Stilbene polyphenols in the brown red wood of *Vitis vinifera* cv. Sangiovese affected by "esca proper"*

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Summary. A number of stilbene polyphenols, dimers, trimers and tetramers of resveratrol (viniferins), which are typical Vitaceae metabolites, were extracted from asymptomatic wood (AW) and symptomatic brown-red discoloured wood (BRW) of *Vitis vinifera* cv. Sangiovese affected by "esca proper", the trunk disease caused by the fungal complex *Phaeomoniella chlamydospora*, *Phaeoacremonium aleophilum* and *Fomitiporia mediterranea*. Resveratrol and the same types of viniferins were found in both AW and BRW, with the exception of α-viniferin, which was only detected in AW, and ampelopsin B, only in BRW. The total concentration of stilbene polyphenols was higher in symptomatic wood (3.7% in BRW vs 1.2% in AW). The absolute variations in molar concentrations of each stilbene polyphenol (i) between BRW and AW ($\Delta^i = C^i_{BRW} - C^i_{AW}$), were higher for ε-viniferin and resveratrol than the other compounds, while the relative variations (Δ^i/C^i_{AW}), were lower for ampelopsin H and isohopeaphenol, and higher for ampelopsin B, hopeaphenol, ampelopsin A, leachianol F and G, pallidol and ε-viniferin (in descending order), than the relative variation for resveratrol. Aspects relating to the biosynthesis of stilbene polyphenols and their role in the host-esca pathogen interaction are discussed.

Key words: *Vitis vinifera*, *Phaeomoniella chlamydospora*, *Phaeoacremonium aleophilum*, viniferins.

Introduction

Phaeomoniella chlamydospora (Pch) and *Phaeoacremonium aleophilum* (Pal) are fungal pathogens that commonly affect grapevine wood; both cause a vascular infection associated with the development of foliar chlorosis and necrosis described as tiger striping (Surico, 2009). The vascular infection can be superimposed or can coexist with other fungal infections of wood such as those caused by *Fomitiporia mediterranea* (Fmed), giving rise to the so-called "esca proper" syndrome, as well as by other disease agents affecting wood (*Eutypa lata* and Botryosphaeriaceae species) (Péros *et al.*, 2008; Úrbez-Torres *et al.*, 2006; Surico, 2009).

When Pch and/or Pal colonize the xylem vessels a common reaction of the infected tissues is to form a brown-red discoloration (BRW) in the affected wood, with necrosis and black streaking (Mugnai *et al.*, 1999). In contrast, infection by Fmed degrades the wood (white rot), which is often surrounded by a thin boundary of blackish tissue (Mugnai *et al.*, 1999).

In earlier work, Amalfitano *et al.* (2000) reported that BRW in Sangiovese vines colonised by Pch and Pal was accompanied by an increase in the concentrations of two stilbene polyphenols, *trans*-resveratrol (3, 5, 4'-trihydroxy-stilbene) and its dimer *trans*-ε-viniferin. Resveratrol and its oli-

gomers (viniferins) are a well known class of secondary metabolites in the Vitaceae, Fabaceae and other plant families, often considered as constitutive compounds, but also known as phytoalexins, antimicrobial substances synthesised in response to fungal infection (e.g. *Botrytis cinerea, Plasmopara viticola*) or in response to other stresses, including injuries and exposure to ultraviolet radiation (Langcake and Pryce, 1976; Langcake and McCarty, 1979; Langcake, 1981; Jeandet *et al.*, 1991; Gottstein and Gross, 1992; Soleas *et al.*, 1997; Bavaresco *et al.*, 1999; Cichewicz and Kouzi, 2002; Jeandet *et al.*, 2002; Borie *et al.*, 2004).

The aim of the present work was to compare the profile of resveratrol oligomers in asymptomatic wood (AW) and BRW of a "Sangiovese" grapevine naturally infected by Pch, Pal and Fmed. The data are discussed in the light of the specific activity of the fungi involved in the disease and the host-pathogen interaction.

Materials and methods

All solvents were of analytical or HPLC grade of purity, and were obtained from Carlo Erba (Milan, Italy). Deionised water was obtained from a MilliQ system (Millipore, Bedford, MA, USA).

One dimensional and two dimensional (COSY and HSQC) ^{1}H and ^{13}C NMR spectra were obtained using a Bruker spectrometer (Karlsruhe, Germany) operating at 400 MHz for H[1] in CD_3OD and/or $(CD_3)_2CO$ solution. Electrospray ionization mass spectra in positive and negative ion mode were recorded on an ESI-MS API 100 LC-MS spectrometer (Perkin Elmer, Norwalk, CT, USA). Optical rotation was measured on a P-1010 polarimeter (Jasco, Tokyo, Japan). The HPLC instrument (Kontron, Milan, Italy) comprised two pumps (model 422), a UV-double channel detector (model 433), and PC Integration pack software.

Isolation and purification of stilbene polyphenols

A vine (*Vitis vinifera,* cv. Sangiovese growing in Tuscany) showing the tiger-stripe necrosis and discolouration of leaves in summer was uprooted early in the following growing season. To isolate the viniferins, a portion of the wood showing brown-red discolouration was taken, the bark removed and discarded, the wood cut into chips and ground in liquid nitrogen as described previously (Amalfitano *et al.*, 2000).

The powder (334.0 g) was extracted using a ratio of 4.5 mL solvent g[-1] wood for 24 h, once in petroleum ether and three times in methanol. The evaporated methanol fraction yielded 13.5 g extract. Approximately 1.5 g of this extract was re-dissolved in 2.5 mL methanol, passed through 0.45 μm Anotop filters (Whatman, Maidstone, UK) and fractionated using HPLC by repeated injections of 20–50 μL of the dissolved extract. A reverse-phase semi-preparative column (LiCrospher 100 RP-18, 250×10 mm i.d., 10 μm particles, Merk, Darmstadt, Germany) fitted with a 2 μm filter (Alltech, Sedriano, Italy) was used. The methanol extract was eluted at a flow rate of 5 mL min^{-1} in a linear H_2O/CH_3CN concentration gradient from 20% to 33% CH_3CN over 90 min; the column was rebalanced to 20% CH_3CN within 5 min and maintained in isocratic conditions at least for a further 5 min before the next injection. The eluate was collected in an automatic fraction collector (HeliRac 2212 LKB, Bromma, Sweden). The UV-detector was set to 210 nm. Homogeneous fractions were combined, partially evaporated in a Rotavapor (Heidolph, Kelheim, Germany) at 35°C, and freeze-dried. Each fraction was then re-dissolved in a minimal volume of methanol, and re-chromatographed using the same conditions until at least 2 mg of each compound was obtained in a highly purified form; these compounds were then subjected to spectroscopic and physical analysis. The purified compounds were used as standards for quantitative HPLC analysis.

Quantitative HPLC analysis of methanol extracts

From the same plant used for isolation of metabolites, samples of BRW and of AW without any trace of black streaking taken from ≈30 cm below from the BRW area were selected. Each wood type (≈0.5 g) was chipped and reduced to powder as described above (Amalfitano *et al.*, 2000), before extraction in petroleum ether using a Soxhlet extractor (8 h). The residue was re-extracted in 10 mL methanol with shaking in subdued lighting for 4 h at room temperature, and the methanol extraction repeated overnight. The combined methanol extracts were dried in a Rotavapor at 35°C, yielding 6.92% organic extract for BRW and 5.57% for AW. An aliquot of residues was re-dissolved in methanol and 20 μL of the solution (containing 1.25 mg

residue mL^{-1}) was injected into the HPLC in duplicate. The following standards were used for the analyses: *trans*-resveratrol (Sigma-Aldrich, Seelze, Germany); *cis*-resveratrol, obtained by *trans*-resveratrol isomerisation induced by three hours irradiation at 365 nm (Trela and Waterhouse, 1996); pterostilbene (3,5-dimetoxy-4'-hydroxystilbene), obtained by synthesis. The pterostilbene was synthesised from 3,5-dimethoxyphenylacetic acid and *p*-hydroxybenzaldehyde (Merck) (Massarini, 1957), purified by preparative TLC (Kieselgel 60 F$_{254}$, 0.5 mm, Merck) and identified by ESI-MS and ^{1}H NMR analyses (Dawidar *et al.*, 1994). The different viniferin standards were isolated from the organic extracts of symptomatic wood. However, it was only possible to obtain a mixture of *cis*- and *trans*-ε-viniferin because the stilbene polyphenols were subject to isomerization during purification, therefore a mixture of *cis*- and *trans*-ε-viniferin in CD$_3$OD was used as the standard for both isomers. The concentrations of each ε-viniferin in the standard solution were calculated based on the total concentration and on the molar ratio in solution of *cis* and *trans* isomers. The molar ratio was determined by the ratio of the integration of ^{1}H NMR signals at 3.84 ppm and 4.22 ppm of the H8a of *cis* and *trans* forms, respectively (Figure 1). A standard solution containing both leachianol G and F was also used because the two compounds have the same retention times in HPLC and could not be purified using this method. For this reason the amounts of these compounds measured in the methanol extracts represent approximations.

HPLC analysis of the methanol extracts was performed using a LiCrospher column (100 RP-18, 250 × 4 mm i.d., 5 μm particle size, Merck) and the same elution gradient used to purify the viniferins, but with a flow rate of 1 mL min^{-1}. Resveratrol and all the viniferins, except pterostilbene, were quantified using this method. For pterostilbene a different elution gradient was necessary: eluent H$_2$O/CH$_3$CN, from CH$_3$CN at 20% up to 50% in 50 min, then to 20% in 5 min, followed by 10 min of isocratic elution. The chromatographic peaks were identified by comparing the average retention times based on two injections (variations ≤1 min) with retention times of the standards, and by the coincidence of peaks in the co-injection of sample extracts with the standards. Calibration curves for quantitative analysis were obtained by injection of standard methanol solutions at a minimum of three to a maximum of five different concentrations. Correlation coefficients (R^2) were ≥ 0.9935. The limit of quantitation (LOQ) was calculated from the standard curves using the equation: LOQ=2SD/s (SD: standard deviation of the calibration curve; s: chromatographic peak area μg^{-1} metabolite at the lower level of linear range) (Amalfitano *et al.*, 2002). The lowest and the highest LOQs were 0.0097 μg (*trans*-ε-viniferin) and 0.2 μg (ampelopsin B), respectively; only hopeaphenol in the AW methanol extract and miyabenol in the AW and BRW methanol extracts were beyond the respective LOQ limits. The chromatograms recorded at 290 nm were integrated for *trans*-resveratrol and ampelopsin B, and those recorded at 210 nm used for the other viniferins.

To support the hypothesis that the extraction procedure used for the isolation and determination of viniferins produced the isomerisation artifacts, *cis*-resveratrol and *cis*-ε-viniferin, a rapid, low temperature extraction of a current year lignified branch of grapevine (≈5 cm long and 1.5 cm diameter) from cultivars Trebbiano, Montepulciano and Sangiovese was carried out using acetone. Samples were cut into coarse discs (≈5 mm thick) and extracted in acetone for 1 hour at 4°C protected from light. Extracts were decanted and concentrated by partial solvent evaporation under nitrogen flux. The extracts were immediately injected into HPLC and eluted as described above.

Results and discussion

Isolation and identification of stilbenic polyphenols

Fourteen stilbene polyphenols were isolated from the diseased Sangiovese grapevine wood (Figure 1). The ^{1}H and ^{13}C NMR spectra and the specific optical rotations obtained here were identical to those reported in the literature. These metabolites have been reported in the Vitaceae, mostly in tissues other than the wood (Cichewicz and Kouzi, 2002). In addition to the *cis* and *trans* isomers of resveratrol (Deak and Falk, 2003), the dimers of this stilbene, (+)-pallidol (Khan *et al.*, 1986; Delaunay *et al.*, 2002), and (+)-*cis* and *trans*-ε-viniferin (Langcake and Pryce, 1977; Kurihara *et al.*, 1990; Bala *et al.*, 2000), were found. Other dimers identified were (+)-ampelopsin A and B (Reniero *et al.*, 1996; Ito *et al.*, 1998), and leachianol G and F
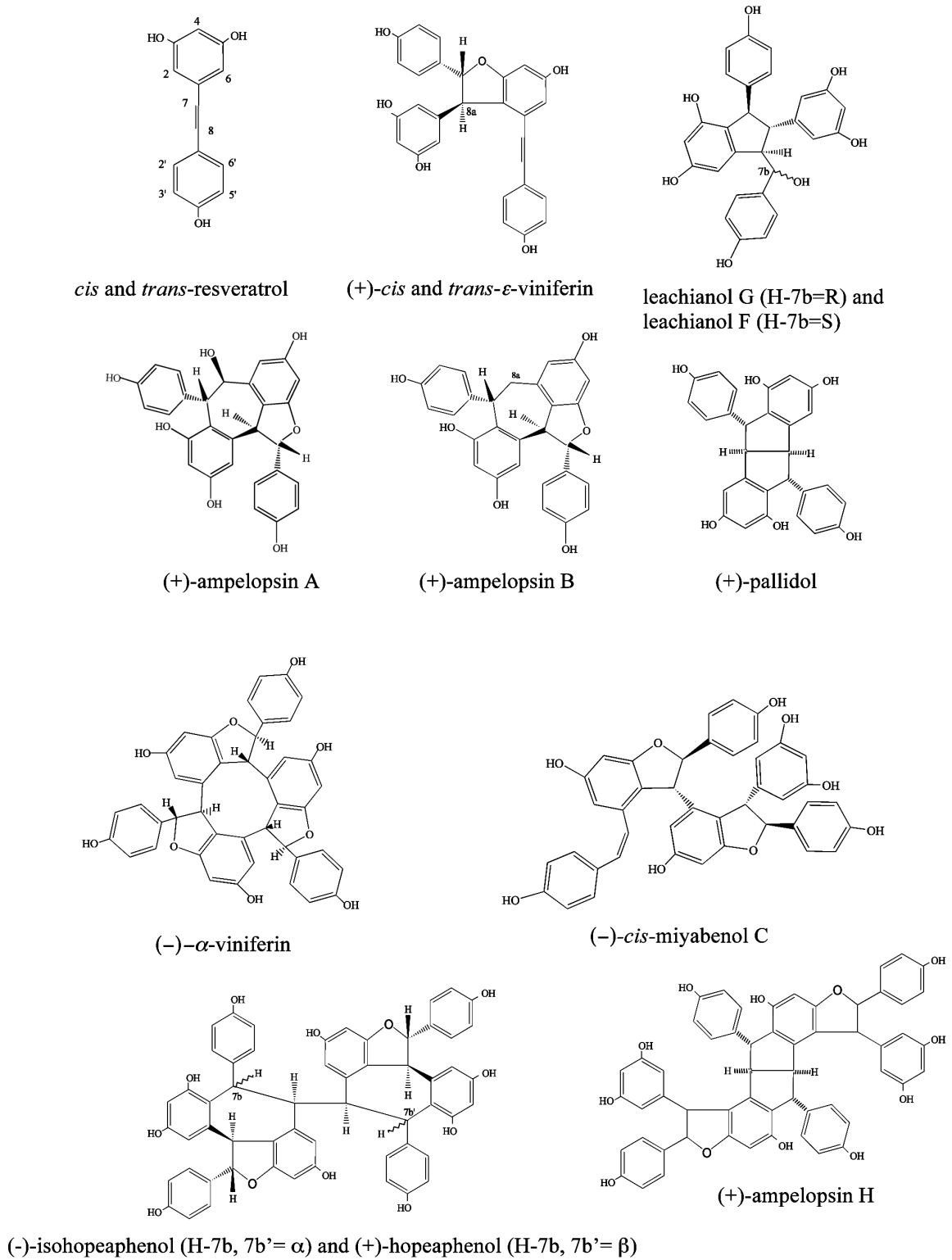
cis and *trans*-resveratrol

(+)-*cis* and *trans*-ε-viniferin

leachianol G (H-7b=R) and
leachianol F (H-7b=S)

(+)-ampelopsin A

(+)-ampelopsin B

(+)-pallidol

(−)−α-viniferin

(−)-*cis*-miyabenol C

(-)-isohopeaphenol (H-7b, 7b'= α) and (+)-hopeaphenol (H-7b, 7b'= β)

(+)-ampelopsin H

Figure 1. Structures of stilbene polyphenols isolated from wood of *Vitis vinifera* cv. Sangiovese.

(Ohyama *et al*., 1995). The latter dimers, with the exception of ampelopsin A, have not been reported in *Vitis vinifera* previously. Also isolated were the trimers (-)-α-viniferin (Pryce and Langcake, 1977; Kitakana *et al*., 1990) and (-)-*cis*-miyabenol C (Kurihara *et al*., 1991; Meng *et al*., 2001; Barjot *et al*., 2007), and the tetramers (+)-hopeaphenol, (-)-isohopeaphenol (Ito *et al*., 1997; Takaya *et al*., 2002) and, for the first time in *Vitis vinifera*, ampelopsin H (Oshima and Ueno, 1993). The absolute configurations of all these metabolites, except for pallidol, α-viniferin and ampelopsin H, are known. Together with the stilbene polyphenols, the flavan epicatechin was isolated from the wood.

Following repeated purifications, (+)-*trans*-ε-viniferin was always obtained together with considerable quantities of (+)-*cis*-ε-viniferin, despite the favourable chromatographic separation conditions (difference in retention times of 7.9 min). The *cis* form detected was probably due to the isomerization of the *trans* form during the extraction and purification processes. This finding suggests that more caution should be exercised during these phases. Actually, the rapid cold acetone extraction of wood from branches of healthy grapevine cultivars (Trebbiano, Montepulciano and Sangiovese), followed immediately by HPLC analysis, revealed only the *trans*-ε-viniferin isomer. Moreover, *cis*-resveratrol was not detected in these extracts, which suggests that, as for *trans*-ε-viniferin, the presence of this stereoisomer in the extracts of woods from the diseased plant can be ascribed to the isomerisation of *trans*-resveratrol.

The fact that resveratrol and ε-viniferin are present in healthy vines indicates that these metabolites are constitutive in the wood tissue and should, therefore, be classified as phytoanticipins in the wood, as they increase in concentration when the plants are under stress, while they can act as phytoalexins in the foliage (Van Etten *et al*., 1994). On the other hand, it is not possible to exclude that the metabolites formed following some form of stress which the plants have undergone, even just the stress that the branch samples were certainly subjected to when they were cut for metabolite extraction. In this case resveratrol and ε-viniferin should be classified as phytoalexins. However, some authors have reported resveratrol and viniferins in tree heartwood and bark (Langcake and Pryce, 1976; Soleas *et al*., 1997; Cichewicz

and Kouzi, 2002), and the formation of these dead tissues is induced by physiological oxidative processes (Plomion *et al.,* 2001), which also characterize stress responses. This process could occur even during wood formation in vine although it does not produce distinct heartwood in the trunk.

HPLC analysis of wood methanol extracts

Chromatograms of the methanol extracts from BRW and AW were qualitatively similar (Figure 2). Pterostilbene was not detected in either type of wood: no significant peak appeared at the retention time corresponding to the standard. This result was confirmed by co-injection of the pterostilbene standard.

Quantities of identified polyphenols detected in BRW and AW are shown in Table 1, where the combined amounts of *trans* and *cis*-ε-viniferin and of *trans* and *cis*-resveratrol were taken to reflect the true amounts of *trans*-ε-viniferin and *trans*-resveratrol, as the *cis* forms were assumed to be artefacts (see Isolation and identification of stilbene polyphenols, above). Data for *cis*-miyabenol C are not reported because its peak in both methanol extracts were lower than the LOQ. Amounts of epicatechin also are not reported due to partial overlapping of the peak for this compound (3.8 min) and the consequent uncertainty regarding integration of the peak.

Overall, higher amounts of the identified polyphenols were found in BRW (3.7% corresponding to 53.1% of the BRW methanol extract) than in AW (1.2%, corresponding to 20.7% of the AW methanol extract). This finding confirms that these typical stress metabolites of grapevine accumulated in the plant in infected wood (Amalfitano *et al*., 2000; Cichewicz and Kouzi, 2002). In BRW resveratrol and ε-viniferin were present in the highest concentrations reaching tens of μmol g^{-1} compared with the other resveratrol oligomers, which were present in μmol g^{-1} quantities. The HPLC method used in the present work detected higher quantities of resveratrol and, in particular, ε-viniferin than those reported in earlier work (Amalfitano *et al*., 2000) in which the two metabolites were isolated and quantified by laborious procedures which probably resulted in significant losses. Quantitative data on resveratrol and ε-viniferin in diseased vinewood are lacking in the literature. However, the concentrations of these polyphenols
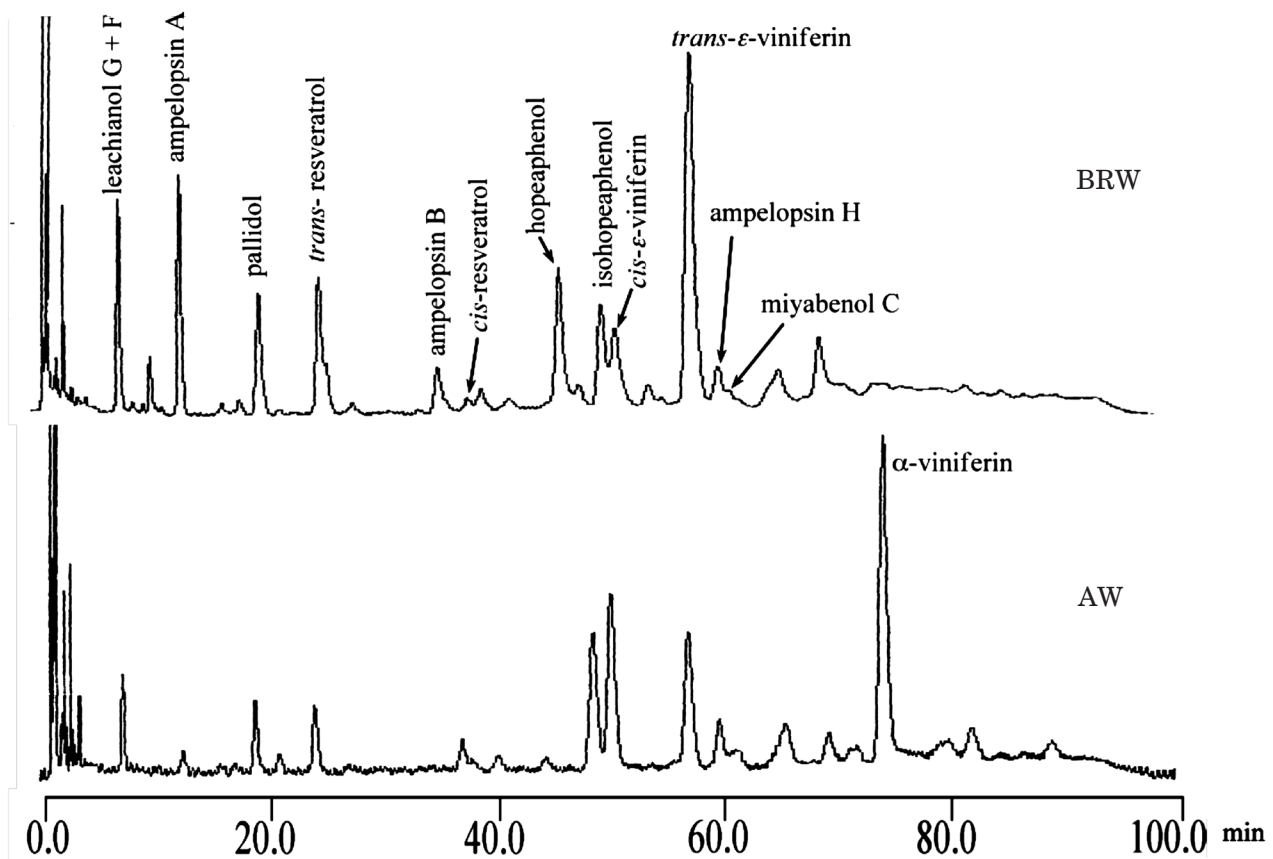
Figure 2. Chromatograms of methanolic extracts of brown-red wood (BRW) and asymptomatic wood (AW).

found in symptomatic wood in this study were from one to three orders of magnitude higher than the amounts of the same polyphenols reported by others in the leaves and berries of *V. vinifera* a few days after inoculation with *Plasmopara viticola, Botrytis cinerea* and other pathogens, or after UV irradiation (Langcake and Pryce, 1976; Langcake and McCarty, 1979; Langcake, 1981; Pool *et al.*, 1981; Sarig *et al.*, 1997).

Ampelopsin B, a reduced dimer of resveratrol at alkyl carbon C8a (Figure 1) occurs in Iris seeds (Keckeis *et al.*, 2000) and was originally isolated from the roots of a *Ampelopsis brevipeduncolata* (Oshima and Ueno, 1993), was found only in the BRW.

As in BRW, in AW the quantities of resveratrol and ε-viniferin were higher than those reported from the more complex isolation procedure (Amalfitano *et al.*, 2000). Resveratrol quantities, however, were similar to those reported in freshly pruned branches of healthy grapevine cv. Müller-Thurgau (Langcake and Pryce, 1976). No data on concentrations present in grapevine wood are available for the other viniferins. However, in order to determine whether the amounts of polyphenols detected in AW are due to a systemic response of the plant to disease (Langcake and McCarty, 1979), comparison with healthy reference vines is required.

In AW α-viniferin was the most abundant amongst the identified viniferins. Langcake (1981) reported that in leaves inoculated with *B. cinerea* and *P. viticola*, α-viniferin, which is more active than resveratrol against growth of the two fungi, begins to accumulate in the infection area only after the first rise in concentrations of resvera-

trol and ε-viniferin. Thus, in the hypothesis that the same pattern may occur in woody tissues, the high α-viniferin content in AW indicated a state of stress in the entire plant that induced its accumulation, although we cannot exclude the possibility that α-viniferin could be a normal viniferin that accumulates in wood. However, the absence of α-viniferin in BRW, in contrast to the general increases in concentrations in other viniferins in the altered wood, is surprising. Anyway, although Langcake (1981) described a general increase in α-viniferin quantities in vine leaves inoculaed with *P. viticola* within four days, the compound was undetectable at the seventh day.

Molar variations in the content of each metabolite (i) between BRW and AW, $\Delta^i = C^i_{BRW} - C^i_{AW}$, and the variation in relative terms, Δ^i/C^i_{AW}, were not homogeneous (Table 1). It is possible that the host has differing abilities to biosynthesize each metabolite, but the values may have been influenced by other factors, including: the relative capacities of each metabolite to spread within the wood; the differing abilities of the pathogens to degrade the viniferins, or, differences in partial immobilisation of the metabolites in wood, rendering them unextractable with the methods used here. However, the hypothesis of a transformation caused by specific activities of the fungi in BRW (Pch and Pal)

Table 1. Concentrations and variations in stilbene polyphenols in asymptomatic wood (AW) and brown-red wood (BRW).

Stilbene	Wood symptom	Concentration in wood		Molar variation [a] $\Delta^i=(C^i_{BRW}-C^i_{AW})$	Relative variation Δ^i/C^i_{AW}
		mg g^{-1}	μmol g^{-1}		
trans+cis-resveratrol	AW	0.78	3.4	13	3.8
	BRW	3.6	16		
trans+cis-ε-viniferin	AW	2.5	5.5	26	4.7
	BRW	14	31		
Leachianol F + G	AW	0.35	0.74	5.1	6.9
	BRW	2.8	5.8		
Pallidol	AW	0.41	0.9	4.8	5.3
	BRW	2.6	5.7		
Ampelopsin A	AW	0.12	0.25	5.5	22
	BRW	2.7	5.7		
Ampelopsin B	AW	nd	nd	1.1	∞
	BRW	0.49	1.1		
α-Viniferin	AW	4.7	6.9	-6.9	-1.0
	BRW	nd	nd		
Hopeaphenol	AW	<0.18	<0.2	>5.3	>27
	BRW	5.0	5.5		
Isohopeaphenol	AW	1.5	1.6	1.9	1.2
	BRW	3.2	3.5		
Ampelopsin H	AW	1.1	1.2	1.6	1.3
	BRW	2.5	2.8		
Total	AW	12	21		
	BRW	37	77		

[a] C^i_{BRW} and C^i_{AW}: the molar concentration of "i" metabolite in BRW and AW, respectively.
nd, Not determined.

is not tenable, because such fungi exhibited limited phenoloxidase activity *in vitro* and were able to utilize phenolic substrates only when carbohydrates were absent in the substrate (Mugnai *et al.*, 1999; Bruno and Sparapano, 2006; Santos *et al.*, 2006), whilst carbohydrates are certainly present in BRW (Agrelli *et al.*, 2009). Furthermore, although it has been demonstrated that the stilbene polyphenols are involved in radical condensation processes in BRW (Agrelli *et al.*, 2009), resulting in transformation to methanol-insoluble compounds, the individual metabolites probably do not differ greatly in reactivity. In addition diffusion of stilbene polyphenols is probably negligible. Thus, the recorded accumulations could reflect the actual ability of the host to biosynthesise these metabolites.

The relative increase in resveratrol in BRW compared to AW was lower than that of ε-viniferin, although high conversion of ε-viniferin presumably did occur, as suggested by the increases detected in concentrations of most other viniferins that probably arose from oxidative condensation of ε-viniferin. Thus, it is likely that an extremely rapid dimerization of resveratrol occurred, to produce ε-viniferin that accumulated in BRW during the host response.

Given the presence in both BRW and AW of the same viniferins with few exceptions (α-viniferin and ampelopsin B), and the absence of evident alteration products from viniferins in the HPLC pattern of the BRW extract, it would appear that the fungi are neither able to block the synthesis, nor to degrade these compounds. It is more likely that the activities of resveratrol synthase and, more significantly, peroxidises (leading to resveratrol condensation to form viniferins) increased in the host, as also commonly occurs in abiotic stresses (Cichewicz and Kouzi, 2002).

Viniferin biosyntheses driven by peroxidases could have increased in response to the stress of infection by means of a higher expression of the enzyme and also by a higher activity of oxygenated radicals (ROS), which typically also act as cell signalling agents in stress responses (Mittler, 2002). However, ROS could react, not only as electron acceptors from stilbene polyphenols, but also as a source of radicals that can cause additional reactions on polyphenols. Thus, increasing amounts of ROS could have caused the high relative increase (Δ^i/C^i_{AW}, Table 1) in the hydroxylated viniferins ampelopsin A, and leachianol G and F. These viniferins derive from the addition of ·OH to intermediate radicals at a level of the olefinic bonds, which originate from ε-viniferin and dimerized resveratrol (C7 and C2 linked to the C7 and C8 of another resveratrol unit) that otherwise would evolve towards hopeaphenol and pallidol, respectively (Takaya *et al.*, 2002; Takaya *et al.*, 2005). Furthermore, ampelopsin B, which is present only in BRW, may be a product of radical addition between the above-mentioned intermediate radical in hopeaphenol synthesis and the ·H extracted from the abundant polyphenols in BRW. However, ampelopsin B could also be the product of an intra-condensation reaction of ε-viniferin in an acidic environment (Takaya *et al.*, 2002), which may arise due to protons and acidic metabolites released by fungi in infected wood (Deacon, 2000). It is worth noting that in the laboratory synthesis of certain viniferins without control for stereospecificity, a prevailing stereoisomer is obtained amongst various reaction products (Takaya *et al.*, 2002; Takaya *et al.*, 2005). Therefore, because of the radical-mediated reactions and the intra-condensation described above, a stereospecific product may result and be evident in the wood even in the absence of any dirigent proteins, which are generally thought to be essential to determine stereospecificity (Cichewicz and Kouzy, 2000).

Despite the accumulation of polyphenols in BRW, the invasion of the host by the fungi Pch and Pal is not blocked. In fact, it has been shown that these fungi can grow on substrates containing various kinds of polyphenols (Mugnai *et al.*, 1999) or even resveratrol itself (Bruno and Sparapano, 2006); it was also found that spores of the fungi germinated in the presence of stilbene polyphenols extracted from the wood of similar diseased grapevine (Amalfitano *et al.*, 2010). Furthermore, despite the high polyphenol concentration, Pch and Pal infection does not induce the formation of necrotic dark barriers, which are phenolic in nature (reaction zones), around the area of browning wood, except in the portion that eventually is exposed to air near to wounds; generally this reaction occurs in pruning wounds on healthy plants, and at the interface with white rot. This latter reaction is conceivably due to the activity of the white rot fungus rather than to Pch and Pal,

given that the dark barrier is also present at the healthy wood-white rot interface (Mugnai *et al.*, 1999). Furthermore, the formation of a dark barrier is typical for wood decay by white rot fungi (Pearce, 1996). This suggests that BRW maintains the ability to respond and does not show a typical hypersensitive response against Pch and Pal, even though the susceptibility of grapevine cells to extracellular substances from Pch and Pal has been ascertained (Luini *et al.*, 2010). On the other hand these fungi, in particular Pch, do not seem to be highly aggressive towards parenchyma cells in the wood tissues, since they preferentially invade the vessel lumen (Troccoli *et al.*, 2001, Valtaud *et al.*, 2009). However, the growth of the fungal hyphae does lead to embolisms in the xylem, which could also extend into the surrounding but still uninfected vessels (Edwards *et al.*, 2007) causing the host response. There the ability of the host to occlude injured vessels will protect the wood tissues from the entry of air. This response, comprising principally the obstructions of vessels with polyphenols and pectic substances (Agrelli *et al.*, 2009), in itself could act as an aid rather than an hindrance to the expansion of fungi in these cavities, since pectin is an excellent substrate for the nutrition of Pch and Pal that show pectinolytic activity (Marchi *et al.*, 2001) and the fungi are not inhibited by the polyphenols. Furthermore, Pch and Pal probably tolerate low oxygen activity, which is likely to occur in this wood following embolism of xylem vessels with the subsequent obstruction of the vessels and oxygen consumption due to the host oxidative response. On the other hand the slow growth rates of Pch and Pal are well known (Mugnai *et al.*, 1999), consistent with low metabolic activities in these fungi.

Although some antagonism between the fungi present in the diseased grapevine has been reported, in particular between Fmed and Pal (Sparapano *et al.*, 2000), it is also likely that until there are high concentrations of free polyphenols in BRW, where mainly Pch and Pal are present, this wood zone will be particularly hostile to invasion by Fmed. Fmed, as with several other Hymenomycetes, can be inhibited by soluble phenolic metabolites (Shortle and Cowling, 1978; Pearce, 1996; Mugnai *et al.*, 1997). However, like other white rot fungi with substantial oxidase activities (Deacon, 2000), Fmed causes darkening of the wood through condensation and precipitation of polyphenols before the definitive invasion of the wood (Shortle and Cowling, 1978); it is no coincidence that many of these fungi are more active on dead wood, in which no host response resulting in production of polyphenols can occur. However, Pch and Pal infection could, over time, facilitate the entry of Fmed into BRW, due to the slow process of decay and complete death of BRW tissue (with the consequent inability of the host to produce polyphenols) and the simultaneous progressive condensation of soluble polyphenols with lignin (Agrelli *et al.*, 2009). It should be noted, however, that condensation of these response polyphenols with lignin could render this biopolymer recalcitrant to attack by Fmed (Agrelli *et al.*, 2009). This process does not imply any Pch and Pal detoxification of polyphenols, and in fact, there was no evidence of degraded stilbene polyphenols in the methanol extract from BRW. However, the ability of Fmed to grow in culture substrates containing polyphenols, after Pch and Pal were grown (Mugnai *et al.*, 1999), as well as the disappearance of resveratrol in growth medium of those fungi (Bruno and Sparapano, 2006) has been demonstrated. Rather than indicating specific degradative activity, however, this finding could reflect the ability of Pch and Pal to induce a local oxidative environment which causes the oxidation and condensation of polyphenols (Agrelli *et al.*, 2009). The release by fungi of H_2O_2 and chelators including secondary metabolites (Evidente *et al.*, 2000; Tabacchi *et al.*, 2000) in the presence of microelements such as iron could promote that process (without taking into account the additional oxidative effects of atmospheric oxygen) (Goodell *et al.*, 1997; Sakihama *et al.*, 2002, Osti and Di Marco, 2010). Instead, *in vivo* the oxidative processes on polyphenols are performed effectively by the oxidative response of the host.

Conclusions

The purpose of the work described here was to determine the secondary metabolites produced in the wood of grapevine plants (cv. Sangiovese) under the stress induced by esca proper. A total of fourteen stilbene polyphenols typical of the Vitaceae were identified in symptomatic brown red wood (BRW), and asymptomatic wood (AW) from the same grapevine plant. Amongst these compounds,

it appeared that *cis*-resveratrol and *cis*-ε-viniferin were extraction artefacts of the respective *trans* forms present in the plant.

The qualitative composition of methanolic extracts from BRW and AW were similar in terms of chromatographic profiles, whereas quantitative analysis demonstrated higher concentrations of the identified stilbene polyphenols in BRW than AW, the sole exception being α-viniferin. ε-Viniferin was the most abundant stilbene polyphenol in BRW (in the order of tens of μmol g^{-1} of wood) followed by resveratrol. The accumulation of polyphenols is a consequence of the host oxidative response in BRW; this response, if not attributable to the specific interaction with the pathogen, is caused at least by xylem embolism which is due to the growth of the tracheomycotic fungi Pch and Pal in the vessels. In addition, because the relative increases in particular viniferins in BRW (such as the tetramer hopeaphenol and the dimers ampelopsin A and B) was higher than in AW, it was deduced that the accumulation of ROS in the oxidative response, leads to radical-mediated reactions between polyphenols (involving in particular the probable precursor ε-viniferin) and the addition of oxygenated radicals on the reaction intermediates of the biosynthesis of resveratrol oligomers.

On the basis of the polyphenolic profile found in grapevine wood, as well as the literature on polyphenol interactions *in vitro* with the esca fungi, and the morphology of decayed wood tissues, conclusions on the mode of action of the pathogens were made. In BRW, Pch and Pal are able to induce the accumulation of stilbene polyphenols but are unable to transform/detoxify these compounds, as supported by the similar qualitative stilbene polyphenol composition of BRW and AW. Furthermore, the fungi essentially tolerate resveratrol and viniferins and do not induce a boundary/reaction zone of condensed polyphenols in the BRW, but merely a general brownish-red discolouration. All these conclusions are consistent with the recognized inability of the host to control the expansion of these fungi.

In addition, it is hypothesized that the BRW, which accumulates resveratrol and viniferins, can resist invasion by fungi that are susceptible to soluble polyphenols, such as the white rot agent Fmed, as long as this wood retains the ability produce large amounts of polyphenols. As BRW degradation proceeds, polyphenol condensation, which invariably develops in BRW, will prevail over polyphenol production and Fmed, although able also to act as a primary pathogen (Surico *et al.*, 2006), will grow more easily in such wood.

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