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RESEARCH PAPERS

Patterns of phytoalexins in the grapevine leaf stripe disease (esca complex)/grapevine pathosystem

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Summary. Vineyards containing vines affected with grapevine leaf stripe disease (GLSD), one of the diseases of the esca complex, suffer losses in grape yield and quality every growing season. To examine the relation between GLSD foliar symptoms and levels of phytoalexins in grapevine, phytoalexin levels were monitored in the leaves of symptomatic, asymptomatic/diseased, and healthy grapevine leaves, at various growth stages, in two vineyards in Italy, over four growing seasons. At the same time, the leaf symptoms of the vines at some of those growth stages were recorded in each vineyard and in each growing season. The compounds extracted and identified were: *trans*-resveratrol, *trans*-pterostilbene, trans-ε-viniferin and trans-δ-viniferin. The most common phytoalexin found was resveratrol. Amounts of all the phytoalexins were generally greater in symptomatic leaves than in asymptomatic/diseased or healthy leaves. In symptomatic leaves, resveratrol levels were greatest at pre-bunch closure, and peaks in pterostilbene occurred at the same time. Leaves of each category (symptomatic, asymptomatic/diseased, healthy) had lower amounts of these compounds at veraison and generally higher amounts at the stages of harvesting and/or the softening of berries. It seems therefore that the formation and pattern over time of the phytoalexins was linked to the growth stage of the vines. Leaf symptoms never occurred before pre-bunch closure, but became much more common from veraison to harvest. This study provides evidence of a relationship between the levels of phytoalexins, grapevine growth stage, and the seasonal pattern of development of GLSD symptoms.

Key words: phytoalexins, GLSD, leaf symptoms.

Introduction

Grapevine leaf stripe disease (GLSD) is one of the grapevine wood diseases that form part of the esca disease complex. GLSD is widespread in European vine-growing areas and causes losses in grape yield and quality (Calzarano *et al.*, 2001, 2004). GLSD is a trachaeomycosis caused by *Phaeoacremonium aleophilum* (Pal), [*P. minimum sensu* Gramaje *et al.* (2015),] and *Phaeomoniella chlamydospora* (Pch). This disease is also known as "young esca" and in combination with the disease caused by the white rot fungus *Fomitiporia mediterranea* (Fmed) gives rise to the disease termed "esca proper" (Surico, 2009). Pal and Pch colonise the vine wood, causing dark streaks and brown or reddish-brown necroses (Marchi *et al.*, 2001; Surico, 2009). During colonization, these pathogens produce toxins, toxic metabolites and wood degradation products (Evidente *et al.*, 2000; Tabacchi *et al.*, 2000) which find their way to the vine crown through the xylem vessels. These are thought to activate a defence response that results in the development of leaf symptoms (Andolfi *et al.*, 2011; Bertsch *et al.*, 2013; Calzarano *et al.*, 2013). However the precise physiological mechanisms which cause leaf symptoms have not been fully elucidated. In some

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vines symptom severity decreases markedly over one or a few growing seasons and this fluctuation is affected by environmental factors. In particular, large amounts of rainfall early in the growing season, increases the incidence of leaf symptoms in the subsequent months (Marchi *et al.*, 2006; Calzarano and Di Marco, 2007). That heavy rainfall increases symptom severity was confirmed by an experiment in which 20-year-old diseased vines grown in pots were overwatered; this led to a significant increase in the leaf symptoms (Surico *et al.*, 2010). However, since vines heavily infected with Pal and Pch sometimes remain completely asymptomatic in a growing season and, conversely, vines that are only slightly infected with these fungi may exhibit serious leaf symptoms, other factors are also likely to cause leaf symptoms (Calzarano *et al.*, 2001, 2004; Calzarano and Di Marco, 2007).

GLSD symptoms initially consist in irregular chlorotic interveinal spots, which then coalesce, leaving only narrow lines of still green tissue along the veins. Subsequently the chlorotic spots become necrotic and assume a characteristic appearance resembling tiger stripes. The leaf symptoms are usually accompanied by localised shrivelling of the shoots and a more or less extensive withering of the grapebunches, which may also display typical black measles (Mugnai *et al.*, 1999). The factors causing these symptoms are complex, deriving both from the plant itself and from the environment. This probably explains why trials designed to artificially reproduce the symptoms have never been entirely successful (Sparapano *et al.*, 2001; Feliciano *et al.*, 2004).

Since the damage caused by the disease is related to the severity of the leaf symptoms (Calzarano *et al.*, 2001, 2004; Bertsch *et al.*, 2013), tests have also been carried out to investigate why symptoms fail to appear in some growing seasons. By varying the mix of nutrients given to the vines it has been shown that nutrients have direct roles in the infection process (Osti and Di Marco, 2010). Nutrients may also have indirect roles, since they affect the physiology of grapevines at different growth stages (Di Marco *et al.*, 2001; Calzarano *et al.*, 2009). Moreover, in studies on the nutritional status of vines suffering from esca proper (*sensu* Surico, 2009), and on how leaf fertilisation affected leaf symptom expression, it was found that calcium concentration was greater in diseased/asymptomatic vine leaves than in symptomatic leaves. Thus calcium may have a role in limiting symptom expression (Calzarano *et al.*, 2009). These findings have led to the development of a nutrient mix that reduces the incidence and severity of leaf symptoms. This mix is based on calcium, magnesium and extracts of algae, and is given during the plant growth period, when it is presumed that the mechanisms that will cause symptoms later in the season are primed (Calzarano *et al.*, 2014).

Knowing how vines responds to infection by Pal and Pch is fundamental to understand how the disease will progress during the rest of the growing season, and hence to design more effective methods of management. In the Vitaceae the most common and characteristic response of plants to fungal infections is production of increased amounts of phytoalexins. These are secondary metabolites produced aspecifically in response to general growth constraints. In grapevine the phytoalexins are produced constitutively or in response to stress, either biotic or abiotic. (Dufour *et al.*, 2013; Saigne-Soulard *et al.*, 2015).

In *Vitis vinifera* the principal stress response phytoalexins are stilbenes, the foremost component of which is resveratrol (*trans*-3,4'.5-trihydroxy-stilbene). Resveratrol gives rise to the other phytoalexin stilbenes, such as pterostilbene (*trans*-3,5-dimethoxi-4'-hydroxy-stilbene), and the viniferins (Cichewicz and Kouzi, 2002; Jeandet *et al.*, 2002). Earlier studies reported the constitutive presence of resveratrol in the wood of grapevine and not in healthy leaves, so it was thought that resveratrol only accumulated in leaves in response to stress (Borie *et al.*, 2004). More recent studies have shown that resveratrol is also a constituent of healthy leaves (Calzarano *et al.*, 2004; 2013; Wang *et al.*, 2010).

In the context of the esca complex, the levels of phytoalexins in the organs of affected vines, or in vines at different growth stages, have been little studied. It is known that resveratrol and ε-viniferin levels rise significantly in grape wood colonised by the esca-complex pathogens (Amalfitano *et al.*, 2000), and that pterostilbene inhibits the growth of Pch *in vitro* (Mazzullo *et al.*, 2000). More recent studies have reported that the levels of resveratrol and other phenolic compounds increase in asymptomatic GLSD-affected vine leaves after the leaf symptoms start to appear (Lambert *et al.*, 2013; Fontaine *et al.*, 2016). Other studies have reported the expression of the genes of phenylalanine ammonia-lysase (PAL) and stilbene-synthase STS, which catalyse the formation of resveratrol and other related compounds, in the

vine leaves before and after the onset of symptoms (Magnin-Robert *et al.*, 2011). This is correlated with the cultivar susceptibility to GLSD (Lambert *et al.*, 2013). Preliminary studies have also examined the occurrence of resveratrol in leaves and berries of GLSD-affected vines, but there are no reports on the levels of phytoalexins accumulated in the epigeal portion of GLSD-affected vine plants over time and at various growth stages (Calzarano *et al.*, 2004, 2008).

The aims of the research reported here were i) to determine the levels and variations over time of phytoalexins during the growing season in leaves of vines affected with GLSD, and in leaves of healthy vines; ii) to record the incidence and severity of the foliar symptoms of GLSD at various plant growth stages over a number of growing seasons. The overall aim was to determine whether there was any correlation between phytoalexin levels and GLSD symptoms. A second objective was to assess effects of various vine growth stages on the seasonal fluctuations in phytoalexin levels, and on the leaf symptoms that appeared.

Materials and methods

Optimising the extraction and clean-up of *trans*-resveratrol and its derivatives in symptomatic leaves of GLSD-affected vines

The extraction and clean-up procedure was optimised using *trans*-resveratrol and *trans*-pterostilbene as target compounds, in order to maximise the extraction of all targeted stilbenic compounds. *Trans*-resveratrol and *trans*-pterostilbene were selected because they are commercially available as standards and can be considered model analytes for the other stilbene derivatives known as viniferins (dimers of resveratrol), since they exhibit similar chemical/physical characteristics.

Sample preparation

Five hundred g of tiger-striped (GLSD-affected) leaves taken from 20 symptomatic vines were subjected to flash freezing by dipping them in liquid nitrogen for 90 s. The leaves were then finely ground (< 5 mm) in a blender (Moulinex, model A 505) at room temperature for 30 s. The powder obtained was immediately subjected to two solid-liquid extraction procedures (E1 and E2; below).

Solid-liquid extraction (E1)

Fifty g of leaf powder was mixed with 150 mL of methanol and stirred for 20 min in an orbital shaker (300 rpm) in the dark. The sample was centrifuged for 5 min at 4800 rpm and 50 mL of supernatant was collected. The extract obtained (E1) was stored at –20°C in the dark. All the instruments used were pre-cooled, and the whole procedure was run in the dark.

Solid-liquid extraction (E2)

Three g of leaf powder was mixed with 15 mL of ethanol/water (80:20, v/v) and homogenised for 1 min with an ultra-turrax. The sample was centrifuged for 3 min at 4800 rpm, and the supernatant transferred to a 50 mL capacity falcon flask (Calzarano *et al.*, 2008). The leaf residues were subjected to another extraction with 15 mL of ethanol/water (80:20, v/v) following the procedure described above. The extracts obtained (E2) were collected in a 50 mL capacity falcon flask and stored at –20°C in the

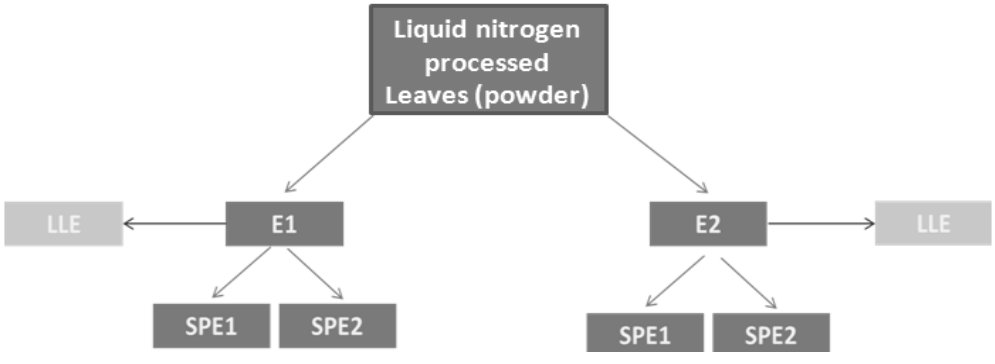

Figure 1. General scheme of the extraction and clean-up treatments.

dark. All the instruments used were pre-cooled and the whole procedure was run in the dark.

Liquid-liquid and solid phase extraction clean-up steps (LLE, SPE1 and SPE2)

The grapevine leaf extracts obtained with the solid-liquid extraction procedures (E1 and E2) were naturally rich in pigments (particularly chlorophylls) and other compounds. To remove these potentially interfering compounds from E1 and E2, three extraction/clean-up procedures were compared: one liquid-liquid extraction and two solid phase extractions (LLE, SPE1 and SPE2). Figure 1 shows the combination of the extraction and clean-up procedures that were carried out.

LLE procedure

Extracts E1 and E2 were each mixed with ethyl acetate (ethyl acetate/extract 1:1, v/v) and stirred for 5 min in an orbital shaker (300 rpm) in the dark. Each sample was then centrifuged for 5 min at 4800 rpm and the supernatant isolated. Subsequently, the supernatant was washed with ethyl acetate, and the phases were separated as reported in the previous step. One mL of the alcoholic (E1) / hydro-alcoholic (E2) fraction obtained was collected (E1-LLE and E2-LLE) for analyses.

SPE1 procedure

Commercially available octadecyl C18 cartridges (1 g, 6 mL) (International Sorbent Technology) were used for the clean-up extraction step, as follows. Extracts E1 and E2 were each diluted with H_2O (1:4, v/v); 20 mL of the solution obtained was loaded (10 × 2 mL) onto a column conditioned with 2 × 5 mL of acetonitrile and 2 × 5 mL of distilled H_2O, and kept in the dark. To eliminate hydrophilic interference, the column was subjected to a washing step with 2 × 5 mL of distilled H_2O. The column was eluted with 4 × 0.5 mL of acetonitrile, and each aliquot was collected separately.

SPE2 procedure

Lab-made cartridges each containing 5 g of C18 sorbent phase (International Sorbent Technology) were used for the clean-up extraction step. In order to concentrate the analytes, three of each extract (E1 and E2) were combined up to reach a final volume of 150 mL. The extract was loaded (10 × 15 mL) onto a column conditioned with 15 × 5 mL of distilled H_2O and kept in the dark. The washing step was carried out with 1.5 mL of acetonitrile, followed by 5 × 15 mL of distilled H_2O. Different combinations of solvents were tested to optimise the elution step. The column was eluted with acetonitrile-ethyl acetate gradients as follows: 2 × 4 mL of acetonitrile: ethyl acetate (100:0, v:v), 2 × 4 mL of acetonitrile: ethyl acetate (90:10, v:v), 2 × 4 mL of acetonitrile: ethyl acetate (70:30, v:v), and 2 × 4 mL of acetonitrile: ethyl acetate (50:50, v:v). Each aliquot was collected separately. The elution finally chosen was 6 mL of acetonitrile-ethyl acetate (70:30, v:v) in one step.

Evaluation of grapevine leaf extraction and clean up

Leaf extracts from the different extraction/clean up procedures were compared using a high-performance liquid chromatograph (Perkin Elmer Series 200 System) equipped with an auto-sampler and a UV–Vis-diode-array detector (DAD), following the method of Pezet *et al*. (2003). This was to determine the levels of *trans*-resveratrol and *trans*-pterostilbene. Each combination of extraction and purification was performed in triplicate. In this HPLC method, a 250 mm C18 Lichrospher column was used (Merck Millipore), diam. 4.6 mm, particle diam. 5 μm. The column was equipped with a Security Guard Cartridge C18 security guard column (Phenomenex). The gradient elution was carried out using the following mobile phases: mobile phase A, consisting of acetonitrile; mobile phase B, consisting of water. The binary elution gradient used was as follows: 1 min at 20:80 (A:B, v:v), linear gradient at 75:25 (A:B, v:v) for 30 min, and at 100:0 (A:B, v:v) for 2 min. The column was kept for 3 min at 100:0 (A:B, v:v), and then switched back to the initial 20:80 (A:B, v:v) for 4 min. The flow rate was set at 1 mL min^{-1} and the UV-Vis-diode array detector was set at 307 nm (Pezet *et al*., 2003).

Determination of stilbene compounds by ultra-high performance liquid chromatography/high resolution mass spectrometry (UHPLC/QTOF-MS)

Chromatographic analyses of the different fractions, including analysis of the unknown chromatographic peaks (resulting from compounds without commercially available standards) were performed using an Agilent UHPLC 1290 Infinity (Agilent Technologies) equipped with Agilent 1290 Infinity autosampler (G4226A) coupled to Agilent 6540

accurate-mass Q-TOF mass spectrometer (nominal resolution 40,000) and Dual Agilent Jet Stream Ionisation source. Three tiger-striped vine leaf samples (ten leaves per sample) were processed with the extraction technique selected (E2), and assayed with the HPLC/QTOF-MS procedure.

Chromatographic separation was performed following the method of Pezet *et al.* (2003) with a modified gradient elution: phase A) acetonitrile; phase B) water acidified with 0.2% (v:v) acetic acid; flow rate 0.6 mL min^{-1}; sample injection 10 µL. Q-TOF conditions: negative ionisation mode; sheath gas: nitrogen 10 L min^{-1} at 400°C; dehydration gas 8 L min^{-1} at 350°C; nebuliser pressure 60 psi, nozzle voltage 0 kV, capillary voltage 3.5 kV. Signals recorded were in the *m*/*z* 100-1700 range. Negative mass calibration was performed with standard mix G1969-85000 (Supelco Inc.) with residual error ± 0.2 ppm for the expected masses. Lock masses were TFA anion at *m*/*z* 112.9856 and HP-0921 (+ formate) at *m*/*z* 966.0007 in negative-ion mode.

Description of the vineyards examined, methods of sampling and calculation of dry weights

The study was carried out in two vineyards in the Province of Teramo, Region of Abruzzo, Italy. The vineyards had the same climatic conditions.

The vineyard of Controguerra was established 38 years previously and was trained to the Geneva Double Curtain (GDC) system. It comprised 740 vines on 5,984 m^{-2} with a vine spacing of 2 m within rows and 4 m between rows, and with an average yield of 13 kg per vine stock, and up to 16.5 kg in peak years. The vineyard of Giulianova was of the same age and was trained to the Tendone system. It had 1,296 vines in an area of 11,016 m^{-2}, with a vine spacing of 3 m × 3 m and producing an average annual yield of 20 kg per vine stock. Both vineyards were grown with the cv. Trebbiano d'Abruzzo on 420A rootstock. The soil of the vineyards had a calcareous and high clay content structure. Trebbiano d'Abruzzo is a very vigorous cultivar, and the training systems used formed large trunks that grew vertically for 2–2.2 m, with two permanent long cordons (GDC), or four large branches (Tendone).

The two vineyards have been under observation for GLSD foliar symptoms for more than 20 years. These multi-year inspections have made it possible to distinguish between asymptomatic vines that certainly had GLSD (because in one or more previous inspection years they exhibited the GLSD symptoms, but were asymptomatic in the season of the experiment) and vines that were healthy, because in all the inspection years they never exhibited any GLSD symptoms. Hence the concentrations of the various phytoalexins were determined on: 1. leaves of diseased/symptomatic vines (i.e. vines exhibiting tiger-striped leaves in the sampling year) on which the stripes had reached their greatest extent, covering 65% of the laminae; 2. leaves of vines known from previous inspections to be diseased, but asymptomatic in the sampling year; and 3. leaves of vines that in all previous inspections had never been symptomatic and were thus assumed to be healthy.

In both vineyards, and in each year of study, six vines were selected from each category (healthy, asymptomatic/diseased, or symptomatic). Twelve leaf laminae per vine were collected from the middle portion of grape-bearing shoots, and opposite a grape bunch. Each sample collection therefore comprised six sub-samples, each comprising 12 leaves, for each of the three categories of vines under study, and each sample consisted of leaves from a single vine. Each vine was identified at the time of the first sampling of each growing season, by its row number and its plant number within the row. In this way it was possible to follow each of these six vines in each category throughout the growing season, and to record any variations in phytoalexin concentrations in each vine. During successive growing seasons the leaves were sampled at different vine growth stages, described following the BBCH classification scheme (Lorenz *et al.*, 1995) as: 'fruit set' (71); 'berries pea-sized' (75); 'berries beginning to touch' (77); 'berries developing colour' (83); 'softening of berries' (85); and 'berries ripe for harvest' (89).

In 2006 and 2007 the vines in both vineyards were sampled at growth stages 77, 83, 85 and 89; in 2012 and 2013 vines were sampled only in the Controguerra vineyard, at growth stages 71, 75, 77, 83, 85, and 89.

In all the study years, in both vineyards, vine leaves from all three categories of plants were sampled to determine their water contents, so that the concentrations of the various compounds extracted from the dry weight of the leaves could be calculated. Twenty-four leaves were sampled, representing six replicates of four leaves each, for each vine category. Sampling was carried out at growth stage 77, and repeated at growth stage 89. Each sample of

four leaves was weighed when fresh, after which the leaves were kept in an oven at 75°C for 24 h and then weighed again to determine their dry weights. The percentage of water in the various categories of leaves was calculated using the formula [(fresh weight – dry weight)/fresh weight] × 100. The concentrations of the different phytoalexins in the leaves was then expressed on a leaf dry weight basis.

Statistical analyses

In each of the years under study, the data were analysed separately for each compound and at each vine growth stage, comparing the concentrations of each of the compounds in the leaves of each of the three categories of vines (six repetitions per vine group, corresponding to the concentrations of six individual vines) using Tukey's honest significant difference (HSD) test at P=0.05. Statistical analyses were carried out using SAS version 9.3 (SAS Institute Inc.).

Detection of leaf symptoms

In each of the test years, 2006, 2007, 2012, and 2013, the incidence and severity of GLSD leaf symptoms were measured in both vineyards, in vines at four successive BBCH growth stages: 75; 77; 83; and 89. These four measurements were made in both vineyards on July 10, July 28, August 24, and 20 September 2006, and on 9 July, 31 July, 24 August, and 19 September 2007. In the Controguerra vineyard these measurements were also made on 12 July, 31 July, 24 August and 21 September 2012, and on 11 July, 29 July, 26 August and 20 September, 2013.

The incidence of GLSD leaf symptoms in each vineyard was calculated by dividing the number of vines with leaf symptoms by the total number of diseased (asymptomatic/diseased and symptomatic) vines, and multiplying by 100. Percentage severity of symptom expression was calculated using the formula SN × 100/(Y × Z), where SN = the sum of the symptom severity values; Y = the number of vines observed (asymptomatic/diseased and symptomatic); and Z = maximum symptom severity value (McKinney, 1923). Leaf symptom severity of a vine (as a percentage of the vine crown affected) was recorded on a disease rating scale of 0 to 5, where 0 = no leaf symptom; 1 = 1–10% of the vine crown symptomatic for GLSD; 2 = 11–30%; 3 = 31–50%; 4 = 51–70%; and 5 = 71–100% symptomatic.

Results

Optimising extraction and clean-up of *trans*-resveratrol and derivatives in symptomatic leaves of GLSD-affected vines

To identify the best procedure for the preparation, extraction and purification of GLSD- symptomatic leaves, different combinations of treatments of leaf samples were tested (Table 1). The optimal procedure was determined using the HPLC/UV-Vis-DAD method according to Pezet *et al.* (2003).

Sample pre-treatment played an important role in optimising extraction efficiency. A brief but effective homogenisation of extract E2 enabled greater amounts of *trans*-resveratrol and *trans*-pterostilbene to be extracted. Extracts E1 and E2 were each subjected to a clean up step, obtaining different treatment/clean-up combinations: E1-SPE1, E1-SPE2, E2-SPE1, E2-SPE2, E1-LLE and E2-LLE. The clean up obtained with the SPE1 and SPE2 procedures gave high *trans*-resveratrol and *trans*-pterostilbene recovery (> 80%), but these procedures did not quantitatively remove the chlorophylls. On the other hand, the LLE procedure quantitatively removed the chlorophylls, but led to a significantly lower recovery of both *trans*-resveratrol and *trans*-pterostilbene (≈ 50%). For this reason, leaf samples were extracted with the E2 liquid-solid extraction without additional treatments to

Table 1. *Trans*-resveratrol and *trans*-pterostilbene concentrations (fresh weight values) in samples of tiger-striped grapevine leaves obtained with different extraction/purification techniques. The values shown in the Table are the averages of three replicates.

Treatment	*trans*-resveratrol (mg kg^{-1})	*trans*-pterostilbene (mg kg^{-1})
E1	12.6 ± 0.8	2.3 ± 0.1
E2	15.4 ± 0.7	4.4 ± 0.3
E1-SPE1	10.1 ± 1.0	1.7 ± 0.1
E1-SPE2	9.5 ± 0.8	1.6 ± 0.2
E2-SPE1	12.6 ± 1.1	2.1 ± 0.1
E2-SPE2	12.0 ± 0.9	1.5 ± 0.2
E1-LLE	6.5 ± 0.5	0.5 ± 0.1
E2-LLE	8.7 ± 0.7	0.8 ± 0.2

remove the chlorophylls; only a C18 guard column (Phenomenex) was used to safeguard the HPLC apparatus. In studies of this nature a high recovery of *trans*-resveratrol and *trans*-pterostilbene is essential since this provides more sensitive indication of any differences in the amounts of phytoalexins detected.

Determination of stilbene compounds by ultra-high performance liquid chromatography/high resolution mass spectrometry (UHPLC/QTOF-MS)

To identify unknown phytoalexins, a HPLC/QTOF-MS analysis was performed. The three grapevine tiger-striped leaf samples were subjected to the selected extraction procedure, followed by the chromatographic analysis selected (E2 coupled with the procedure of Pezet *et al*., 2003). In these samples different trans and cis stilbenes were identified, such as *trans*-resveratrol, *cis* and *trans*-ε-viniferin, *trans*-δ-viniferin, and *trans*-pterostilbene. These are listed in Table 2 with their respective mass and retention times. Figure 2 shows a LC-MS chromatogram of a symptomatic grapevine leaf extract.

The compounds were identified using the standards commercially available (*trans*-resveratrol and *trans*-pterostilbene), on the accurate masses measured, and by performing multiple mass spectrometry (MS/MS) analyses as previously described (Flamini *et al*., 2013) (Table 2).

The retention time of each compound was used to identify the corresponding stilbene derivative in the grapevine leaf samples assayed by HPLC. Thus, considering the analytical data, the whole set of data of the field experiment was obtained using extraction procedure E2 coupled to HPLC (Pezet *et al*., 2003).

Levels and patterns over time of phytoalexins extracted from GLSD-affected vine leaves.

The water levels determined in all the investigated years, in the vine leaves studied were used to calculate the concentrations of phytoalexins on the basis of the leaf dry weight. These yielded uniform data in the replicates of all three categories of leaves, in both growth stages studied. Phytoalexin concentrations had to be calculated on the dry weight of the leaves to adjust for differences due to different water concentrations in symptomatic leaves on the one hand, and asymptomatic/diseased and healthy leaves on the other. The water concentration was on average of 65% in symptomatic (tiger-striped) leaves, and 72% in both healthy and asymptomatic/diseased leaves, at both growth stages and in both vineyards. Analysis of the leaves of healthy and diseased vines in the years of study revealed higher levels of *trans*-resveratrol and lower levels of *trans*-pterostilbene, *trans*-ε-viniferin, and *trans*-δ-viniferin, (in what follows, the prefix *trans*- is omitted). In the growing seasons under study, resveratrol levels in the three categories of vines ranged between the minimum and maximum values shown in Table 3. The other compounds were not detected in asymptomatic/diseased and healthy vine leaves in different growth stages, particularly at stage 71 and at stage 75.

The seasonal peaks resveratrol levels in symptomatic leaves were reached at stage 77 in both vine-

Table 2. Chromatographic retention times, and theoretical and experimental masses of pseudo-molecular ions of *trans*-resveratrol and stilbene derivatives identified in a symptomatic grapevine leaf extract prepared using the procedure selected (E2; see text).

Peak	RT min	Compound	Theoretical mass $[M-H]^-$ *m/z*	Experimental mass $[M-H]^-$ *m/z*	Δ ppm
1	5.64	*trans*-resveratrol	227.0714	227.0712	-0.88
2	8.36	*cis*-ε-viniferin	453.1344	453.1349	1.10
3	9.00	*trans*-ε-viniferin	453.1344	453.1348	0.88
4	10.68	*trans*-δ-viniferin	453.1344	453.1346	0.44
5	16.51	*trans*-pterostilbene	255.1027	255.1029	0.78

Table 3. Ranges of average concentrations of phytoalexins extracted from the leaves of different categories of grapevine plants growing in the Controguerra and Giulianova vineyards, during the growing seasons studied.

Category of leaves	Level	*trans*-resveratrol	*trans*-pterostilbene mg kg^{-1} d wt	*trans*-ε-viniferin	*trans*-δ-viniferin
Healthy	min	1.38	0.00	0.00	0.00
	max	13.38	2.41	2.67	1.92
Asymptomatic	min	2.57	0.00	0.00	0.00
	max	25.26	3.00	4.11	1.63
Symptomatic	min	3.75	0.31	0.36	0.37
	max	50.49	5.41	8.75	3.23

yards, and in all the investigated years, with mean concentrations of 50.49 mg kg^{-1} d wt at Controguerra, and 46.96 mg kg^{-1} d wt at Giulianova in 2006, and 23.40 mg kg^{-1} d wt (Controguerra) and 21.62 mg kg^{-1} d wt (Giulianova) in 2007, and with concentrations of 22.70 mg kg^{-1} d wt in 2012, and 22.10 mg kg^{-1} d wt in 2013, in the Controguerra vineyard. At stage 77, resveratrol levels were slightly, though not significantly, higher in asymptomatic/diseased vine leaves than in healthy vine leaves in 2006 and 2012, and were significantly higher in the Controguerra vineyard in 2013 (Figure 3). In all categories of vine plants, resveratrol

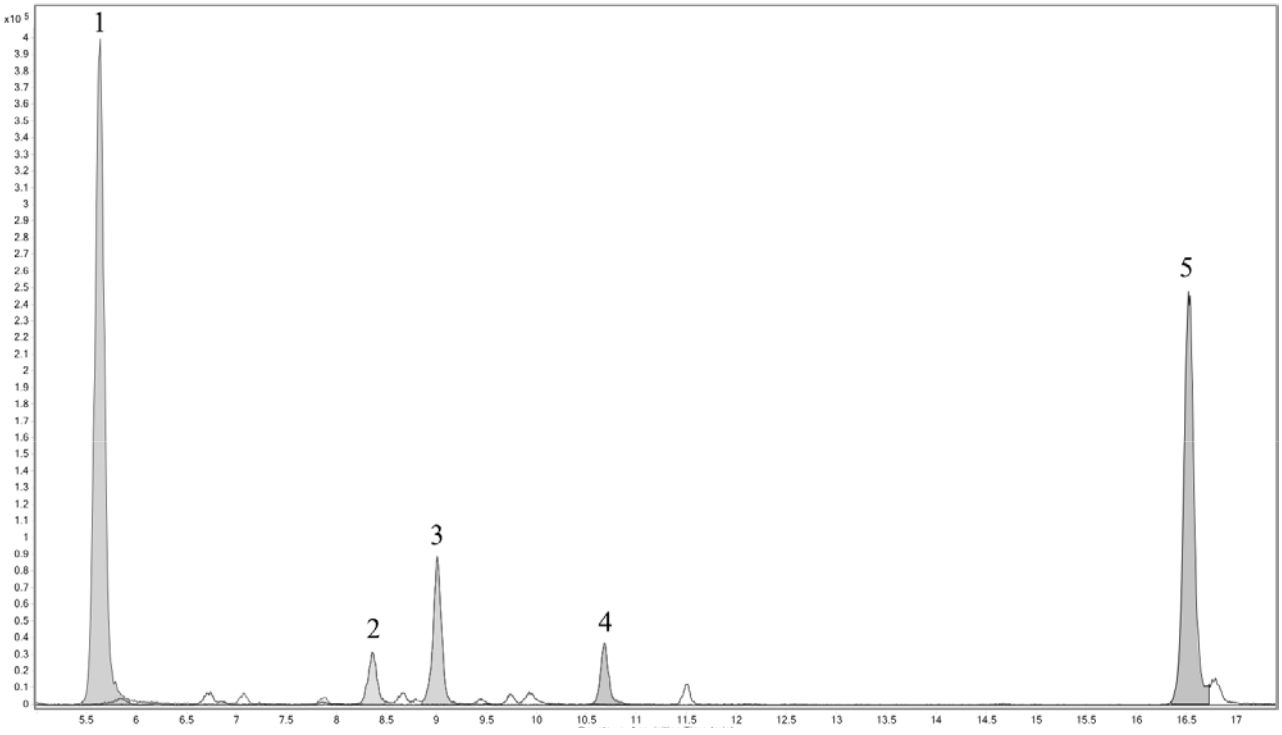

Figure 2. UHPLC/QTOF - Extract ion chromatogram of a symptomatic grapevine leaf extract prepared using the procedure selected (E2; see text). 1, *trans*-resveratrol; 2, *cis*-ε-viniferin; 3, *trans*-ε-viniferin; 4, *trans*-δ-viniferin; 5, *trans*-pterostilbene.

concentrations decreased at stage 83, and the differences in resveratrol concentrations between leaf-categories also decreased, though the differences between symptomatic and other (asymptomatic/diseased, healthy) leaves still remained significant in some cases (Giulianova in 2006 and 2007, and Controguerra in 2013). At later growth stages, resveratrol levels increased again in all categories of leaves, and the differences in resveratrol levels between the categories of leaves also increased. These differences reached statistical significance at growth stage 85 and/or at stage 89. At these stages, the tiger-striped leaves in particular differed significantly from the other leaves, while the asymptomatic/diseased leaves had intermediate resveratrol levels between symptomatic and healthy leaves, but did not always differ significantly from healthy leaves (Figure 3).

In tiger-striped leaves, whenever resveratrol levels peaked at stage 77, pterostilbene levels also peaked. Mean amounts of pterostilbene were 4.24 mg kg^{-1} d wt at Controguerra, and 4.17 mg kg^{-1} d wt at Giulianova in 2006, 1.82 mg kg^{-1} d wt at Controguerra and 2.06 mg kg^{-1} d wt at Giulianova in 2007, and 4.90 and 5.41 mg kg^{-1} d wt in 2012 and 2013 at Controguerra (Figure 4). At this growth stage, pterostilbene levels were significantly higher in tiger-striped leaves than in asymptomatic/diseased or healthy leaves. In tiger-striped leaves, pterostilbene levels then always

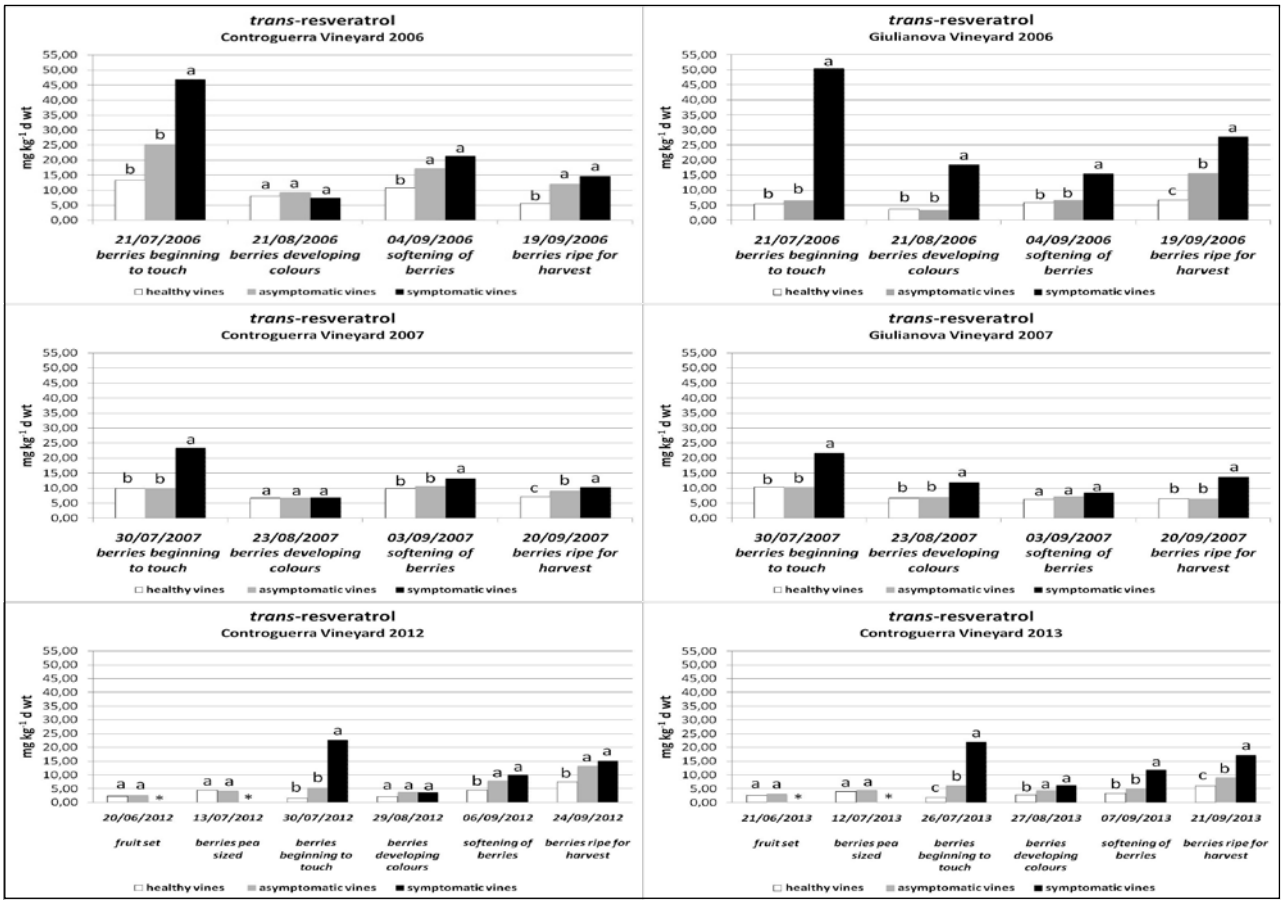

Figure 3. Levels of *trans*-resveratrol in the leaves of vines affected with GLSD and in leaves of healthy grapevines at various growth stages in the 2006, 2007, 2012 and 2013 growing seasons in the Controguerra and Giulianova vineyards. Statistical analyses were performed according to Tukey's honest significant difference (HSD) test. Each grapevine category (symptomatic, asymptomatic and healthy) comprised six replications of leaf data (one replication = one grapevine plant) in each growth stage, in each year and in each vineyard. Different letters represent significant differences at P=0.05. * = no symptomatic vines found.

decreased considerably at growth stage 83, but after this they tended to increase again in the last growth stages. This again increased the difference between symptomatic and healthy leaves, and sometimes also between symptomatic and asymptomatic/diseased leaves. Pterostilbene levels in healthy and asymptomatic/diseased leaves were similar in all vine growth stages and in both vineyards in 2006, and also in the first four growth stages of 2012 and 2013, when this compound was quite lacking in these two categories of leaves. In 2007, in all growth stages and in both vineyards, pterostilbene levels were often significantly higher in asymptomatic/diseased leaves than in healthy leaves, and were similar to pterostilbene levels in tiger-striped leaves. This was also the case at growth stage 85 at the Controguerra vineyard in 2012 and 2013 (Figure 4).

As regards ε-viniferin levels, in the Controguerra vineyard in 2006, and in both vineyards in 2007, tiger-striped leaves exhibited reductions at growth stage 83, but then ε-viniferin levels increased again at stages 85 and 89, to higher levels very similar to those seen earlier at stage 77. In the Giulianova vineyard in 2006, on the other hand, ε-viniferin levels of tiger-striped leaves did not differ much between any of the growth stages examined. In any case, in 2006 and 2007 mean ε-viniferin amounts did not exceed 1.85 mg kg^{-1} d wt, which was much less than the

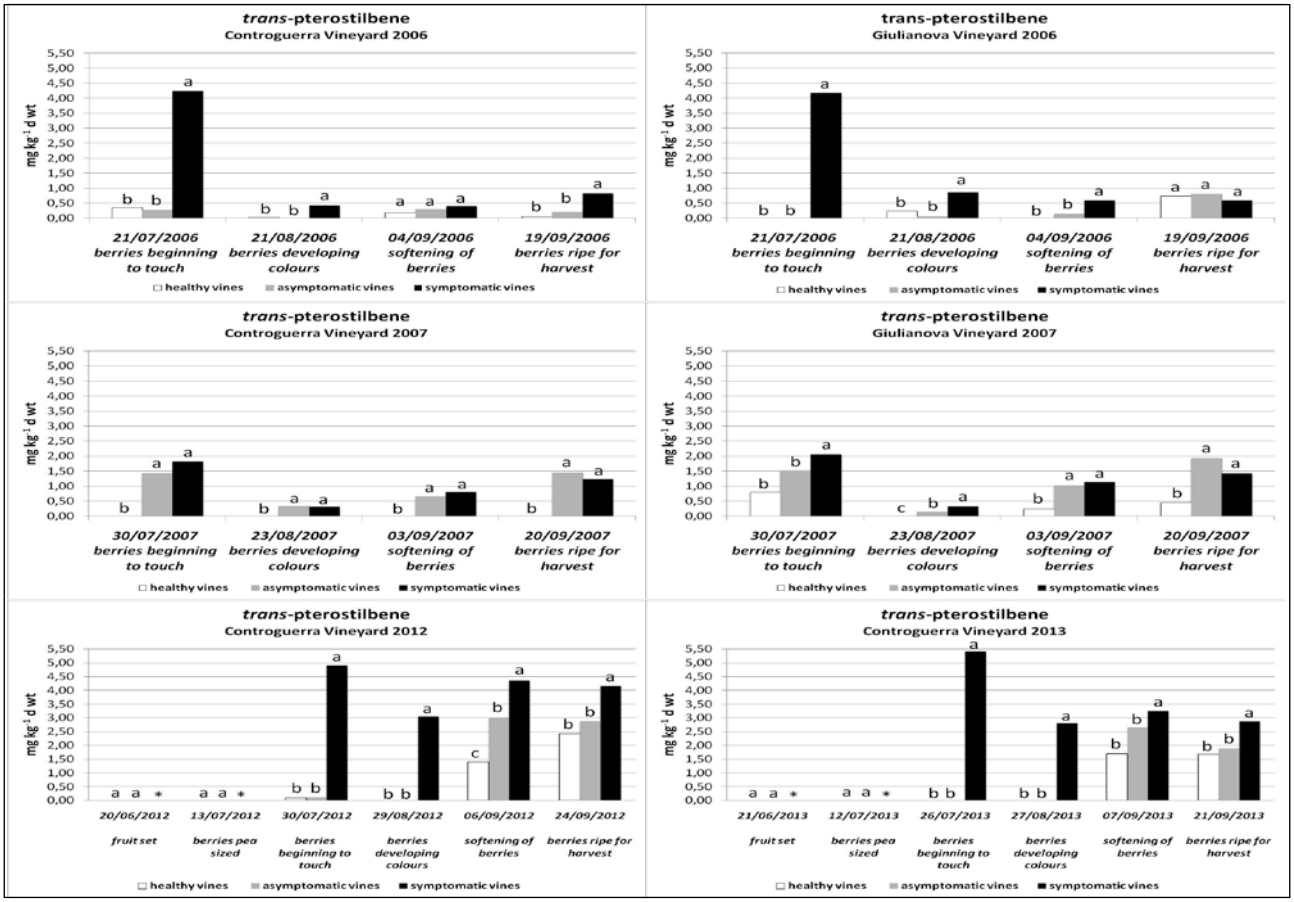

Figure 4. Levels of *trans*-pterostilbene in the leaves of vines affected with GLSD and in leaves of healthy grapevines at various growth stages in the 2006, 2007, 2012 and 2013 growing seasons in the Controguerra and Giulianova vineyards. Statistical analyses were performed according to Tukey's honest significant difference (HSD) test. Each grapevine category (symptomatic, asymptomatic and healthy) comprised six replications of leaf data (one replication = one grapevine plant) in each growth stage, in each year and in each vineyard. Different letters represent significant differences at P=0.05. * = no symptomatic vines found;

ε-viniferin levels found in 2012 and 2013 at Controguerra. In this vineyard during those years, levels in tiger-striped leaves were much higher at stages 85 and 89 than at stage 77. At stage 85 and 89, mean amounts of ε-viniferin were similar, ranging between 7.72 and 8.75 mg kg^{-1} d wt, with significant differences between the three categories of leaves. In the asymptomatic/diseased leaves ε-viniferin levels were low in both vineyards in 2006, and nil in 2007, until growth stage 83. They then increased in the last two growth stages, often to a level intermediate between the levels found in tiger-striped and healthy leaves. At the last two growth stages (85 and 89), ε-viniferin levels of asymptomatic leaves were sometimes higher at stage 85, as they were in 2006 and 2007 at Giulianova, and sometimes also higher at stage 89, as in 2006 and 2007 at Controguerra. Otherwise, they always remained very similar, as in 2012 and 2013 at Controguerra. In healthy leaves, ε-viniferin levels were low or nil in both vineyards in 2006 and 2007, but in 2012 and 2013 in the Controguerra vineyard they tended to increase in the last two growth stages, as they did in the other categories of leaves. In 2007, ε-viniferin was hardly ever found in healthy leaves in either vineyard (Figure 5).

In 2006 and 2007, patterns of δ-viniferin amounts in tiger-striped leaves at the growth stages studied were very similar to the resveratrol patterns, in both

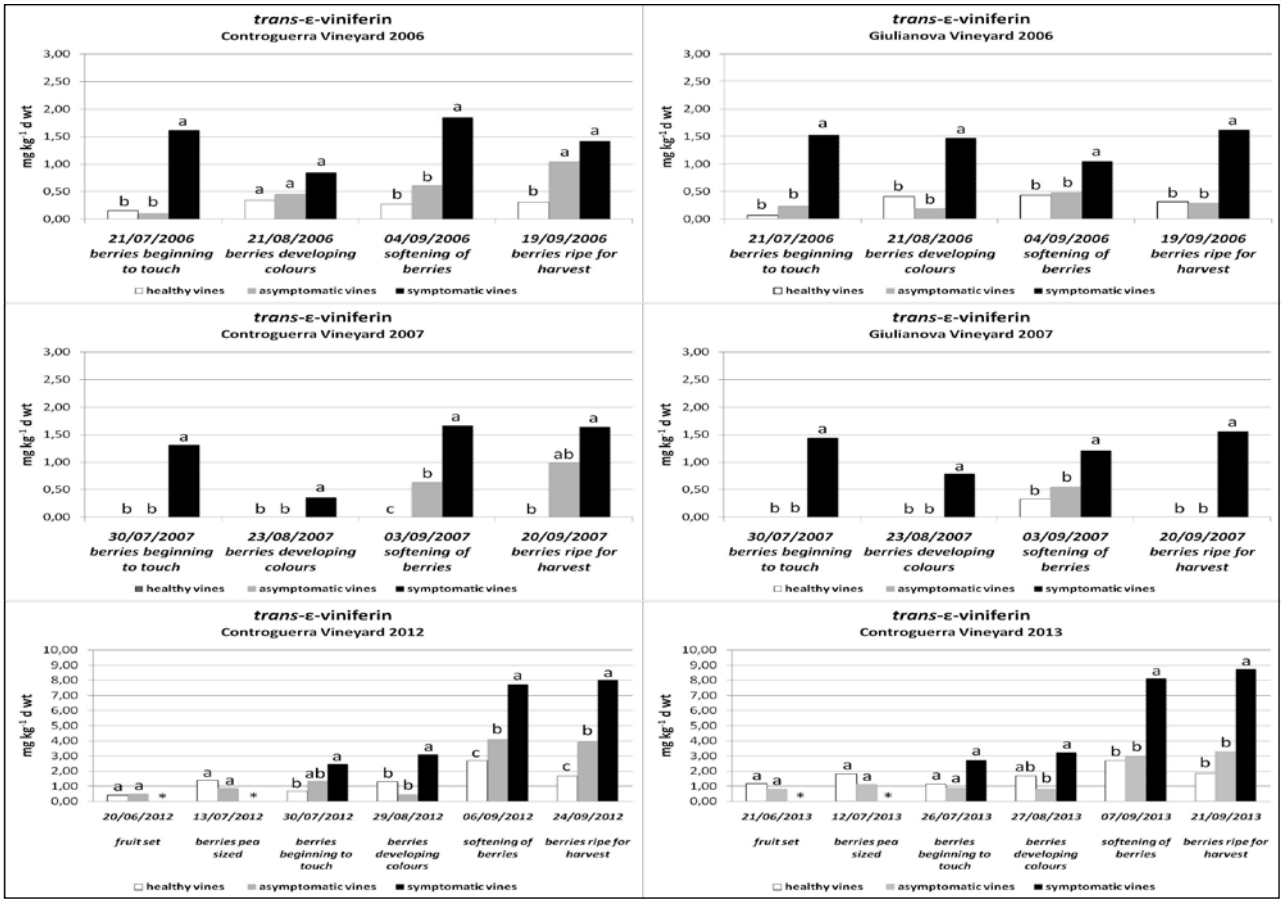

Figure 5. Levels of *trans*-ε-viniferin in the leaves of vines affected with GLSD and in leaves of healthy vines at various growth stages in the 2006, 2007, 2012 and 2013 growing seasons in the Controguerra and Giulianova vineyards. Statistical analyses were performed according to Tukey's honest significant difference (HSD) test. Each grapevine category (symptomatic, asymptomatic and healthy) comprised six replications of leaf data (one replication = one grapevine plant) in each growth stage, in each year and in each vineyard. Different letters represent significant differences at P=0.05. * = no symptomatic vines found;

vineyards. The δ-viniferin levels went up at stage 77, and went down again at stage 83. There was a tendency towards higher levels at growth stages 83 or 89. Mean amounts of δ-viniferin were similar to, or only slightly below, ε-viniferin levels in the same leaves, and ranged from 0.37 to 1.87 mg kg^{-1} d wt (Figure 6). In 2012 and 2013, in the tiger-striped leaves from the Controguerra vineyard, the highest δ-viniferin levels were found at stage 85; they then decreased at stage 89 though still remaining higher than they had been at stages 77 and 83. In 2006 and 2007 δ-viniferin levels in asymptomatic/diseased and healthy leaves were low, and often similar, and this compound was mostly completely lacking in 2007. In 2012 and 2013, no δ-viniferin was recorded in asymptomatic and in healthy leaves, in the first two growth stages in 2012 and in the first four growth stages in 2013. In all subsequent stages, however, the levels of δ-viniferin increased, particularly at stages 85 and 89. Mean amounts were never greater than 1.87 mg kg^{-1} d wt, recorded in healthy leaves, and with no significant differences between healthy and asymptomatic leaves (Figure 6).

In 2012 and 2013, in the two growth stages 71 and 75, before stage 77, no leaf symptoms were found, so all vines were asymptomatic diseased or healthy. In the leaves of these last two categories of vines, levels of the various compounds under study were

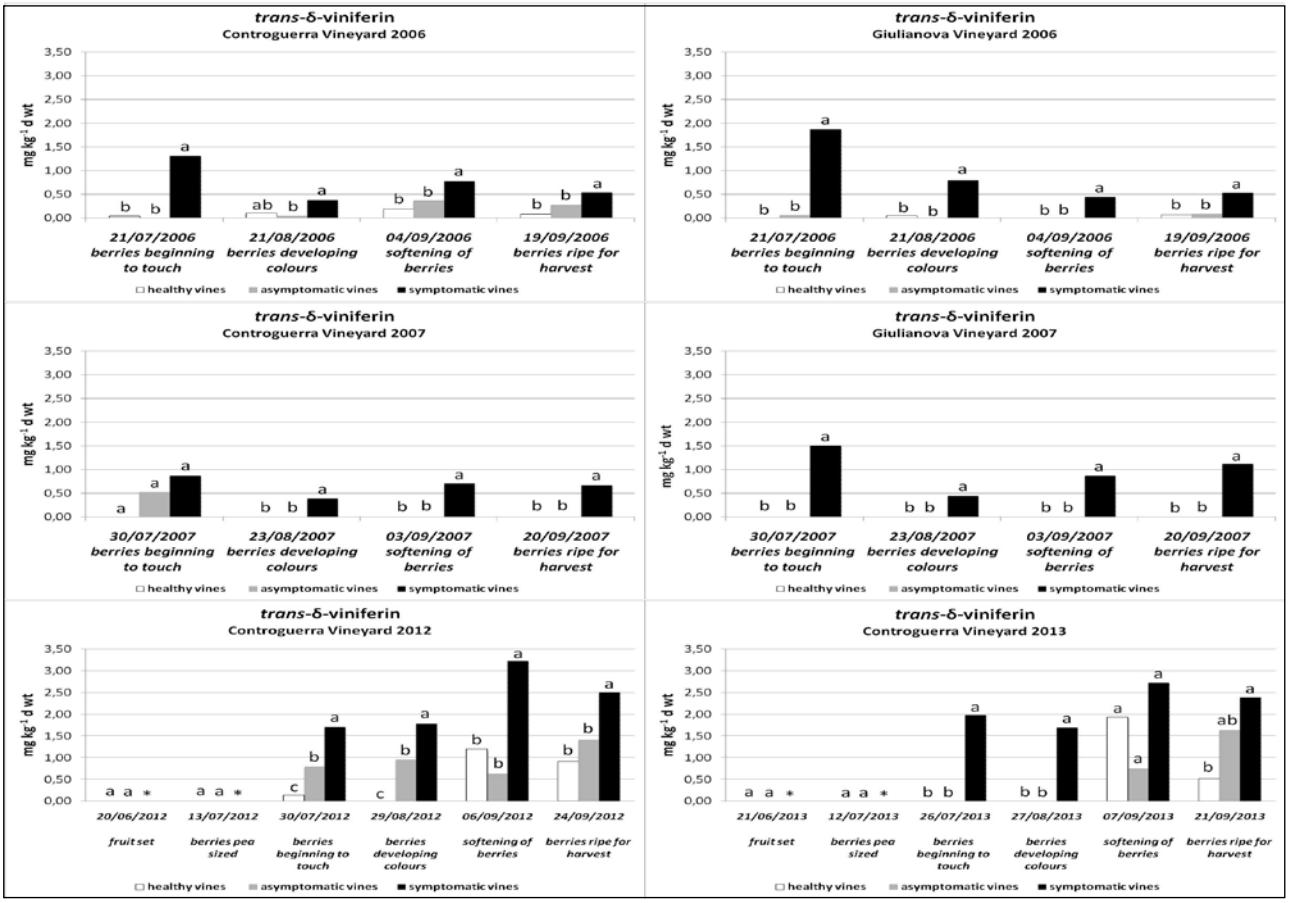

Figure 6. Levels of *trans*-δ-viniferin in the leaves of vines affected with GLSD and in leaves of healthy vines at various growth stages in the 2006, 2007, 2012 and 2013 growing seasons in the Controguerra and Giulianova vineyards. Statistical analyses were performed according to Tukey's honest significant difference (HSD) test. Each grapevine category (symptomatic, asymptomatic and healthy) comprised six replications of leaf data (one replication = one grapevine plant) in each growth stage, in each year and in each vineyard. Different letters represent significant differences at P=0.05. * = no symptomatic vines found;

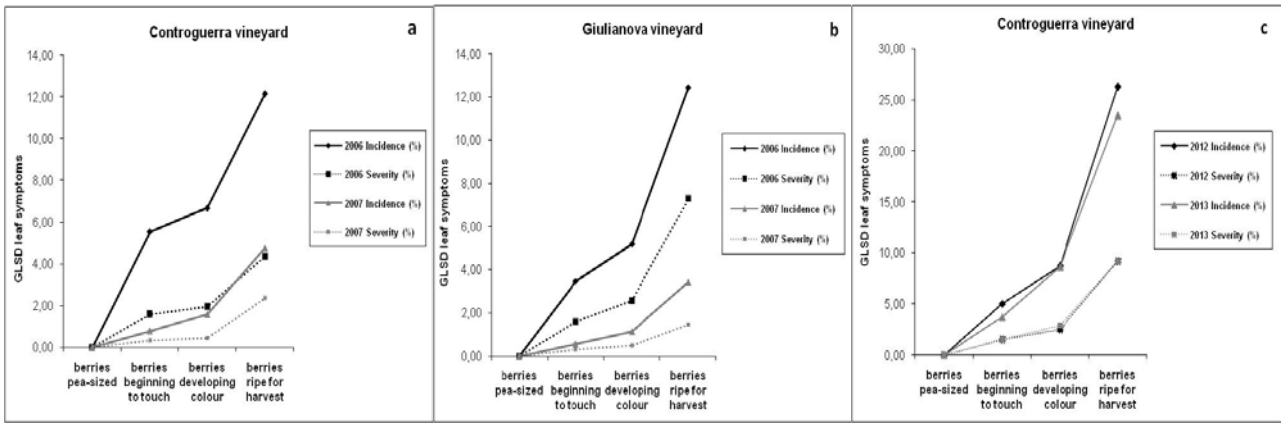

Figure 7. Incidence and severity of GLSD leaf symptoms in four growth stages in the 2006, 2007 (a, b), 2012 and 2013 (c) growing seasons, in the Controguerra and Giulianova vineyards

uniformly low and not dissimilar, or they were nil. Maximum mean resveratrol amounts were 5.29 mg kg^{-1} d wt, ε-viniferin amounts were 1.79 mg kg^{-1} d wt, and there was a complete lack of pterostilbene and δ-viniferin (Figures 3, 4, 5 and 6).

Detection of leaf symptoms

The leaf symptoms, recorded in the two vineyards in the same growing seasons in which the phytoalexins were measured, exhibited similar patterns in each growing season and in each vineyard. Leaf symptoms were absent at growth stage 75, and both the incidence and severity of symptoms were still limited at stage 77 (Figure 6). From stage 77 until stage 83, symptoms increased only moderately, but from stages 83 to 89 symptom expression increased markedly, to reach a peak at the time of harvesting (Figure 7).

Discussion

In this study, leaves generally showed higher levels of resveratrol than other stilbenes, independently of the presence or absence of the symptoms of GLSD. In particular, symptomatic leaves showed greater increases of resveratrol compared with increases of pterostilbene and viniferins. In the absence of biotic pathogenic agents in the leaves, as for GLSD, this finding is consistent with that of Pezet *et al*. (2004). This supports the view that the esca leaf symptoms on grapevines are primarily formed in response to toxic metabolites produced in the wood by pathogens that do not occur in the leaves (Mugnai *et al.*, 1999; Evidente *et al.*, 2000; Tabacchi *et al.*, 2000; Surico, 2009). On the other hand, pterostilbene and the viniferins are mainly produced in the plant tissues that harbour the pathogens, because these compounds possess greater antimicrobial activity (Langcake and McCarthy, 1979; Jeandet *et al.*, 2002; Pezet *et al.*, 2004).

The levels of resveratrol and other phytoalexins at various growth stages in different growing seasons revealed that amounts of these compounds varied during vegetative growth and ripening both in leaves of GLSD-affected vines and healthy vines. All the phytoalexins occurred at significantly higher levels in symptomatic than in asymptomatic/diseased and healthy leaves. This confirms earlier preliminary studies on resveratrol, carried out by our team (Calzarano *et al*., 2013). In those studies, the greatest amounts of resveratrol in symptomatic vines occurred at growth stage 77 (berries beginning to touch) or at stage 89, (berries ripe for harvest), and these levels differed significantly between the three categories of leaves. In most cases, however, levels then became much attenuated by stage 83 (berries developing colour), when resveratrol amounts tended to decrease overall. In the present study, in asymptomatic/diseased leaves, in some cases, some phytoalexins occurred at stage 77 (berries beginning to touch) at concentrations intermediate between the concentrations in the other two categories of leaves. In all the other cases, however, asymptomatic/dis-

eased leaves exhibited similar amounts of these compounds as healthy leaves, in which the phytoalexins occurred only sporadically and at low levels in the initial growth stages, to increase in the final growth stages, during ripening.

All categories of leaves exhibited uniformly lower levels of all phytoalexins at stage 83 (berries developing colour) and generally higher levels during ripening, at stages 85 (softening of berries) and 89 (berries ripe for harvest). Despite the differences mentioned between the different categories of leaves at different growth stages, which were separated by at least 2 week intervals, the phytoalexins detected increased or decreased in uniform patterns. These variations in compound concentrations were consistent with previous results, where the phytoalexins are formed out of resveratrol, and that the process of formation takes place within a few days (Keller *et al.*, 2000; Commun *et al*., 2003; Slaughter *et al.*, 2008).

The patterns of the phytoalexins over time in symptomatic leaves were similar in all the growing seasons studied, irrespective of considerable variations in the phytoalexin levels and in the incidence and severity of the leaf symptoms from one year to the next. In years when symptoms were more severe (2006, 2012, and 2013), phytoalexin concentrations also increased, especially resveratrol in 2006, and ε-viniferin in 2012 and 2013. The same factors that determined symptom severity therefore also affected the amount of phytoalexins produced, but not their variations over the growing season. Variations were more closely linked to the plant growth stage. In both vineyards, variations in the extent of leaf symptoms, and in the amount of phytoalexins produced, were very similar among years. This emphasises the importance of environmental variables (Marchi *et al*., 2006) for such variations, since the vineyards were located in an area that had the same weather/climate. The links between the amounts of phytoalexins produced and the severity of foliar symptoms are also shown by fact that variations in phytoalexin levels reflected variations in the extent of foliar symptoms within each growing season.

Variations in carbohydrate reserves in vinewood during the growing season may affect the amount of toxins released by the pathogenic fungi and hence the expression of GLSD symptoms. In the measurements taken over time in this study, GLSD symptoms were first recorded at the growth stage 77 (berries beginning to touch), when carbohydrate reserves were also at their lowest. This may have stimulated release of toxins by the pathogenic fungi in the vinewood (Sumarah *et al*., 2005; Eaton *et al*., 2015). From the stage 77 (berries beginning to touch) onwards the leaves produced carbohydrates, which were translocated to the wood to reconstitute the carbohydrate reserves, and this process ended shortly before stage 83 (berries developing colour) (Lebon *et al.*, 2008). It is likely, therefore, that in the interval between these two growth stages the fungi were less stimulated to produce toxins. Between stages 77 and 83, the incidence and severity of the leaf symptoms recorded did not greatly increase. However, after the carbohydrate reserves had been reconstituted, from stage 83 until stage 89 (berries ripe for harvest), other factors again increased the extent of GLSD symptoms, such as the prolonged susceptibility of pruning wounds (Serra *et al.*, 2008; Rolshausen *et al.*, 2010). These increased the probability of new infections in the course of the growing season, probably leading to greater amounts of toxins being produced by the fungi colonising the vinewood near the shoots and leaves (Mugnai *et al*., data unpublished). At growth stage 89 (berries ripe for harvest), increases of leaf symptoms were concomitant with increases of phytoalexins in symptomatic vines, compared to the previous stage 83 (berries developing colour). This increase of phytoalexins was observed in the leaves of all the types of vines, but in symptomatic vines the increases were more consistent compared to those observed in the leaves of the other two types of vines. At stage 89, symptomatic vines, significantly differentiated again from the other type of vines, as observed at stage 77 (berries beginning to touch).

The same factors that affect variations in the leaf symptoms may also affect the phytoalexin levels recorded during the various growth stages, but the extent to which these factors cause the leaf symptoms and produce higher levels of phytoalexins remains to be clarified.

In the present study, low levels of *trans*-resveratrol and other phytoalexins were found in asymptomatic/diseased vines in the first two growth stages monitored in 2012 and 2013: stage 71 (fruit set) and stage 75 (berries pea-sized). In both these stages, no GLSD symptoms were detected, and the levels of phytoalexins in asymptomatic/diseased leaves were low and did not differ from those in healthy leaves. In the next stage monitored, 77 (berries beginning to touch), GLSD symptoms began to appear and at the

same time that levels of some of these compounds (*trans*-resveratrol in 2012 and 2013, and δ-viniferin in 2012), began to increase, in asymptomatic/diseased leaves more greatly than in healthy leaves. These findings were consistent with previous studies which reported that resveratrol and other phenolic compounds increased in asymptomatic/diseased leaves once symptoms began to appear (Magnin-Robert *et al.*, 2011; Valtaud *et al.*, 2011; Lambert *et al.*, 2013). In other studies no alterations in photosynthesis in completely asymptomatic vine-shoots were detected, but they were found by Christen (2006) in asymptomatic leaves of vine-shoots bearing symptoms. These findings, together with those reported in the present study, on asymptomatic/diseased vines at growth stages 71 (fruit set) and 75 (berries pea-sized), justify the conclusion that phytoalexins are not involved in inhibiting the leaf symptoms that occur until stage 77 (berries beginning to touch). Rather, from the 77 growth stage, the higher levels of resveratrol and the other phytoalexins in tiger-striped than in asymptomatic/diseased leaves, indicate that these compounds only form in response to already existing lesions. These compounds probably do not prevent lesions that are in the process of being formed by toxic metabolites produced by wood-colonising fungi, and which are translocated to the leaves, similar to any biotic or abiotic stress factor that injures leaves (Smith, 1996; Bavaresco and Fregoni, 2001). The expression of the *PAL* and *STS* genes, which is induced in esca-diseased vine leaves even before symptoms appear, was suppressed in the green portions of the leaves, after the chlorotic/necrotic lesions appeared. Expression began only in the chlorotic areas, indicating that the host defence responses were low immediately before or during the onset of the symptoms (Magnin-Robert *et al.*, 2011). This shows that the phytoalexins found in the present study were synthesised after the onset of the leaf lesions, and not before. These lesions therefore resemble a hypersensitivity reaction, confirming what is reported in the literature about the synthesis of antimicrobial compounds after such lesions have formed (Heath, 2000).

Measurement of the phytoalexins in the present study made it possible to ascertain the pattern of their concentration over time, in diseased vines. This pattern was uniform from one growing season to the next, independently of the extent of symptoms that occurred, or variations in the amount of the phytoalexins extracted, which varied from one season to the next. The fact that phytoalexin levels were greater at growth stages 77 (berries beginning to touch) and 89 (berries ripe for harvest) in symptomatic leaves than they were in healthy leaves, indicates that host plant defence responses depend more on the particular physiological condition of the plant at those stages than on the translocation of toxins from pathogen-colonised wood. Plant growth variations, in particular the synthesis of carbohydrates, changes in the rate of carbohydrate translocation at the different growth stages, and the onset of new infections during the growing season, may affect the production of toxins released by the pathogens, the onset of symptoms, and the synthesis of stilbenes as a plant defence response. The stilbenes are produced as a defence response at higher concentrations in symptomatic leaves, and after the onset of symptoms, but their production is not sufficient to limit the severity of the symptoms. This would be consistent with the conclusions of Magnin-Robert *et al.* (2011) and Spagnolo *et al.* (2012), who reported that the anti-oxidant system of diseased vines was unable to cope with the oxidative stress produced by the onset of the leaf symptoms.

Literature cited

Amalfitano C., A. Evidente, G. Surico, S. Tegli, E. Bertelli and L. Mugnai, 2000. Phenols and stilbene polyphenols in the wood of esca-diseased grapevine. *Phytopathologia Mediterranea* 39, 178–183.

Andolfi A., L. Mugnai, J. Luque, G. Surico, A. Cimmino and A. Evidente, 2011. Phytotoxins produced by fungi associated with grapevine trunk diseases. *Toxins* 3, 12, 1569–1605.

Bavaresco L. and C. Fregoni, 2001: Physiological role and molecular aspects of grapevine stilbenic compounds. In: *Molecular Biology and Biotecnology of the grapevine* (K.A. Roubelakis-Angelakis Ed.), Kluwer Acad. Publ., Dordrecht, The Netherlands, 153–182.

Bertsch C., M. Ramírez-Suero, M. Magnin-Robert, P. Larignon, J. Chong, E. Abou-Mansour, A. Spagnolo, C. Clèment and F. Fontaine, 2013. Grapevine trunk diseases: complex and still poorly understood. *Plant Pathology* 62, 2, 243–265.

Borie B., P. Jeandet, A. Parize, R. Bessis and M. Adrian, 2004. Resveratrol and Stilbene Synthase mRNA Production in Grapevine Leaves Treated with Biotic and Abiotic Phytoalexin Elicitors. *American Journal of Enology and Viticulture* 55, 1, 60–64.

Calzarano F., A. Cichelli and M. Odoardi, 2001. Preliminary evaluation of variations in composition induced by esca on cv. Trebbiano D'Abruzzo grapes and wines. *Phytopathologia Mediterranea* 40, Supplement, S443–S448.

Calzarano F., L. Seghetti, M. Del Carlo and A. Cichelli, 2004.

Effect of esca on the quality of berries, musts and wines. *Phytopathologia Mediterranea* 43, 125–135.

Calzarano F. and Di Marco S., 2007. Wood discoloration and decay in grapevines with esca proper and their relationship with foliar symptoms. *Phytopathologia Mediterranea*, 46, 96–101.

Calzarano F., V. D'Agostino and M. Del Carlo, 2008. Trans-resveratrol extraction from grapevine: application to berries and leaves from vines affected by esca proper. *Analytical Letters* 41, 1–13.

Calzarano F., C. Amalfitano, L. Seghetti, and V. Cozzolino, 2009. Nutritional status of vines affected with esca proper. *Phytopathologia Mediterranea* 48, 20–31.

Calzarano F., V. D'Agostino, F. Osti and S. Di Marco, 2013. Levels of *trans*-resveratrol in leaves of vines affected with "esca". In: *Protection and Production in Viticulture, IOBC/WPRS Bulletin* Vol. 85, 129–136.

Calzarano F., S. Di Marco, V. D'Agostino, S. Schiff and L. Mugnai, 2014. Grapevine leaf stripe disease (esca complex) are reduced by a nutrients and seaweed mixture. *Phytopathologia Mediterranea* 53, 3, 543–558.

Christen D., 2006. *Towards an Integrative Management of Eutypa Dieback and Esca Disease of Grapevine*. Thesis of the Swiss Federal Institute of Technology, Zurich, Switzerland.

Cichewicz R.H. and S.A. Kouzi, 2002. Resveratrol oligomers: structure, chemistry, and biological activity. In: *Studies in Natural Products Chemistry*, Atta-ur-Rahman (Ed), Elsevier Science B.V., 26, 507–579.

Commun K., M.C. Mauro, Y. Chupeau, M. Boulay, M. Burrus, and P. Jeandet, 2003. Phytoalexin production in grapevine protoplasts during isolation and culture. *Plant Physiological Biochemistry* 41, 317–323.

Di Marco S., F. Osti, A. Mazzullo and A. Cesari, 2001. How iron could be involved in esca fungi development. *Phytopathologia. Mediterranea* 40, S449–S452.

Dufour M.C., C. Lambert, J. Bouscaut, J.M. Mérillon and M.F. Corio-Costet, 2013. Benzothiadiazole-primed defence responses and enhanced differential expression of defence genes in *Vitis vinifera* infected with biotrophic pathogens *Erysiphe necator* and *Plasmopara viticola*. *Plant Pathology* 62, 370–382.

Eaton C. J., P. Y. Dupont, P. Solomon, W. Clyton, B. Scott, and M. P. Cox, 2015. A core gene set describes the molecular basis of mutualism and antagonism in *Epichloë* spp. *Molecular Plant-Microbe Interactions*. 28(3), 218–231.

Evidente A., L. Sparapano, A. Andolfi and G. Bruno, 2000. Two naphthalenone pentaketides from liquid cultures of *Phaeoacremonium aleophilum*, a fungus associated with esca of grapevine. *Phytopathologia Mediterranea* 39, 162–168.

Feliciano A.J., A. Eskalen, and W.D. Gubler, 2004. Differential susceptibility of three grapevine cultivars to *Phaeomoniella chlamydospora* in California. *Phytopathologia Mediterranea* 43, 66–69.

Flamini R., M. De Rosso, F. De Marchi, A. Dalla Vedova, A. Panighel, M. Gardiman, I. Maoz and L. Bavaresco, 2013. An innovative approach to grape metabolomics: stilbene profiling by suspect screening analysis. *Metabolomics,* 9, 1243–1253.

Fontaine F., C. Pinto, J. Vallet, C. Clément, A. C. Gomes, and A. Spagnolo, 2016. The effects of grapevine trunk diseases (GTDs) on vine physiology. *European Journal of Plant Pathology*, 144(4), 707–721.

Gramaje D., L. Mostert, J. Z. Groenewald, and P. W. Crous, 2015. *Phaeoacremonium*: from esca disease to phaeohyphomycosis. *Fungal biology*, 119(9), 759–783.

Heath M.C., 2000. Hypersensitive response-related death. *Plant Molecular Biology* 44, 321–334.

Jeandet P., A. C. Douillet-Breuil, R. Bressis, S. Debord, M. Spaghi, and M. Adrian, 2002. Phytoalexins from the Vitaceae: Biosynthesis, phytoalexin gene expression in transgenic plants, antifungal activity, and metabolism. *Journal of Agricultural and Food Chemistry* 50, 2731–2741.

Keller M., C.C. Steel and G.L. Creasy, 2000. Stilbene accumulation in grapevine tissues: developmental and environmental effects. *Acta Horticolturae* 514, 275–286.

Lambert C., I.L.K. Khiook, S. Lucas, N. Télef-Micoleau, J.M. Mérillon and S. Cluzet, 2013. A faster and stronger defense response: one of the key elements in grapevine explaining its lower susceptibility to esca? *Phytopathology*, 103(10), 1028−1034.

Langcake P. and W.V. McCarty, 1979. The relationship of resveratrol production to infection of grapevine leaves by *Botrytis cinerea*. *Vitis* 18, 244–253.

Lebon G., G. Wojnarowiez, B. Holzapfel, F. Fontaine, N. Vaillant-Gaveau, and C. Clément, 2008. Sugars and flowering in the grapevine (*Vitis vinifera* L.). *Journal of Experimental Botany* 59(10), 2565–2578.

Lorenz D.H., K.W. Eichhorn, H. Bleiholder, R. Close, U. Meier and E. Weber, 1995. Phenological growth stages of the grapevine (*Vitis vinifera* L. ssp. *vinifera*). Encoding and description of the phenological stages of the grapevine according to the extended BBCH scheme. *Australian Journal of Grape and Wine Research* 1(2), 100–103.

Magnin-Robert M., P. Letousey, A. Spagnolo, F. Rabenoelina, L. Jacquens, L. Mercier, C. Clément and F. Fontaine, 2011. Leaf stripe form of esca induces alteration of photosynthesis and defence reactions in presymptomatic leaves. *Functional Plant Biology* 38, 856–866.

Marchi G., I.G. Pascoe and J. Edwards, 2001. Young esca in Australia. *Phytopathologia Mediterranea* 40 (3), 303-310.

Marchi G., F. Peduto, L. Mugnai, S. Di Marco, F. Calzarano and G. Surico, 2006. Some observations on the relationship on manifest and hidden esca to rainfall. *Phytopathologia Mediterranea* 45, 117–126.

Mazzullo A., S. Di Marco, F. Osti and A. Cesari, 2000. Bioassays on the activity of resveratrol, pterostilbene and phosphorous acid towards fungi associated with esca of grapevine. *Phytopathologia Mediterranea* 23(3), 357–365.

McKinney H.H., 1923. Influence of soil temperature and moisture on infection of wheat seedlings by *Helminthosporium sativum*. *Journal of Agricultural Research* 26, 195–218.

Mugnai L., A. Graniti and G. Surico, 1999. Esca (black measles) and brown wood streaking: two old and elusive diseases of grapevines. *Plant Disease* 83, 5, 404-418.

Osti F. and S. Di Marco, 2010. Iron-dependent, non-enzymatic processes promoted by *Phaeomoniella chlamydospora* and *Phaeoacremonium aleophilum*, agents of esca in grapevine.

Physiological and Molecular Plant Pathology 74, 309–316.
Pezet R., C. Perret, J.B. Jean-Denis, R. Tabacchi, K. Gindro and Viret O., 2003. δ-Viniferin, a resveratrol dehydrodimer: one of the major stilbenes synthesized by stressed grapevine leaves. *Journal of Agricultural and Food Chemistry* 51, 18, 5488–5492.
Pezet R., K. Gindro, O. Viret and H. Richter, 2004. Effects of resveratrol, viniferins and pterostilbene on *Plasmopara viticola* zoospore mobility and disease development. *Vitis* 43, 145–148.
Rolshausen P.E., J.R. Úrbez-Torres, S. Rooney-Latham, A. Eskalen, R.J. Smith, and W.D. Gubler, 2010. Evaluation of pruning wound susceptibility and protection against fungi associated with grapevine trunk diseases. *American Journal of Enology and Viticulture*, 61, 113–119.
Saigne-Soulard C., A. Abdelli-Belhadj, M. Télef-Micouleau, J. Bouscaut, S. Cluzet, M.F. Corio-Costet, and J. M. Mérillon, 2015. Oligosaccharides from *Botrytis cinerea* and elicitation of grapevine defense. *Polysaccharides: Bioactivity and Biotechnology* 939–958.
Serra S., M.A. Mannoni and V. Ligios, 2008. Studies on the susceptibility of pruning wounds to infection by fungi involved in grapevine wood diseases in Italy. *Phytopathologia Mediterranea* 47, 234–246.
Slaughter A.R., M. Md. Hamiduzzaman, K. Gindro, J.M. Neuhaus and B. Mauch-Mani, 2008. Beta-aminobutyric acid-induced resistance in grapevine against downy mildew: involvement of pterostilbene. *European Journal of Plant Pathology* 122, 185–195.
Smith C.J. 1996; Tansley review No. 86. Accumulation of phytoalexins: defence mechanism and stimulus response system. *New Phytologist* 132, 1–45.
Spagnolo A., M. Magnin-Robert, T.D. Alayi, C. Cilindre, L. Mercier, C. Schaeffer-Reiss and F. Fontaine, 2012. Physiological changes in green stems of *Vitis vinifera* L. cv. Chardonnay in response to esca proper and apoplexy revealed by proteomic and transcriptomic analyses. *Journal of Proteome Research*, 11(1), 461–475, doi:10.1021/pr200892g.
Sparapano L., G. Bruno and A. Graniti, 2001. Three-year observation of grapevines cross-inoculated with esca-associated fungi. *Phytopathologia Mediterranea* 40, 376–386.
Sumarah M.W., J.D. Miller and B.A. Blackwell, 2005. Isolation and metabolite production by *Penicillium roqueforti*, *P. paneum* and *P. crustosum* isolated in Canada. *Mycopathologia*, 159(4), 571–577.
Surico G., 2009. Towards a redefinition of the diseases within the esca complex. *Phytopathologia Mediterranea* 48, 5–10.
Surico G., G. Marchi, L. Mugnai and F. Peduto, 2010. Espressione dei sintomi fogliari dell'esca in funzione della disponibilità di acqua nel suolo e germogliamento delle gemme. In: *Il Mal dell'Esca della Vite: Interventi di Ricerca e Sperimentazione per il Contenimento della Malattia. Progetto MesVit*. (Surico G., Mugnai L., ed.), Arsia Regione Toscana, Firenze, Italy, 276–291.
Tabacchi R., A. Fkyerat, C. Poliart and G. Dubin, 2000. Phytotoxins from fungi of esca of grapevine. *Phytopathologia Mediterranea* 39, 156–161.
Valtaud C., F. Thibault, P. Larignon, C. Berstch, P. Fleurat-Lessard and A. Bourbouloux, 2011. Systemic damage in leaf metabolism caused by esca infection in grapevines. *Australian Journal of Grape and Wine Research* 17(1), 101–110, doi:10.1111/j.1755-0238.2010.00122.x.
Wang W., K. Tang, H.R. Yang, P.F. Wen, P. Zhang, H.L. Wang, 2010. Distribution of resveratrol and stilbene synthase in young grape plants (*Vitis vinifera* L. cv. Cabernet Sauvignon) and the effect of UV-C on its accumulation. *Plant Physiology and Biochemistry* 48, 142–152.

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