<i>PR6</i> (serine proteinase inhibitor)	5'-AGGGAACAATCGTTACCCAAG-3' 5'-CCGATGGTAGGGACACTGAT-3'	AY156047	leaf
<i>Gluc</i> (glucanase)	5'-TCAATGGCTGCAATGGTGC-3' 5'-CGGTCGATGTTGCGAGATTA	AF 239617	leaf green stem
<i>fascAGP</i> (fasciclin-like arabinogalactan protein)	5'-CGAAACCCCAAAGCCTAAGAA-3' 5'-GAAAACACAAAGGGGTGCA-3'	XM_002280793	green stem
<i>PIP2.2</i> (aquaporin plasma membrane intrinsic protein 2-2)	5'-GGTTCAGTCTCCATTGCACATG-3' 5'-TTGGCAGCACAGCAGATGTAT-3'	XM_002271336	leaf green stem
<i>Lac17</i> (laccase like 17)	5'-GGACCCAATGGGACAAAGTTT-3' 5'-CCATTGATTGCCAGAGAAG-3'	XM_002284437	leaf
<i>STS</i> (stilbene synthase)	5'-AGGAAGCAGCATTGAAGGCTC-3' 5'-TGCACCAGGCATTCTACACC-3'	X76892	green stem
<i>PPO</i> (polyphenol oxidase)	5'-TGGTCTTGCTGATAAGCCTAGTGA-3' 5'-TCCACATCCGATCGACATTG-3'	XM_002272606	green stem

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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 (mm²).

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. Aragonéz (= Tempranillo) by isolates of *Neofusicoccum parvum* (Np) and *Diplodia seriata* (Ds) eight months after inoculation.

Isolates	Grapevines with foliar symptoms (%) ^x			
	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 *Phyllosticta ampelicida* (Gb), *Cladosporium* and *Penicillium* three months after inoculation in green stems: width (mm), length (mm), area (mm²).

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
<i>Cladosporium</i>	5.1 ± 0.9 a	5.2 ± 0.8 a	20.9 ± 5.1 a
<i>Penicillium</i>	5.5 ± 0.8 a	5.4 ± 1.0 a	23.3 ± 5.3 a
<i>Phyllosticta ampelicida</i> (Gb17)	11.0 ± 2.2 b	16.0 ± 2.3 b	137.4 ± 33.0 b
<i>Phyllosticta ampelicida</i> (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 ($\mu\text{g g}^{-1}$ FW)	Control	Np 19		Np 67		Np AR		Ds 99-7		Ds 98-1	
		AP	SP	AP	SP	AP	SP	AP	SP	AP	SP
<i>trans</i> -piceid	202.0 \pm 46.0	200.0 \pm 5.0	253.0 \pm 53.0	233.0 \pm 51.0	240.0 \pm 41.0	205.0 \pm 19.0	265.0 \pm 24.0	347.0 \pm 68.0	291.0 \pm 57.0	228.0 \pm 75.0	201.0 \pm 45.0
<i>trans</i> -resveratrol	3.0 \pm 0.0	12.0 \pm 5.0	12.0 \pm 6.0	3.0 \pm 2.0	11.0 \pm 2.0	13.0 \pm 11.0	7.0 \pm 2.0	9.0 \pm 2.0	30.0 \pm 17.0*	4.0 \pm 1.0	11.0 \pm 4.0
<i>trans</i> - <i>e</i> -viniferin	7.0 \pm 1.0	7.0 \pm 4.0	18.0 \pm 9.0	16.0 \pm 3.0	15.0 \pm 1.0	7.0 \pm 1.0	17.0 \pm 12.0	29.0 \pm 17.0	33.0 \pm 11.0*	11.0 \pm 2.0	17.0 \pm 5.0
<i>trans</i> -vitisin A	5.0 \pm 1.0	nd	6.0 \pm 3.0	5.0 \pm 2.0	7.0 \pm 0.0	3.0 \pm 1.0	6.0 \pm 2.0	5.0 \pm 1.0	6.0 \pm 3.0	1.0 \pm 1.0	6.0 \pm 4.0
<i>trans</i> -vitisin B	5.0 \pm 1.0	nd	nd	nd	3.0 \pm 3.0	nd	nd	2.0 \pm 2.0	nd	1.0 \pm 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 ($\mu\text{g g}^{-1}$ FW)	Control	Np 19		Np 67		Np AR		Ds 99-7		Ds 98-1	
		AP	SP	AP	SP	AP	SP	AP	SP	AP	SP
<i>trans</i> -piceid	nd	nd	11.0 \pm 10.0	0.021 \pm 0.003	nd	39.0 \pm 6.0	nd	nd	nd	1.0 \pm 1.0	26.0 \pm 7.0
<i>trans</i> -resveratrol	nd	31.0 \pm 12.0	30.0 \pm 5.0	51.0 \pm 2.0	22.0 \pm 6.0	nd	87.0 \pm 19.0*	nd	nd	28.0 \pm 10.0	67.0 \pm 18.0
<i>trans</i> - <i>e</i> -viniferin	13.0 \pm 2.0	840.0 \pm 386.0	907.0 \pm 159.0	1615.0 \pm 265.0	863.0 \pm 267.0	2847.0 \pm 503.0	1387.0 \pm 404.0	510.0 \pm 36.0	146.0 \pm 24.0	785.0 \pm 251.0	1188.0 \pm 341.0
<i>trans</i> -vitisin A	nd	nd	12.0 \pm 11.0	166.0 \pm 37.0	32.0 \pm 9.0	195.0 \pm 61.0	nd	nd	nd	34.0 \pm 19.0	41.0 \pm 13.0
<i>trans</i> -vitisin B	nd	120.0 \pm 82.0	174.0 \pm 58.0	258 \pm 35.0	150.0 \pm 31.0	919.0 \pm 14.0	263.0 \pm 6.0*	78.0 \pm 21.0	nd	115.0 \pm 65.0	383.0 \pm 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.

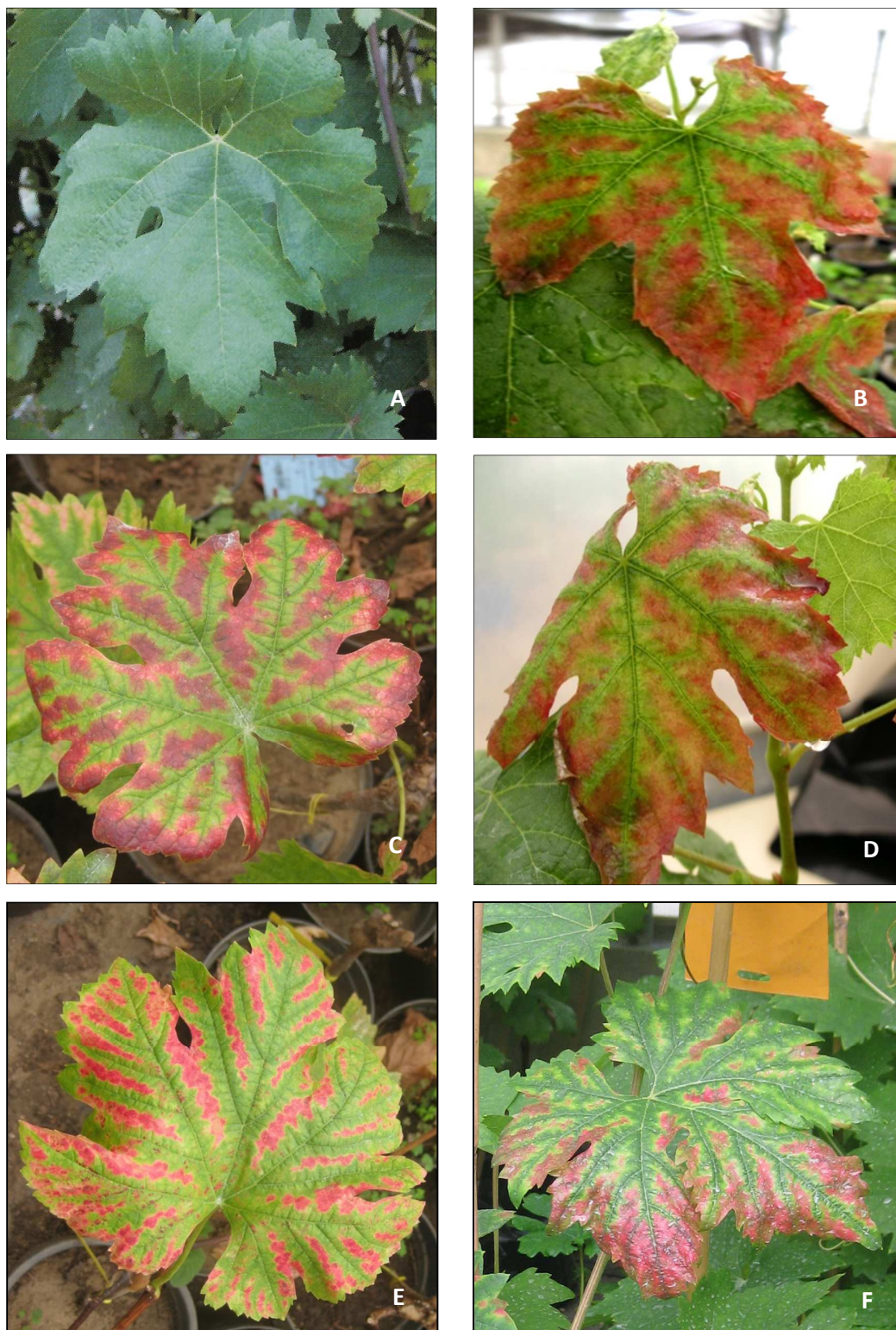


Figure 2.

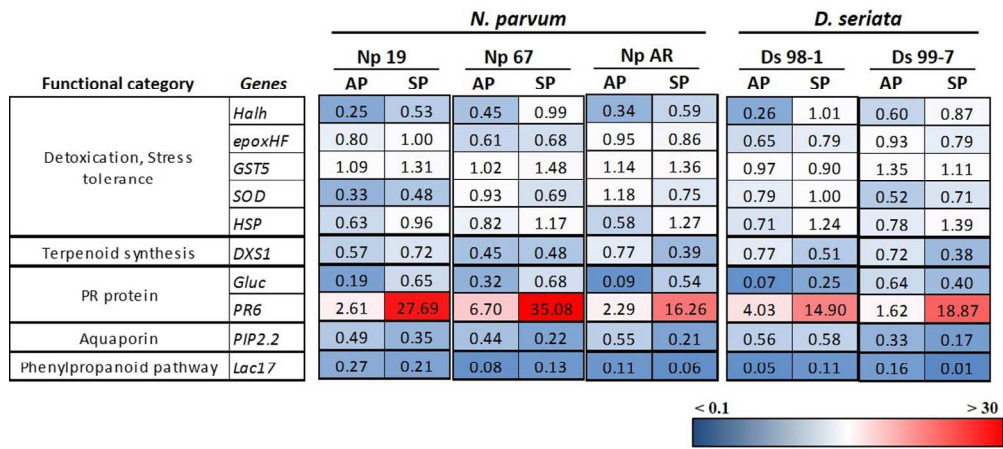


Figure 3.

Fig 3
208x114mm (150 x 150 DPI)

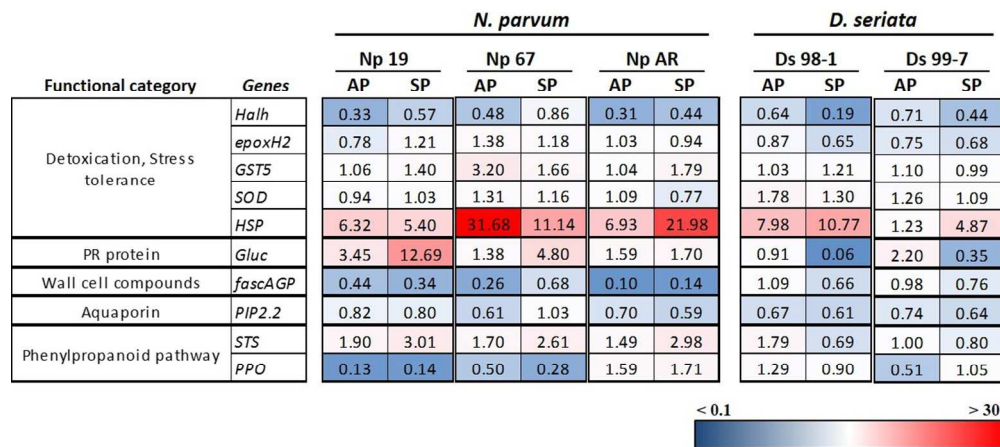


Figure 4.

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.