The biosynthetic origin of E-resveratrol accumulation in grape canes during post-harvest storage
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Resveratrol biosynthesis in pruned vine-wood.

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Abstract (100-150 words)

Grape canes are vineyard waste products containing valuable phytochemicals of medicine and agriculture interest. Grape canes storage is critical for the accumulation of these bioactive compounds. In the present study, we investigated the changes in stilbenoid phytochemical composition during grape cane storage and the influence of temperature on final concentrations. A strong increase in the concentration of the monomer \( E \)-resveratrol (approx. 57-fold) was observed during the first six weeks of storage at 20°C in eight different grape varieties without any change in oligomer concentrations. The \( E \)-resveratrol accumulation was temperature dependent with an optimal range at 15-20°C. A 2h heat-shock treatment aiming at protein denaturation inhibited \( E \)-resveratrol accumulation. The constitutive expression of key-genes involved in the stilbene precursor biosynthesis along with an induction of STS (stilbene synthase) expression during the first weeks of storage contribute to a \textit{de novo} biosynthesis of \( E \)-resveratrol in pruned wood grapes.

Keywords: stilbenoids, grape cane, postharvest storage, stilbene synthase
INTRODUCTION

Stilbenoids are non-flavonoid polyphenols derived from the phenylpropanoid pathway. From phenylalanine, the first three steps are successively catalyzed by the phenylalanine ammonia lyase (PAL), cinnamate 4-hydroxylase (C4H) and 4-coumarate CoA ligase (4CL) yielding p-coumaroyl-CoA. This product is an important metabolic intermediate acting as a substrate for the generation of several phenylpropanoid subclasses such as ubiquitous lignin or flavonoids but also stilbenoids which are present in a few species including grapevine. In these plants, p-coumaroyl-CoA is condensed by stilbene synthase (STS) to form E-resveratrol, the first stilbenoid product. In grapevine, STS is induced under both biotic and abiotic conditions in accordance with stilbenoid functions in plant resistance. For instance, antimicrobial activities of E-resveratrol and its dimer E-ε-viniferin have been reported for downy mildew, powdery mildew and gray mold. E-Piceatannol, a resveratrol analog, is induced by fungal elicitors and under UV irradiations. Additionally, resveratrol oligomers display interesting activities against several grape fungal diseases. A growing interest in stilbenoids arises due to their potential impact on human health. Several reviews have indicated that E-resveratrol possesses a number of important pharmacological activities and is known to provide a level of protection against both metabolic and cardiac disorders together with anti-aging, anti-carcinogenic and anti-inflammatory properties. Furthermore, the less studied E-piceatannol also exhibits a wide range of biological activities including suppression of tumor proliferation and chemopreventive activities. Recently, bioassays testing oligomers and analogs of E-resveratrol highlighted their broad pharmacological potential.
Given the pharmacological importance of stilbenoids, a growing demand is expected for diverse applications in health, nutraceutical, food and cosmetic sectors. Currently, *E*-resveratrol is primarily extracted from field-grown Japanese knotweed (*Fallopia japonica*) and several alternative production strategies have been proposed including biotechnological\(^{23}\) and synthetic chemistry strategies.\(^{24}\) Whereas the synthesis of *E*-resveratrol and its derivatives remained challenging,\(^{25}\) the bioproduction by yeast, bacterial and plant cell cultures requires scalable methods.\(^{23,26,27}\) Additionally, by-products of the wine industry have been recently proposed as a valuable source of stilbenoids.\(^{28}\)

Grape canes represent a large source of waste derived from the viticulture industry with an estimated volume of 1-5 tons/hectare/year depending on plantation density, climate and vigor of the grape variety. Currently, no or limited valorization of grape canes exists since they are usually burnt in the field or composted.\(^{29}\) Recently, pruned wood from vineyard has been shown to accumulate a broad spectrum of stilbenoids ranging from *E*-resveratrol to complex stilbene oligomers.\(^{11,28,30}\) Therefore, grape canes represent a promising bioresource containing valuable bioactive compounds with potential applications in medicine due to multiple pharmacological properties of stilbenoids\(^ {31}\) and in agriculture as alternative source of anti-phytopathogenic substances.\(^ {12}\)

Despite recent advances in the evaluation of stilbenoid composition of grape canes,\(^ {31-33}\) varying results have been reported. Several factors can explain this variability. First, the variety and the provenance of the grape can greatly influence stilbenoid composition.\(^ {11,30,34}\) To note, *E*-resveratrol ranged from 0.022 mg kg\(^{-1}\) DW in Turkish cultivars\(^ {35}\) to 6533 mg kg\(^{-1}\) DW in canes from Chile.\(^ {30}\) Secondly, the extraction method is critical to ensure a good recovery of stilbenoids\(^ {32,36}\) and the range of extraction methods tends to bias the results.
Furthermore, an accurate quantification of stilbenoids relies on calibration curves with pure stilbenoid standards since the quantification of stilbenoids as resveratrol equivalent leads to an underestimation of the concentrations of resveratrol oligomers. Additionally, a disparity in stilbenoid composition of “Pinot Noir” grape canes has been observed during storage i.e. the time elapsed between vine pruning and the phytochemical analyses. An increase in stilbenoid concentration (up to fivefold) after six months at 20°C was observed. Canes were enriched in E-resveratrol whereas the level of E-ε-viniferin did not vary. Although an increase in stilbenoid concentrations has been found in “Pinot Noir” canes in response to storage, the determining factors controlling this accumulation remain unknown and the effect of storage on other grape varieties has not been determined systematically.

The aim of this study was to better characterize stilbenoid accumulation in pruned grape canes and particularly to i) determine the optimal storage period for 8 grape varieties representative of the Loire valley, ii) evaluate the influence of temperature storage on stilbenoid accumulation and iii) assess the potential biosynthetic activity in pruned grape wood.

MATERIALS AND METHODS

Plant material. Grape canes of *Vitis vinifera* varieties, “Cabernet Franc”, “Chardonnay”, “Chenin”, “Malbec” (Côt), “Gamay”, “Grolleau”, “Pinot Noir” and “Sauvignon Blanc” were collected in vineyards at the wine school of Amboise (France) in December 2012. The canes were cut into 10 cm long sections and stored in a controlled chamber at 20°C for 10 weeks in the dark. In the same way, grape canes of *Vitis vinifera* var. “Malbec” were stored in additional controlled chambers at -20°C, 5°C, 15°C, 20°C and 28°C for 10 weeks in dark. Heat shock treatment with intent to denature enzymes consisted of a treatment at 65°C for 2
hours immediately after cane harvest and followed by storage at 20°C. After storage, samples were ground for 2 min with a cooled analytical grinder (Ika-Werke, Staufen, Germany), frozen in liquid nitrogen and stored at -80°C for stilbenoid and gene expression analysis. For time point zero, canes were cut in the vineyards, transported to the laboratory (1 hour) and immediately ground as described above.

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

**Stilbenoid analysis.** A second grinding step was performed to obtain a powder with an average particle size of 1 mm using a cutting mill (Polymix PX-MFC 90 D, Kinematica AG, Switzerland). Stilbenoid extraction was adapted from Karacabey and Mazza. Fifty mg of lyophilized powder were extracted in 1 mL ethanol/water solution (60:40; v/v) and shaken for 30 min at 1400 rpm at 83°C and centrifuged at 18 000 x g for 5 min. HPLC apparatus consisted in a Waters system equipped with a gradient pump (Waters 600 controller, Milford, MA), a cooled autosampler (Waters 717 plus) and a UV–visible photodiode-array detector (Waters 996) set to acquire data from 200 to 400 nm. Empower 2 software from Waters was used for instrument control, data acquisition and data processing. Analyses were performed on a column packed with 3 μm particles (250 × 4 mm, Multospher 120 RP18HP; CS-Service, Langerwehe, Germany) at 24°C. The mobile phase consisted in aqueous phosphoric acid (0.1% w/v; eluent A) and acetonitrile (eluent B) pumped at 0.5 mL min-1 into the HPLC system. The gradient started at 5% B and increased linearly to 72.5% in 60
min, followed by washing and reconditioning the column. Compounds in extracts were identified according to their UV spectra and retention time by comparison with external standards. Quantification was performed using pure reference standards of \( E \)-resveratrol, \( E \)-piceatannol, \( E \)-\( \varepsilon \)-viniferin, \( Z/E \)-vitisin B, ampelopsin A and hopeaphenol and 5-point calibration curves (0-100 ppm) using the Maxplot detection mode.

**Gene expression analysis.** Total RNA extractions were performed on ground grape canes (approximately 100 mg) using the improved RNA extraction protocol for woody plants,\(^{38}\) with RNeasy Plant Mini Kit (Qiagen, UK). RNA quality and concentration were assessed both spectrophotometrically and on agarose gel. Reverse transcription of RNA was carried out using ThermoScript RT (Invitrogen) according to manufacturer’s instructions. First-strand cDNA was synthesized using total RNA (200ng) and oligo \( dT \) primer (2.5 \( \mu \)M) in presence of RNaseOUT (Invitrogen). Gene expression was analysed by quantitative real-time PCR on retro-transcribed RNA using the following primer pairs: 5’-GAAACCCTCAATTCCGAACC-3’ (forward) and 5’-GCACTGACAATCCAGAGAG-3’ (reverse) for phenylalanine ammonia lyase (PAL), 5’-AAAGCTCTGTTAGCGGTGTTCTG-3’ (forward) and 5’-TCAGGTGTTCAATCCC-3’ (reverse) for cinnamate 4-hydroxylase (C4H), 5’-CCTGAAATTCATCTCCAACC-3’ (forward) and 5’-GCTTTTGCGAGAAATGAGGTG-3’ (reverse) for 4-coumaric acid:CoA ligase (4CL), 5’-CAGCAGCCCAACATTTATTCC-3’ (forward) and 5’-GTTCCAATCGCTAATCCAAGTG-3’ (reverse) for stilbene synthase (STS) and 5’-CTTCTCCTGTATGGGAGCTGCG-3’ (forward) and 5’-GCCATAACCACTGGTACAATCGAC-3’ (reverse) for VATP16 reference gene chosen according to Gamm et al.\(^{39}\) Real-time PCR was run on a CFX96 Touch Real-Time PCR System (Bio-Rad) using the SYBR Green I technology. Each PCR mixture of 15 \( \mu \)L total reaction volume contained equal amount of cDNAs, 0.2 \( \mu \)M.
forward and reverse primers and 1x SsoAdvanced SYBR Green Supermix (Bio-Rad). The reaction was initiated by a denaturation step (95°C, 7 min), followed by 40 cycles at 95°C for 10 s and 60°C for 30 s. Melt curve analysis was performed on the end products of PCR, to determine the specificity of reactions. Absolute quantification of transcript copy number was performed using calibration curves (plasmid dilution series from $10^8$ to $10^4$ copies). The results are expressed as copy number of cDNA normalized using VATP16 reference gene.

**Statistical analysis.** Data were analysed with Statistica, version 6.0 (StatSoft Inc., Tulsa, USA). Statistical significance from different treatments was revealed after one-way analysis of variance (ANOVA) followed by Tukey's test.

**RESULTS AND DISCUSSION**

Ten compounds were identified in grape cane extracts from the varieties, “Cabernet Franc”, “Chardonnay”, “Chenin”, “Malbec” (Côt), “Gamay”, “Grolleau”, “Pinot Noir” and “Sauvignon blanc” corresponding to the flavonoids; catechin and epicatechin, and the stilbenoids; ampelopsin A, $E$-piceatannol, $E$-resveratrol, hopeaphenol, isohopeaphenol, $E$-ε-viniferin, $E$-miyabenol C and $Z/E$-vitisin B (Figure 1). HPLC chromatograms of “Sauvignon blanc” grape cane extracts before and after 6-week storage period show striking changes in stilbenoid composition, particularly for $E$-resveratrol and $E$-piceatannol (Figure 2). To confirm those observations, a survey of stilbenoid accumulation in pruned wood was expanded to eight grape varieties stored for 8 weeks at 20°C (Figure 3). The initial concentration of $E$-resveratrol contained in branches before any treatment was determined prior storage. For this, the grape canes were ground and frozen at -80°C within 1 hour (maximum) after pruning in the vineyards. The $E$-resveratrol contents measured at $t_0$ in all varieties were
shown to be very low (101 ± 77 mg kg\(^{-1}\) DW) and corresponded to the values previously reported for unstored grape canes.\(^{37}\) By contrast, an accumulation of \(E\)-resveratrol was observed in the eight tested grape varieties during storage reaching a peak after six weeks (Figure 3A). On average a 57-fold increase in \(E\)-resveratrol was observed with a range from 19-fold for “Pinot Noir” to 102-fold for “Chenin” (Table 1). At the same time, \(E\)-piceatannol increased in a similar fashion but on a smaller scale (Figure 3B). The accumulation of \(E\)-resveratrol during storage in these eight varieties confirms previous results obtained for “Pinot Noir”\(^{37}\) with an additional increase in \(E\)-piceatannol (3-hydroxyresveratrol). Such an induction of monomeric stilbenoids was also observed in other postharvest plant materials.\(^{40}\) Indeed, \(E\)-resveratrol and \(E\)-piceatannol were shown to accumulate in sugarcane billets within seven days after harvest. Furthermore, it has been shown that red wines and table grapes can be enriched in stilbenoids by postharvest UV-C irradiation of grapes.\(^{41-43}\) As for other stilbenoid-rich products, the present results highlight the importance of the postharvest process to obtain grape canes with enriched contents in \(E\)-resveratrol and \(E\)-piceatannol.

Nevertheless, the origin of the accumulated stilbenoids was not elucidated. They might be formed from the degradation of oligomeric stilbenoids or by a biosynthetic activity. For this reason, the concentrations of resveratrol oligomers (ampelopsin A, hopeaphenol, isohopeaphenol, \(E\)-\(\epsilon\)-viniferin, \(E\)-miyabenol C and \(Z/E\)-vitisin B) were individually quantified during the storage period but no significant changes were observed (data not shown). In summary, Figure 3C presents the total concentration of resveratrol oligomers which do not significantly vary during storage in any of the varieties tested. Therefore, the possibility that \(E\)-resveratrol and \(E\)-piceatannol originated from the degradation of resveratrol oligomers was ruled out. To test the hypothesis that the accumulation of \(E\)-resveratrol originated from
a de novo biosynthesis, the influence of temperature storage on $E$-resveratrol accumulation was tested on the “Cabernet franc” variety over 10 weeks (Figure 4). Firstly, when the canes were stored at either 5, 15 or 20°C, $E$-resveratrol concentration increased to approximately 6384 ± 456 mg kg$^{-1}$ DW, however the accumulation rate of $E$-resveratrol was shown to be delayed at lower temperatures. Secondly, at 28°C $E$-resveratrol accumulated rapidly during the first two weeks up to a peak of 3923 ± 584 mg kg$^{-1}$ DW, however no further accumulation was observed in the weeks after. Finally when a short heat shock treatment (2 hours at 65°C) was applied to grape canes, no $E$-resveratrol accumulation was observed. Such a treatment with intent to denature proteins inhibits the accumulation of $E$-resveratrol during the storage period and suggests the involvement of active enzymes in this phenomenon. Similarly, when canes were stored at -20°C, $E$-resveratrol was not induced. The temperature during the storage process of grape canes is therefore a determining factor for optimal $E$-resveratrol accumulation (observed at 20°C). Moreover, whereas excessive temperature (up to 28°C) rapidly inhibits $E$-resveratrol accumulation, a decrease in the storage temperature only slows down the induction rate and the optimal $E$-resveratrol content is still achieved, albeit at a later time period (Figure 4). These data prompt us to assume that the biosynthetic enzyme activities, and particularly those involved in the stilbene pathway, persist during storage and that the optimal period of storage is directly related to the storage temperature.

Given these data, we investigated by real-time PCR the transcriptional activity of key-genes involved in the stilbene biosynthesis during the six weeks of $E$-resveratrol accumulation in pruned grape wood of the “Cabernet franc” variety stored at 20°C (Figure 5). The transcript abundance of the structural genes PAL, 4CL, C4H and STS were assayed by quantitative RT-PCR (Figure 5). PAL, 4CL and C4H the three genes of the general phenylpropanoid pathway
were constitutively expressed over the six weeks of storage and in addition STS (forming E-
resveratrol) was induced during the first four weeks of the storage. These results suggest
that grape pruned wood is still transcriptionally active during the storage period.

In planta, E-resveratrol is a well-known phytoalexin i.e. an anti-microbial substance that
rapidly accumulate at the pathogen infection site. E-Resveratrol is induced in response to
biotic and abiotic stresses such as elicitors\textsuperscript{44} UV-C light,\textsuperscript{45} ozone,\textsuperscript{46} anoxia\textsuperscript{47} and wounding.\textsuperscript{48}
Following pathogenic infection, the STS transcripts are induced within 16-48 hours\textsuperscript{3,39} and
the peak value for E-resveratrol accumulation occurs around 24-72 hours.\textsuperscript{6} By contrast, the
induction of stilbene metabolism in postharvest material is poorly documented and available
data are restricted to grape berries. Pioneering work showing the induction of stilbene
metabolism during drying process of postharvest grape berries was reported by Versari et
al.\textsuperscript{49} Recently, complementary studies showed that both STS transcripts and E-resveratrol
biosynthesis are induced in the epicarp of postharvest grape berries and the induction level
depends on the dehydratation rate.\textsuperscript{50} Our study highlights a similar induction of stilbene
biosynthesis in postharvest grape canes where the drying process during storage might be
perceived as a stress signal by living tissues. Stilbene metabolism is thus induced and E-
resveratrol and E-piceatannol accumulate until the tissues are dry. Further investigations will
help to evaluate the influence of the hygrometric conditions in relation to temperature on
stilbenoid accumulation during grape canes storage.

In conclusion, at the pruning stage, grape canes contain high levels of oligomeric stilbenoids
previously accumulated during the growing season but almost no monomeric stilbenoids.
During the first six weeks of storage, E-resveratrol and E-piceatannol accumulate at various
rates depending on storage temperature. This E-resveratrol accumulation resulted from a de
novo biosynthesis with induction of STS expression during storage. Grape canes appear to be an original plant by product containing valuable stilbenoids. Because this pruned wood is still metabolically active during the drying process, the storage conditions have to be controlled to obtain stilbene-enriched material for further medicinal or agronomical applications. To further characterize the variability of stilbenoids in grape canes, the impact of grape diseases during the growing season will be required.

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Notes

The authors declare no competing financial interest.

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REFERENCES


**FIGURE LEGENDS**

**Figure 1.** Selected structures of typical stilbenoids present in grape canes.

**Figure 2.** HPLC chromatograms measured in maxplot detection of hydroalcoholic cane extracts of *Vitis vinifera* “Sauvignon blanc”. Grape canes were harvest in the vineyard and stored for six weeks at 20°C (A) or were immediately ground for stilbenoid extraction (B). (1) catechin, (2) epicatechin, (3) ampelopsin A, (4) *E*-piceatannol, (5) *E*-resveratrol, (6) hopeaphenol, (7) isohopeaphenol, (8) *E*-ε-viniferin, (9) *E*-miyabeno C, (10) Z/E-vitisin B.
Figure 3. Changes in stilbene concentration during storage of grape canes from eight varieties. The grape varieties are presented by lines with “Cabernet Franc”, “Chardonnay”, “Chenin”, “Malbec”, “Gamay”, “Grolleau”, “Pinot Noir” and “Sauvignon blanc”. The stilbene compounds are presented by column with \( E \)-resveratrol (A), \( E \)-piceatannol (B) and the sum of oligomers (C; i.e. addition of concentrations of ampelopsin A, hopeaphenol, isohopeaphenol, \( E \)-\( \varepsilon \)-viniferin, \( E \)-miyabenol C and \( Z/E \)-vinis B).

Figure 4. Effect of temperature on \( E \)-resveratrol accumulation during cane storage of \( Vitis vinifera \) “Cabernet franc”. Grape canes were stored for a period of 10 weeks at -20°C, 5°C, 15°C, 20°C and 28°C. Heat shock consisted of a treatment at 65°C for a period of 2 hours immediately after harvest and followed by storage at 20°C.

Figure 5. Transcript abundance of the stilbene biosynthesis pathway genes PAL, 4CL, C4H and STS measured by quantitative RT-PCR during cane storage of \( Vitis vinifera \) “Cabernet franc”. Grape canes were collected in the vineyard and directly frozen at -80°C (\( t_0 \)) or stored at 20°C for 2, 4 and 6 weeks. Transcript abundance of each gene was normalized against VATP16 reference gene. Different letters in the same group of bars indicate significant differences (\( P < 0.05 \)).

Table 1. Changes in \( E \)-resveratrol concentration in grape canes from eight varieties after a 6-week storage period at 20°C.
<table>
<thead>
<tr>
<th>variety</th>
<th>$E$-resveratrol concentration (mg kg$^{-1}$ DW)</th>
<th>fold induction</th>
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<tbody>
<tr>
<td></td>
<td>0 weeks</td>
<td>6 weeks</td>
</tr>
<tr>
<td>Sauvignon blanc</td>
<td>60 (31)</td>
<td>2908 (552)</td>
</tr>
<tr>
<td>Chardonnay</td>
<td>96 (16)</td>
<td>3175 (508)</td>
</tr>
<tr>
<td>Côt</td>
<td>158 (23)</td>
<td>3316 (762)</td>
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<td>Grolleau</td>
<td>41 (18)</td>
<td>3913 (704)</td>
</tr>
<tr>
<td>Chenin</td>
<td>45 (15)</td>
<td>4631 (926)</td>
</tr>
<tr>
<td>Pinot noir</td>
<td>250 (112)</td>
<td>4725 (897)</td>
</tr>
<tr>
<td>Cabernet franc</td>
<td>57 (15)</td>
<td>4762 (952)</td>
</tr>
<tr>
<td>Gamay</td>
<td>100 (33)</td>
<td>5100 (867)</td>
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<tr>
<td>Means</td>
<td>101</td>
<td>4066</td>
</tr>
</tbody>
</table>
(A) Six weeks of storage at 20°C

(B) $t_0$
Figure 3

(A) E-resveratrol (mg kg^{-1} DW)

(B) E-piceatannol (mg kg^{-1} DW)

(C) Sum of oligomers (mg kg^{-1} DW)

Time of storage (weeks)

Cabernet franc
Chardonnay
Chenin
Malbec
Gamay
Grolleau
Pinot Noir
Sauvignon blanc

0 2 4 6 8 ...

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