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Phenols and stilbene polyphenols in the wood of esca-diseased grapevines

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Summary. *Trans*-resveratrol and ϵ -viniferin, already described as stress metabolites produced by the leaves of *Vitis vinifera* in response to fungal infection, UV irradiation, and incubation by chemicals, have been detected in the wood of healthy as well as in the brown-red wood of esca-diseased grapevines. Brown-red wood is considered an initial symptom of esca in grapevine. Resveratrol was the predominant component although ϵ -viniferin was also accumulated in appreciable quantities. The biological significance of the production of resveratrol and ϵ -viniferin is discussed.

Key words: *Vitis vinifera*, esca, resveratrol, ϵ -viniferin, stress metabolites.

Introduction

Resveratrol (3,5,4'-trihydroxy stilbene, **1** in Fig. 1) and biosynthetically related compounds, generally called viniferins (Langcake and Pryce, 1977), are a well-known class of stress metabolites found at different concentrations in all parts of various *Vitis* plants (Langcake and Pryce, 1976, 1977; Pryce and Langcake, 1977; Langcake *et al.*, 1979; Hart, 1981). The viniferins are dimers and oligomers of resveratrol deriving from an oxidative dehydrogenation condensation process. All these compounds are fungitoxic.

Resveratrol and viniferin production in the

leaves and berries of the cultivated grapevine (*Vitis vinifera*) has been investigated mainly in relation to resistance to the pathogens *Botrytis cinerea* (grey mould) and *Plasmopara viticola* (downy mildew) (Langcake and McCarthy, 1979; Langcake, 1981; Jeandet *et al.*, 1991). No work has yet been published examining whether these compounds are produced *in vivo* in response to infection leading to esca.

In the trunk of grapevines the first reaction of the woody tissues to invasion by fungi associated with esca (*Phaeoacremonium aleophilum*, *P. chlamydosporum* and *Fomitiporia punctata*) is the formation of black streaks and brown-red wood (Mugnai *et al.*, 1996, 1999). It is thus possible that in infected tissue, and in particular in brown-red wood, the presence of antifungal compounds, including resveratrol, may interfere with the activi-

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ty of the above-mentioned fungi and therefore with the disease process. In this paper we present preliminary information on the accumulation of stress metabolites, acting as phytoalexins, in the brown-red wood of esca-diseased vines.

Materials and methods

General experimental procedures

The UV-visible and IR spectra were recorded, respectively, in a methanol solution on an UV-visible Lambda 3B, and by thin layer method with KBr window on an FT-IR 1720X, both Perkin Elmer (Norwalk, CT, USA) instruments. The ^1H and ^{13}C NMR spectra were recorded at 400 and 500 MHz and at 100 and 125 MHz respectively on Bruker (Karlsruhe, Germany) instruments in CD_3OD used as internal standard. Carbon multiplicities were determined by DEPT [Distortion Enhancement by Polarisation Transfer] experiments. Mass spectra EI-MS and ES-MS were taken at 70 eV on a Fisons Trio-2000 (Rodano, Italy) and a Perkin Elmer API 100 LC-MS respectively. FAB-MS spectra were recorded on a ZAB 2SE VG, (Manchester, UK) in a glycerol/tioglycerol matrix. Analytical and preparative thin-layer chromatography (TLC) was performed on silica gel Kieselgel 60 F_{254} , 0.25 and 0.5 mm from Merck (Darmstadt, Germany), and a reverse-phase Stratocrom, KC-18, 0.20 mm, from Whatman (Clifton, NJ, USA) plates. The TLC spots were detected by UV light and developing colours by spraying plates with 10% H_2SO_4 and then with 5% phosphomolybdic acid methanol solutions, followed by heating for 10 min at 110°C , and, specifically for phenols, by spraying with a 1% ferric chloride aqueous solution and then heating at 110°C . All organic solvents were of analytical grade and supplied by Carlo Erba (Milan, Italy); the water used was purified with a Milli-Q system (Millipore, Bedford, MA, USA). Analytical quantitative data are the means of triplicate procedures.

Wood sample collection and polyphenols extraction

Wood from two esca-diseased grapevine plants, A and B, grown in Tuscany (Italy), was used. The trunk was sectioned longitudinally and small pieces of brown-red wood were carefully excised with the aid of a scalpel. From the same plants portions of healthy wood were also taken. The wood samples were lyophilised and then milled under nitro-

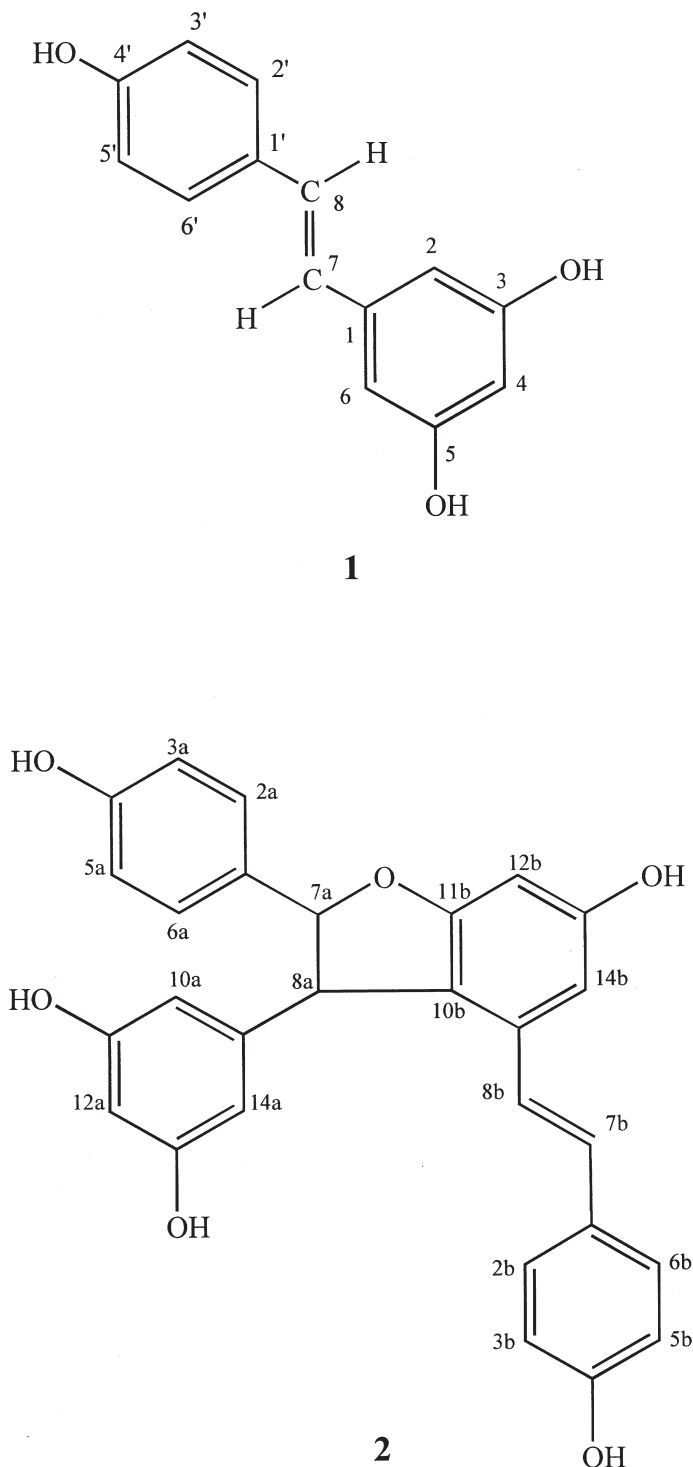


Fig. 1. Structural formulae of *trans*-resveratrol (1) and ϵ -viniferin (2).

gen to obtain about 2 g fine powders of both brown-red (BRW) and healthy (HW) wood. A check for the best solvent mixture to extract polyphenols was carried out using methanol-water and ethanol-water mixtures in different ratios on small portions of the wood powders. Polyphenols were extracted twice (the first time for 3 h and the second time overnight in the dark) in a ratio of 70 ml solvent mixture to 5 g wood. Phenol contents were colorimetrically determined using the Folin-Ciocalteu reagent with resveratrol as standard. On the basis of these tests, equal amounts of HW and BRW (1.50 g from plant A and 1.75 g from plant B) were extracted with the ethanol-water mixture in an 8:2 ratio. The extracts were filtered and evaporated under a rotating vacuum apparatus at 45°C. The residues were then re-dissolved in water and lyophilised to obtain the amount of extract shown in Table 1.

The residues were re-dissolved in water (5 mg/3 ml) and extracted three times with equal volumes of ethyl acetate. The organic extracts were mixed and dried with anhydrous sodium sulphate and then filtered. Water residues were partially evaporated in a rotating vacuum apparatus at 45°C and then lyophilised. All absolute yields are shown in Table 1.

Isolation and purification of polyphenols

Both water and organic extracts of HW and BRW were analysed by silica gel and reverse phase TLC using different elution mixtures. The organ-

ic extract was definitively eluted by chloroform-methanol 8:2 (eluent A) and water residue by butanol-acetic acid-water 6:1.5:2.5 (eluent B), on silica gel TLC. On the basis of such analyses and for the reasons discussed below, two chromatographic bands of organic extract were purified on preparative silica gel TLC at R_fs 0.58 and 0.37 by elution once or twice with eluent A. The pure constituent corresponding to the band at R_f 0.58 was identified as resveratrol (**1** in Fig. 1) obtained in the amounts shown in Table 1. The mixture corresponding to the band at R_f 0.37 was purified by reverse phase-TLC using elution with water-methanol in a 4:6 ratio (eluent C). It consisted of two different compounds for HW (R_fs 0.35 and 0.40) and three compounds for BRW (R_fs 0.35, 0.40 and 0.47) of which the compound at R_f 0.35 was identified as ϵ -viniferin (**2** in Fig. 1); identification of the other two, at R_fs 0.40 and 0.47, named **a** and **b**, is in progress. All the absolute yields of these compounds are shown in Table 1.

Resveratrol identification

Resveratrol (**1** in Fig. 1) was isolated as small white crystals. Its UV λ_{\max} nm (log ϵ): 306 (4.33) and 318 (4.32). IR ν_{\max} cm⁻¹: 3250, 1587, 1513, 1324, 1266, 1154, 964, 833. EI-MS m/z : 228 [M]⁺, 147 [M - 2xC₂H₂ - CO - H]⁺ and 107 [M - C₂H₂ - C₅H₃O₂]⁺. FAB-MS m/z : 229 [MH]⁺, 201 [MH - CO]⁺ and 197 [MH - CO - 4xH]⁺. ¹H and ¹³C NMR data are given in Table 2.

Table 1. Absolute amounts (mg) of extracts, residues and purified compounds from healthy wood (HW) and brown-red wood (BRW).

Extracts and purified compounds	Plant A		Plant B	
	HW (1.50 g) ^a	BRW (1.50 g) ^a	HW (1.75 g) ^a	BRW (1.75 g) ^a
EtOH-H ₂ O extract (mg)	71.2	86.5	88.9	94.1
EtOAc extract	17.3	70.3	21.3	70.4
H ₂ O residue	42.4	20.1	64.9	22.7
Resveratrol	0.2	8.5	0.3	8.2
ϵ -viniferin	0.7 ^b	4.0	0.7 ^b	7.1
a		5.1		0.8
b		0.2		1.2
	Not detected		Not detected	

^a Weight of sample.

^b Represents the total amounts of ϵ -viniferin plus **a**.

Table 2. ^{13}C and ^1H NMR data of resveratrol.

C	δC (ppm)	m	δH (ppm)	m	J (Hz)
1	139.9	s			
2,6	104.5	d	6.48	d	2.1
3,5	158.2	s			
4	101.3	d	6.20	t	2.1
7	128.1	d	6.98	d	16.3
8	125.7	d	6.82	d	16.3
1'	128.9	s			
2',6'	127.4	d	7.38	d	8.5
3',5'	115.1	d	6.80	d	8.5
4'	156.9	s			

Identification of ϵ -viniferin

ϵ -viniferin (**2** in Fig. 1) was isolated as a homogeneous amorphous brown solid. UV λ_{max} nm (log ϵ): 224 (4.02), sh 286 (3.65), sh 310 (3.78) and 325 (3.82). IR ν_{max} cm^{-1} : 3332, 2930, 1605, 1513, 1448, 1243, 1171, 1003 and 834. FAB-MS m/z : 455 $[\text{MH}]^+$, 429 $[\text{MH} - \text{C}_2\text{H}_2]^+$, 399 $[\text{MH} - 2\text{xCO}]^+$ and 307 $[\text{MH} - \text{C}_7\text{H}_6\text{O}_2 - \text{C}_2\text{H}_2]^+$. ES-MS m/z : 455 $[\text{MH}]^+$ and 327 $[\text{MH} - \text{H}_2\text{O} - \text{C}_6\text{H}_6\text{O}_2]^+$. ^1H and ^{13}C NMR data are given in Table 3.

Results and discussion

The yields of EtOH- H_2O -soluble components from HW and BRW (Table 1), 5% as mean value (8% standard deviation), were very similar and reproducible for both plants, suggesting that the esca fungi did not modify by their activity the EtOH- H_2O -soluble components in the examined samples of wood. However, the lipophylic component of the EtOH- H_2O -soluble fraction increased significantly in BRW. Yields of EtOAc extracts from BRW (4.6% and 4.0% BRW from plants A and B) were almost four times as high as those from HW (1.2% HW from both plants) (Table 1).

In addition, silica gel TLC analysis of the water residues of HW performed with eluent B, which has a high eluent power, showed four clearly distinguishable spots at R_f s no higher than 0.6, whilst the same TLC analysis of BRW showed only a diffuse spot at R_f 0.9. This suggested a substantial difference in the components of water extracts between HW and BRW, the former consisting of more polar components than the latter. However, all

water residues did not give a positive response to the phenol ferric chloride reagent so that such fractions were not further investigated.

The EtOAc extracts from both HW and BRW were well eluted on silica gel TLC by eluent A giving several spots, most of which were positive to the phenol assay with ferric chloride. Among these only two, at R_f s 0.58 and 0.37, were common to both extracts, but those from HW were markedly less intense than those from BRW, suggesting that the increase of the compounds corresponding to such R_f s in BRW was a consequence of infection and the suspect they were phytoalexin-like compounds of phenolic nature, as many phytoalexins are. As a result our investigations were focused on such compounds. The compounds corresponding to other spots of organic extract from HW are possibly other typical phenol compounds, such as flavonoids, antocianes, lignans etc. (Di Stefano and Maggiorotto, 1995). It is noteworthy that such compounds were not present in the organic extract from BRW, for which other spots were recorded. This could be attributed to modification and/or partial or total inhibition of the biosynthesis of the compounds present in HW as well as to other activat-

Table 3. ^{13}C and ^1H NMR data of ϵ -viniferin.

C	δC (ppm)	m	δH (ppm)	m	J (Hz)
1a	133.9	s			
2,6a	128.2	d	7.02	d	8.5
3,5a	116.3	d	6.64	d	8.5
4a	158.6	s			
7a	94.8	d	5.24	d	6.6
8a	58.3	d	4.22	d	6.6
9a	147.4	s			
10,14a	107.5	d	6.04	d	2.2
11,13a	160.1	s			
12a	102.2	d	6.06	t	2.2
1b	130.1	s			
2,6b	128.8	d	6.92	d	8.7
3,5b	116.4	d	6.53	d	8.7
4b	158.4	s			
7b	130.6	d	6.70	d	16.3
8b	123.7	d	6.30	d	16.3
9b	136.9	s			
10b	120.1	s			
11b	162.8	s			
12b	96.8	d	6.13	d	2.0
13b	159.9	s			
14b	104.3	d	6.45	d	2.0

ed biosynthetic pathways, all as a consequence of the activity of fungi involved in esca. Furthermore, an effect as contribution of compounds released in the brown-red wood area by the fungi themselves cannot be excluded

The compounds corresponding to the above mentioned two silica-gel TLC spots were purified, yielding a pure component **1** and a mixture of polyphenols. The pure compound **1** was identified as *trans*-resveratrol (Fig. 1), since all the spectroscopic data collected agreed with those reported in the literature (UV, EIMS and NMR) (Langcake and Pryce, 1976), and other data (IR and FABMS) also supported such an identification. For HW the yields (Table 1) were 0.013-0.017% (plants A and B). Such amounts were of the same order of magnitude of those found (0.07% fresh weight) in the lignified stem tissue of grapevine (prunings) of *Vitis vinifera* cv. Muller-Thurgau by Langcake and Pryce (1976), who suggested that resveratrol might be considered as an apparently normal constituent in such tissues, but not in the leaves, where it was not found even after cutting injury. In BRW resveratrol yields were 0.57-0.47% (plants A and B, Table 1) confirming that it occurred in amounts more than one order of magnitude greater in diseased wood than in healthy wood. Although *trans*-resveratrol has been shown to have generally weak antifungal activity in *in vitro* tests carried out on some fungi, in experiments on the leaves of *Vitis vinifera* (Langcake, 1981) it was found to have a tendency to increase in the area surrounding fungal infection, and also initially in the immediate infection area. Values in both areas increased by two or three order of magnitude, although they were calculated on fresh weight, and hence were lower than those here recorded for BRW. After 4 days these values again decreased. It is noteworthy that, as with BRW due to esca, in experiments conducted with *Botrytis cinerea* a browning of infected grapevine tissues *in vivo* or of cultures *in vitro* was also recorded. This has been attributed to the oxidation of resveratrol by a laccase-like stilbene oxidase of *B. cinerea* (Adrian *et al.*, 1998 and literature cited therein). And indeed, with regard to esca, *F. punctata* has shown to produce in culture a laccase-like oxidase while the production of phenoloxidase and peroxidase has been detected in some strains of *P. aleophilum* (Mugnai *et al.*, 1997).

The polyphenol mixture purified by reverse phase-TLC gave three pure phenolic compounds for BRW, **2**, **a** and **b**. Compound **2** was identified as ϵ -viniferin (Fig. 1) since all spectroscopic data recorded agreed with those reported in the literature (UV, EIMS and NMR) (Langcake and Pryce, 1977), and with others (IR, FABMS and ESMS). By comparison with silica gel TLC *R_f*, the presence principally of compound **2** (ϵ -viniferin) was also recorded in HW, although it was there found together with the compound **a** which showed as a light TLC spot. Unfortunately ϵ -viniferin and compound **a** could not be purified because of their very small quantity. However, assuming that the greatest part of the total yields (*ca.* 0.04% HW both plants) consisted of ϵ -viniferin, this amount would be more or less equal to the 0.05% fresh weight obtained from lignified stem tissue of grapevine (prunings) of *Vitis vinifera* cv. Muller-Thurgau by Langcake and Pryce (1976). The yields of ϵ -viniferin from BRW were 0.27% and 0.41% for plants A and B respectively (Table 1), indicating a marked increase in concentration in deteriorated tissue. However, such concentration was one order of magnitude lower than that found in the rotted areas of *Botrytis cinerea*-infected grapevine leaves, and it did not exceed resveratrol concentration, as shown in some experiments carried out after a relatively short period of infection (3-4 days) but sufficient for developing lesions (Langcake and McCarthy, 1979; Langcake, 1981). While *trans*-resveratrol has been demonstrated to exercise weak fungitoxicity, ϵ -viniferin appears to be more active against pathogenic fungi such as *B. cinerea* and *P. viticola* (Langcake, 1981).

Compounds **a** and **b** were isolated in very low amounts from BRW (Table 1) and their total characterization or identification is still in progress. Preliminary data suggest that they are other resveratrol-derived dimers. The collected spectroscopic data of compound **a** closely resemble those of ϵ -viniferin, but a signal on ^{13}C NMR at δ 57 suggested a methoxilate dimer involving pterostilbene (Langcake *et al.*, 1979) rather than resveratrol. The available data on compound **b** suggest a dimer form of resveratrol lacking of any olefin bond.

Biological assays for antifungal activity of the isolated polyphenols showed that these compounds were toxic to *Cladosporium cucumerinum*, a fungus normally used in such assays (Langcake, 1981).

Assays for antifungal activity of the isolated polyphenols against *P. aleophilum*, *P. chlamydosporum* and *F. punctata* are in progress.

Conclusions

The two esca-diseased grapevine plants under study accumulated at least resveratrol and ϵ -viniferin in the brown-red wood. Both these substances are also present in healthy wood but at concentrations much lower than in deteriorated wood. It is thus possible that a defence response from grapevine cells is elicited and that resveratrol and related compounds play a role in the regulation of the esca fungi-grapevine interaction. Because several fungi may be isolated from the brown-red wood (*P. chlamydosporum* and *P. aleophilum* in great amount, but also *F. punctata*, *Stereum hirsutum*, *Eutypa lata* and others) it will be of interest to see which fungus or fungi are more efficient in triggering such a defence response, and which fungus or fungi derive more advantage from the possible transformation (oxidation) of resveratrol and/or viniferins into less fungitoxic compounds. This will shed some light on the first steps of esca development.

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