Composition and Tissue-Specific Distribution of Stilbenoids in Grape Canes Are Affected by Downy Mildew Pressure in the Vineyard

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ABSTRACT: Grape canes are byproducts of viticulture containing valuable bioactive stilbenoids including monomers and oligomers of E-resveratrol. Although effective contents in stilbenoids are known to be highly variable, the determining factors influencing this composition remain poorly understood. As stilbenoids are locally induced defense compounds in response to phytopathogens, this study assessed the impact of downy mildew infection during the growing season on the stilbenoid composition of winter-harvested grape canes. The spatial distribution between pith, conducting tissues, and cortex of Vitis vinifera, E-resveratrol, E-ε-viniferin, ampelopsin A, E-miyabenol C, Z/E-vitisin B, hopeaphenol, and isohopeaphenol in grape canes from infected vineyards was strongly altered. In conducting tissues, representing the main site of stilbenoid accumulation, E-ε-viniferin content was higher and E-resveratrol content was lower. These findings suggest that the health status in vineyards could modify the composition of stilbenoids in winter-harvested grape canes and subsequently the potential biological properties of the valuable extracts.

KEYWORDS: grape canes, stilbenoids, downy mildew, tissue distribution

INTRODUCTION

Stilbenoids are plant polyphenolic compounds defined by a 1,2-diphenylethyleno unit and are biosynthetically derived from the general phenypropanoid pathway with stilbene synthase (STS) as the entry enzyme. Stilbenoids are widespread in the plant kingdom but occur in a limited number of families such as Vitaceae. † Vitis vinifera L. represents the best known source of stilbenoids by the large number of original structures isolated from grape vines and because this plant species constitutes the principal nutritional source of stilbenoids as wine and table grapes. ‡ Stilbenoids are valuable natural products due to their potential health benefit effects. These compounds exhibit numerous pharmacological activities including antidiabetic, † antiproliferative, † life-prolonging, † antifungal, † anti-inflammatory, † antitumor, † antiatherogenic, † antiviral, † and neuroprotective effects. †

In planta stilbenoids are both constitutive and inducible chemical defenses, and their levels in grape are associated with plant disease resistance. 11,12 The biotic stress-dependent regulation of stilbenoid metabolism in grape is well characterized with an induction of STS gene expression followed by an accumulation of E-resveratrol and its oligomeric derivatives. 12 These defense compounds are locally induced in nonwoody plant organs (leaves, flowers, berries) after pathogen infection, 14-17 wounding, 18 elicitors, 19 UV treatment, 20 and insect attack. 21 Environmental factors such as climatic variations are also known to influence stilbenoid accumulation. 22

A developmental regulation of stilbenoid biosynthesis is mainly described for grape berries with an accumulation of E-resveratrol in the exocarp throughout ripening. 23-26 Nevertheless, E-resveratrol and its derivatives are also constitutively accumulated in vegetative organs such as roots, stems, buds, and leaves. 20 Comparative studies on the spatial repartition of stilbenoids in young grape plants showed that resveratrol accumulation is predominant in stems and that its accumulation herein persists after grape harvest. 27 Therefore, winter pruning of grape vine represents an annual step producing stilbenoid-rich wastes. The important volume of pruned wood (1-5 tons/hectare/year) and the world’s grape growing surface (7.5 million hectares) make the grape canes a prominent source of stilbenoids. Grape cane extracts containing bioactive stilbenoids have recently been proposed as a source of alternative fungicides for agronomy 30 and pharmacological products for medicine 31 as well as health-promoting compounds for nutraceutic and cosmetic sectors. 32

The effective content in stilbenoids is known to be highly variable, but the determining factors influencing this composition remain poorly understood. 29,33 Although the impact of environmental cues on grape stilbenoid metabolism has been well described for leaves and berries, the influence of biotic stress during spring and summer on the stilbenoid composition of winter-pruned grape canes is unknown. In the present study, we considered the influence of downy mildew during the growing season on stilbenoid accumulation in grape canes. Particularly, the change in spatial distribution of E-
resveratrol and its derivatives, E-piceatannol, E-ε-viniferin, ampelopsin A, E-miyabenol C, Z/E-vitisin B, hopeaphenol, and isohopeaphenol (Figure 1), between conducting tissues, cortex, and pith has been studied in grape canes from healthy and downy mildew-infected vineyards.

**MATERIALS AND METHODS**

**Plant Material.** The study was conducted on 35-year-old vineyards of the Bourgueil district in the Loire Valley region (France) in vintage 2011. Vineyards were planted with V. vinifera cv. Cabernet franc (clone 327 grafted onto 3309 rootstock) on a clay–silica–limestone soil at a spacing of 1 m (within row) × 2 m (between rows) corresponding to 5000 vines ha⁻¹. Two adjacent vineyard parcels (plot A, 47°17′28.82″ N, 0°13′28.51″ E; plot B, 47°17′49.07″ N, 0°14′34.46″ E) were selected for the study, covering areas of 0.18 and 0.233 ha, respectively. Both plots were subjected to organic management with similar practices except for the copper-based fungicide treatments. In plot A, solutions of copper were applied corresponding to 1 kg ha⁻¹ of the copper treatments were prohibited during the entire growing season. Then, grape canes were stored for 10 weeks at 20 °C in the dark as 10 cm long sections. After storage, five lots were constituted within grape canes originating from plots A and B to compare the stilbenoid compositions from healthy and infected vineyards. Wood sections were manually dissected to separate cortex, pith, and conducting tissues.

**Stilbenoid Analysis.** Whole and dissected tissues of grape canes were ground for 2 min with a cooled analytical grinder (Ika-Werke, Staufen, Germany). A second grinding step was performed using a cutting mill (Polymix PX-MFC 90 D, Kinematica AG, Switzerland) to obtain a powder with an average particle size of 1 mm. Freeze-drying was performed at 0.01 mbar and −20 °C for 48 h with a Christ Alpha 1-5 freeze-dryer. For stilbenoid extraction, 50 mg of lyophilized powder was extracted in 1 mL of an ethanol/water solution (60:40; v/v), shaken for 30 min at 1400 rpm and 83 °C, and centrifuged at 18000g for 5 min. HPLC analyses were performed on a Waters system (Waters 600 controller, Milford, MA, USA) equipped with a UV–visible photodiode array detector (Waters 996) and a column packed with 3 μm particles (250 × 4 mm, Multispher 120 RP18HP; CS-Service, Langerwehe, Germany) at 24 °C. The mobile phase was aqueous phosphoric acid (0.1% w/v; eluent A) and acetonitrile (eluent B) pumped at 0.5 mL min⁻¹. The gradient started at 5% B and increased linearly to 72.5% after 60 min, followed by washing and reconditioning of the column. Compounds in extracts were identified according to their UV spectra and retention time by comparison with external standards. Quantification was performed using reference standards of E-resveratrol, E-piceatannol, E-ε-viniferin, Z/E-vitisin B, ampelopsin A, E-miyabenol C, and hopeaphenol and five-point calibration curves (0–100 ppm) using the Maxplot detection mode.

**Chemicals.** E-Resveratrol and E-piceatannol were purchased from Sigma-Aldrich (St. Louis, MO, USA). E-ε-Viniferin, Z/E-vitisin B, ampelopsin A, E-miyabenol C, and hopeaphenol were extracted from grape canes as previously described. Acetonitrile and ethanol were purchased from Thermo Fisher Scientific (Courtaboeuf, France). Ultrapure water was obtained from a Millipore Milli-Q water purification system (Bedford, MA, USA).

**Microscopic Analysis.** Free-hand cross sections of freshly pruned grape canes from Cabernet franc variety were observed with an epifluorescence microscope (Olympus BX51; Olympus Optical, Tokyo, Japan) equipped with a digital camera (Olympus DP71) and the corresponding software (SIS Cell). The autofluorescence was observed with a UV excitation filter set (Olympus WU2, 330–385 nm excitation filter, 420 nm long pass emission filter).

**Fluorescence Spectroscopy.** Pure standards of E-Resveratrol and E-ε-Viniferin in methanol solution (2 μg mL⁻¹) and 1:100 dilution of grape cane extracts prepared as described above (whole tissues, pith, cortex, or conducting tissues) were used. Emission and excitation spectra were acquired with a spectrometer (Hitachi F-4500; Hitachi, Ltd., Tokyo, Japan). The excitation spectra were acquired with an emission at 390 nm, and the emission spectra were acquired with an excitation at 320 nm.

**Statistical Analysis.** Data were analyzed with Statistica, version 6.0 (StatSoft Inc., Tulsa, OK, USA). Statistical significance from different treatments was revealed after one-way analysis of variance (ANOVA) followed by post hoc Tukey’s honestly significant difference (HSD) test.

**RESULTS AND DISCUSSION**

Two adjacent vineyard plots planted with the Cabernet franc grape variety both on a clay–silica–limestone soil were selected for this study to avoid stilbenoid changes due to genetic or pedo-climatic variations. The two parcels were subjected to similar agricultural practices with the exception of the copper-based fungicide treatments that were applied to plot A (1 kg ha⁻¹) and absent in plot B. As a result, mildew disease intensities at midvarena were drastically contrasted at 5 and 95% in plots A (healthy) and B (infected), respectively. Specifically in plot B, 95% of the leaves were infected by downy mildew with at least 50% of leaf area being necrotic as well as...
the occurrence of clusters turning brown. During winter, grape canes from both parcels were randomly pruned across the total area of plot A and B to overcome intraplot variation and stored for 10 weeks at 20 °C in the dark as 10 cm long sections to allow postpruned stilbenoid accumulation.\textsuperscript{29} HPLC chromatograms of grape canes from healthy (plot A) and infected (plot B) vineyards showed differences in stilbenoid composition (Figure 2). E-Resveratrol and E-ε-viniferin, the two prominent compounds, were strongly influenced by downy mildew infection. Surprisingly, the concentration of E-resveratrol, a well-characterized grape phytoalexin in leaf and berries, decreased in infected grape canes, whereas E-ε-viniferin, the dimer of resveratrol, increased (Figure 3). Interestingly, hopeaphenol, the third most accumulated compound, remained unchanged, whereas other minority stilbenoids either significantly decreased (ampelopsin A, E-piceatannol, E/Z vitisin B) or increased (isohopeaphenol, E-miyabenol C) in grape canes from downy mildew-infected vineyards.

Under favorable conditions, \textit{Plasmopara viticola}, the etiological agent of downy mildew, infects leaves via stomata, spreads within the leaf tissue through the intercellular space of the spongy mesophyll, and later forms haustoria that penetrate host cells. Previous studies showed an induction of stilbenoids in necrotic areas after downy mildew infection,\textsuperscript{35} but the effect on grape cane stilbenoids was not investigated. The change in stilbenoid composition observed in grape canes might affect the antifungal activity of grape cane extracts. Indeed, the toxicity of ε-viniferin against downy mildew is 2-fold higher than that of resveratrol,\textsuperscript{35,36} and the level of resveratrol dimerization is considered as a marker of grapevine resistance to downy mildew.\textsuperscript{37,38} The resistance level of grapevine cultivars depends on their ability to rapidly induce high concentrations of stilbenoids at the infection site.\textsuperscript{39} Among the \textit{V. vinifera} Cabernet franc clone collection, the resistance level to downy mildew was linked to higher production of stilbenoid phytoalexins in leaves including ε-viniferin and δ-viniferin.\textsuperscript{40} δ-Viniferin was not detected in the grape cane extracts of either the present or previous studies.

The stilbenoid composition of stored grape canes results from a sequential accumulation of oligomers and monomers.\textsuperscript{29} Oligomers accumulated before pruning and their levels remain unchanged during storage, but monomers (E-resveratrol and E-piceatannol) are mainly biosynthesized during storage along with an induction of stilbene synthase. In the present study, the stilbenoid composition was affected, but the total concentration was not significantly different between plot A (10792 ± 180 mg kg\textsuperscript{-1} DW) and plot B (10666 ± 331 mg kg\textsuperscript{-1} DW). The high level of E-ε-viniferin in grape canes from infected vineyards was offset by a limited accumulation of monomers during postpruning storage, suggesting a tight regulation of stilbenoid metabolism in grape canes. Resveratrol dimerization involves peroxidase isoenzymes located both in the vacuoles and in cell walls.\textsuperscript{41} The accumulation of E-ε-viniferin in grape canes from downy mildew infected vineyards might result from a local conversion in stems or biosynthesis in leaves at the local infection site followed by phloem-mediated relocation.

To further assess the impact of downy mildew infection during the growing season on winter-harvested grape canes, the change in spatial distribution of stilbenoids was investigated. Stilbenoids emit a blue fluorescence under UV light with an excitation peak around 320 nm and an emission peak at 390 nm.\textsuperscript{42} Therefore, fluorescence analyses were used to investigate the spatial distribution of stilbenoids in healthy grapevine tissues. A free-hand cross section of freshly pruned grape canes from Cabernet franc variety was prepared and observed by fluorescence microscopy. Figure 4 reveals the microscopical structure of a healthy grape cane including pith, conducting tissues, and cortex. The blue fluorescence observed in conducting tissues may rely on lignin fluorescence but also suggests a major accumulation of stilbenoids in vascular tissues of grape canes. No changes were observed between healthy and infected grape canes either in the blue fluorescence intensity or in the tissue structure (data not shown). To evaluate the contribution of stilbenoid fluorescence in the observed blue fluorescence, in vitro fluorescence spectra of pure solutions (E-resveratrol and E-ε-viniferin) and grape cane extracts were compared. Panels A and E of Figure 5 present respectively the

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**Figure 2.** HPLC chromatograms measured in maxplot detection of hydroalcoholic cane extracts of \textit{Vitis vinifera} cv. Cabernet franc from 5% (A) and 95% (B) downy mildew-infected vineyards. Peaks: (1) catechin, (2) epicatechin, (3) ampelopsin A, (4) E-piceatannol, (5) E-resveratrol, (6) hopeaphenol, (7) isohopeaphenol, (8) E-ε-viniferin, (9) E-miyabenol C, (10) Z/E-vitisin B.

**Figure 3.** Stilbenoid composition in total tissues of grape canes from 5 and 95% downy mildew-infected vineyards. Asterisks indicate significant differences between the two infection levels (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$). NS, no significant differences.

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peak between 380 and 420 nm (Figure 5B,F). These results are in agreement with HPLC stilbenoid analyses, where E-resveratrol and E-ε-viniferin were the major accumulated compounds (Figure 3). Fluorescence analyses have been reported as a rapid technique to localize the stilbenoid distribution in grape leaves.34,43 Here this technique points out that conducting tissues represent the main site of accumulation for stilbenoids in grape canes. After manual dissections of cortex, pith, and conducting tissues of grape canes, the spatial distribution of stilbenoids from healthy and downy mildew-infected vineyards was evaluated. The emission and excitation spectra indicate that conducting tissues represent the main site of stilbenoid accumulation in grape canes (Figure 3C,G). The emission maximum around 400 nm for conducting tissues and that around 420 nm for cortex and pith suggest a predominance of monomers in conducting tissues and oligomers in cortex and pith. Moreover, fluorescence spectra suggest that downy mildew affects the spatial repartition of stilbenoids with an increase in the cortex and a decrease in the pith (Figure 3C,D,G,H). To ascertain these global trends, HPLC analyses were performed on the corresponding dissected tissues (Figure 6). Striking differences of stilbenoid composition were revealed in the different grape cane tissues. Although the pith represented only 2% of the whole tissues, it contained high levels of oligomers, particularly hopeaphenol, isohopeaphenol, and E-ε-viniferin, compared to other tissues and was poor in monomers. Conducting tissues represented 88% of the overall tissues, and as a consequence the specific stilbenoid composition of conducting tissues mainly reflected the composition of the whole grape canes (Figure 3). The cortex representing 10% of the whole tissues was poor in stilbenoids, with hopeaphenol and E-ε-viniferin as major compounds. The HPLC tissue-specific analyses allowed a fine description of downy mildew impact on spatial distribution of stilbenoids. Indeed, the levels of all stilbenoid compounds were massively decreased in the pith of canes from infected plants whereas some oligomers were increased in conducting tissues and cortex. It should be noted that there was a drastic increase of E-ε-viniferin in conducting tissues (× 2) and cortex (× 3.4) following downy mildew infection.

The spatial characterization of stilbenoids in grape canes as well as the impact of downy mildew on stilbenoid distribution was unprecedented in the literature. Several studies are in agreement with the presence of stilbenoids in conducting tissues of grape canes. In young grape plants, resveratrol was shown to accumulate mainly in the stem.27 HPLC analyses of xylem sap collected during the bleeding period revealed the presence of resveratrol.24 Interestingly, in the xylem sap of vines infected by esca-associated fungi, a decrease of resveratrol was observed as in downy mildew-infected grape canes. In Norway spruce bark, the stilbenoids accumulate mainly in phloem parenchyma cells,45 and after infection with the bark-beetle-associated fungi Ceratocystis polonica, an increase in stilbenoid dimers was observed accompanied by the loss of monomers. In grape canes from downy mildew-infected vineyards, resveratrol dimerization might be induced in conducting tissues for transport purpose, whereas in pith, a tissue with storage functions, the oligomer accumulation might be limited. The strong increase of E-ε-viniferin in the cortex, a tissue notably involved in protection against mechanical damage and microbial attack, might result from a systemic defense response to protect the plant against upcoming infections. It should be mentioned that a potential effect of copper treatments in plot A...
on the stilbenoid contents in grape canes cannot be excluded because an elicitation has been reported on excised grape leaves of in vitro grown plants.\textsuperscript{16} In conclusion, this study indicates that stilbenoid composition in winter-pruned grape canes was greatly affected by downy mildew infection over the growing season. A change in spatial distribution of stilbenoids was observed, particularly in conducting tissues, where an increase in E-\(\epsilon\)-viniferin and a decrease in E-resveratrol were seen. These findings suggest that the health status in vineyards could modify the composition of stilbenoids in winter-harvested grape canes. As vineyard management contributes to wine quality, it might also influence the composition of bioactive compounds of grape canes and the resulting potential biological properties of these valuable extracts. Further studies are still required to evaluate the influence of pedo-climatic factors on the variations of stilbenoid contents.


