


Stilbenes from *Vitis vinifera* L. Waste: A Sustainable Tool for Controlling *Plasmopara Viticola*

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Supporting Information

ABSTRACT: Stilbene-enriched extracts from *Vitis vinifera* waste (cane, wood, and root) were characterized by UHPLC-MS. Eleven stilbenes were identified and quantified as follows: ampelopsin A, (*E*)-piceatannol, pallidol, (*E*)-resveratrol, hopeaphenol, isohopeaphenol, (*E*)-*ε*-viniferin, (*E*)-miyabenol C, (*E*)-*ω*-viniferin, *r*2-viniferin, and *r*-viniferin. The fungicide concentration inhibiting 50% of growth of *Plasmopara viticola* sporulation (IC₅₀) was determined for the extracts and also for the main compounds isolated. *r*-Viniferin followed by hopeaphenol and *r*2-viniferin showed low IC₅₀ and thus high efficacy against *Plasmopara viticola*. Regarding stilbene extracts, wood extract followed by root extract showed the highest antifungal activities. These data suggest that stilbene complex mixtures from *Vitis vinifera* waste could be used as a cheap source of bioactive stilbenes for the development of natural fungicides.

KEYWORDS: cane, wood, root, UHPLC-MS, grapevine waste, mildew, antifungal activity

■ INTRODUCTION

Grapevine downy mildew, which is caused by the oomycete *Plasmopara viticola*, is a devastating disease worldwide. Most commercially important cultivars of the European grapevine (*Vitis vinifera*) are highly susceptible to this pathogen. Therefore, adequate control by fungicides is crucial, particularly in rainy climates. In France, field observations have shown that downy mildew infection was rated as severe for 25 of the last 50 years, and losses in Europe due to this disease have been huge. The greatest losses to American viticulturists from this disease occurred in the Northern United States, where in some localities from 25% to 75% of the vineyard was destroyed.¹

Plasmopara viticola attacks flower clusters, leaves, and young berries, and it is controlled with frequent applications of chemical fungicides to avoid yield and quality losses. Depending on meteorological conditions, 8–12 fungicide applications are necessary to control it. Currently, *Plasmopara viticola* control is based on the use of copper, but this can cause environmental problems owing to its accumulation in the soil.¹ Despite its unfavorable ecotoxicological profile, the use of copper is still tolerated in acknowledgment of its unique properties as a wide-spectrum fungicide and bactericide.

However, copper compounds have been included in Annex I to Directive 91/414/EEC² (concerning the placing of plant protection products on the market). “It is necessary that Member States introduce monitoring plans in vulnerable areas, where the contamination of the soil compartment by copper is a matter of concern, in order to set, where appropriate, limitations as maximum applicable rates.” Therefore, the future of viticulture is currently

dependent on the availability of copper unless alternatives are identified. The use of microorganisms for biocontrol has been proposed as a powerful alternative. *Aureobasidium pullulans* provides minimal protection;³ meanwhile, *Trichoderma harzianum* T39 treatment enhances the expression of defense-related genes.⁴ However, in the recent past, microbial biocontrol agents have not shown good and consistent activity against *Plasmopara viticola* in the field.¹

Several molecules that induce resistance reactions in grapevine have been shown to increase resistance to downy mildew in susceptible grapevines, such as laminarin,⁵ oligosaccharides,⁶ β -aminobutyric acid (BABA), linoleic acid, benzothiadiazole-7-carbothioic acid (*S*)-methyl ester (BTH),^{3,7} and thiamine.⁸ One of the defense mechanisms induced in plants after treatment with these molecules is the biosynthesis of stilbenes, which are phytoalexins with high antimicrobial properties.⁹

The application of plant extracts, which usually contain these molecules, can also induce resistance to downy mildew in grapevine.^{3,10} Recently, cane extracts were proposed as a potentially active raw material for developing a natural fungicide.^{11,12} They contain stilbene compounds, thereby justifying their bioactivity. Numerous studies have been published on the antifungal activity of stilbenes (mainly (*E*)-

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resveratrol, pterostilbene, ϵ -viniferin, and δ -viniferin) on *Plasmopara viticola*.¹³ Isohopeaphenol and miyabenol C have been described as stress metabolites in *Plasmopara viticola*-infected leaves.¹⁴ Moreover, oligomers such as miyabenol C, isohopeaphenol, r -viniferin, and $r2$ -viniferin are known to reduce the growth of the fungus related to grape trunk diseases.¹⁵

On the other hand, wine production generates a large amount of waste with low added value.¹⁶ Grapevine cane represents a large source of waste derived from the wine industry, with an estimated volume between 2 and 5 tons per hectare per year.

Likewise, when the vineyard is too old or vineyard removal is required for any reason, grapevine wood and roots also constitute a huge source of waste with low added value and high stilbene content.^{17,18} The surface area of vineyards has been reduced dramatically in recent years, especially in Europe. For example, France has recently decreased its vineyard surface area by 13,500 ha/year,¹⁹ leading to an estimated 1.75 million tons of wastes including 79% canes and 21% wood and roots.²⁰ Currently the surface area of vineyards worldwide has stabilized, although old grapevines are constantly being replaced by new ones.

This study tested three stilbene extracts from different vineyard waste sources (cane, wood and root) for their ability to fight *Plasmopara viticola* in grapevine under control conditions. The bioactivity of the main stilbene compounds present in the extracts was also tested. The data may help in selecting the most suitable grapevine waste for developing natural antifungal agents.

MATERIALS AND METHODS

Plant Material. Grapevine plants of *Vitis vinifera* cv. Cabernet Sauvignon, kindly supplied by Dr. Corio-Costet (UMR SAVE, INRA, Villenave d'Ornon, France), were propagated from wood cuttings in a greenhouse. After 3 weeks, rooted cuttings were potted in a sandy soil and were grown under controlled conditions at 25/20 °C day/night temperature with 75% relative humidity and a 15/9 h light/dark photoperiod. Downy mildew assays were performed with two-month-old plants with 10–12 leaves by collecting leaves from the upper part of the shoots (fourth leaf below the apex).

Pathogen Material. *Plasmopara viticola* isolate (ANN-01) collected in 2015 on *Vitis vinifera* cultivar Ugni-blanc in a commercial vineyard located in Charente (France) was multiplied and inoculated as previously described.²¹ Briefly, 15 μ L drops of a 5000 sporangia mL⁻¹ spore suspension were incubated for 7 days at 22 °C with a 16 h day/8 h night photoperiod. The isolate was sensitive to quinone outside inhibitor fungicides (QoI, mitochondrial Cytochrome b inhibitor) and carboxylic acid amid fungicides (CAA, cellulose synthase inhibitor) but resistant to metalaxyl (RNA synthesis inhibitors). The isolate was subcultured weekly on fresh grapevine leaves (*V. vinifera* cv. Cabernet Sauvignon).

Stilbene Extracts from *Vitis vinifera*. Extract of grapevine cane (*Vitis vinifera*), named Vineatrol, was kindly provided by Actichem (Montauban, France). Grapevine canes were harvested in the region of Bordeaux (France) and were composed of a mixture of Cabernet Sauvignon and Merlot cultivars. They were dried in open air for at least two months until their moisture content was <20%. Extract was prepared according to the patented protocol.²² Briefly, extraction was carried out on grapevine canes with ethanol as solvent. A purification step on crude extract was then performed with a hydro-alcoholic mixture to precipitate any apolar impurities, which were then removed by centrifugation. The solid obtained after removal of the solvent was a brown powder (Vineatrol). This extract has been recently tested in greenhouse and vineyard against downy mildew,¹² and it was used in the current study as a reference.

A mixture of Cabernet Franc and Tannat grapevine wood (*Vitis vinifera*) was harvested in a vineyard located in the Southwest region of France (Gers) and was supplied by Actichem (Montauban, France). It was dried at room temperature for two months in conditions with no light and then crushed in powder. Extraction was carried out in our lab with an ethanol–water mixture (85/15, v/v) under agitation at 60 °C. Ethanol was removed by evaporation under reduced pressure, and the aqueous phase was lyophilized, producing a brown powder.

Grapevine roots were harvested in the “Saint Christoly de Blaye” vineyard in the region of Bordeaux (France). Grapevine root was SO4 rootstocks (*Vitis riparia* \times *Vitis berlandieri*). As previously described, roots were dried, crushed, and extracted with an ethanol–water mixture (85/15, v/v) under agitation at 60 °C in our lab. After ethanol evaporation in vacuo, the water phase was lyophilized to produce a brown powder.

Chemicals and Standards. The following compounds were isolated and purified: (*E*)-resveratrol, (*E*)-piceatannol, pallidol, ampelopsin A, (*E*)- ϵ -viniferin, (*E*)- ω -viniferin, (*E*)-miyabenol C, hopeaphenol, isohopeaphenol, $r2$ -viniferin, and r -viniferin, from *Vitis vinifera* root and cane extracts as described in isolation by preparative high-performance liquid chromatography (HPLC) section. The identity and the purity of these compounds were analyzed by ultrahigh-performance liquid chromatography–ultraviolet–diode array detector (UHPLC-UV-DAD), LC-MS, and quantitative NMR as described below and compared to data from the laboratory.^{15,23,24} In addition, UV–vis spectra of each stilbene were recorded on a Cary 300 Bio UV–visible spectrophotometer in MeOH. The purity of the compounds was estimated to be $\geq 95\%$.

For UHPLC-MS analyses and isolation of compounds by preparative HPLC, water was purified using an Elga water-purification system (Bucks, U.K.). LC-MS-grade acetonitrile (VWR, Fontenay-sous-bois, France) and formic acid (Fisher Scientific, Loughborough, U.K.) were used for LC-MS analysis. For preparative HPLC, HPLC-grade acetonitrile, trifluoroacetic acid (TFA), and HPLC-grade methanol were purchased from Sigma-Aldrich (St Louis, MO, U.S.A.).

Identification and Quantification by UHPLC-MS. An UHPLC Agilent 1290 Series from Agilent Technologies (Santa Clara, CA, U.S.A.) was used to identify and quantify the stilbenes. It consisted of an autosampler module, a binary pump with degasser, a column heater/selector, and an UV–vis-DAD. An Agilent Zorbax SB-C18 (100 mm \times 2.1 mm \times 1.8 μ m) column was used for analysis. The solvent system consisted of water acidified with 0.1% formic acid as solvent A and acetonitrile acidified with 0.1% formic acid as solvent B. Elution was performed at a flow rate of 0.4 mL·min⁻¹ with the following gradient: 10% B (from 0 to 1.7 min), 10–20% B (from 1.7 to 3.4 min), 20–30% B (from 3.4 to 5.1 min), 30% B (from 5.1 to 6.8 min), 30–35% B (from 6.8 to 8.5 min), 35–60% B (from 8.5 to 11.9 min), 60–100% B (from 11.9 to 15.3 min), 100% B (from 15.3 to 17 min), 100–10% B (from 17 to 17.3 min). An Esquire 6000 ion trap mass spectrometer using an ESI source (Bruker-Daltonics, Billerica, MA, U.S.A.) was coupled to the UHPLC. Alternating negative and positive modes with a range of m/z 100–1200 were used to obtain ion chromatograms. Nitrogen was used as the drying gas at 10 L·min⁻¹ with nebulizer pressure at 40 psi and temperature at 365 °C. Capillary voltage was 3100 V, capillary exit voltage was –118.3 V, skimmer voltage was –40 V, and trap drive was 58.1. Extracts were dissolved in methanol–water mixture (1/1, v/v) at 1 mg·mL⁻¹, filtered on 0.45 μ m polytetrafluoroethylene (PTFE), and injected at 1 μ L. Analyses were performed in triplicate. Pure standards produced by the laboratory were injected at several concentrations (0, 25, 50, 100, 250, 500, and 1000 μ g·mL⁻¹) in independent triplicate to obtain calibration and equation curves. Data were analyzed with Bruker Data Analysis 3.2 software. The limit of detection (LOD) and the limit of quantification (LOQ) were evaluated at the maximum wavelength of absorption of each compound. The linearity of the standard responses was determined by plotting the peak area versus the compound concentrations.

Stilbene Isolation by Preparative HPLC. Stilbenes were purified from extracts by a Varian Pro Star equipped with an Agilent Zorbax SB-C18 PrepHT column (250 mm \times 21.2 mm, 7 μ m) and online

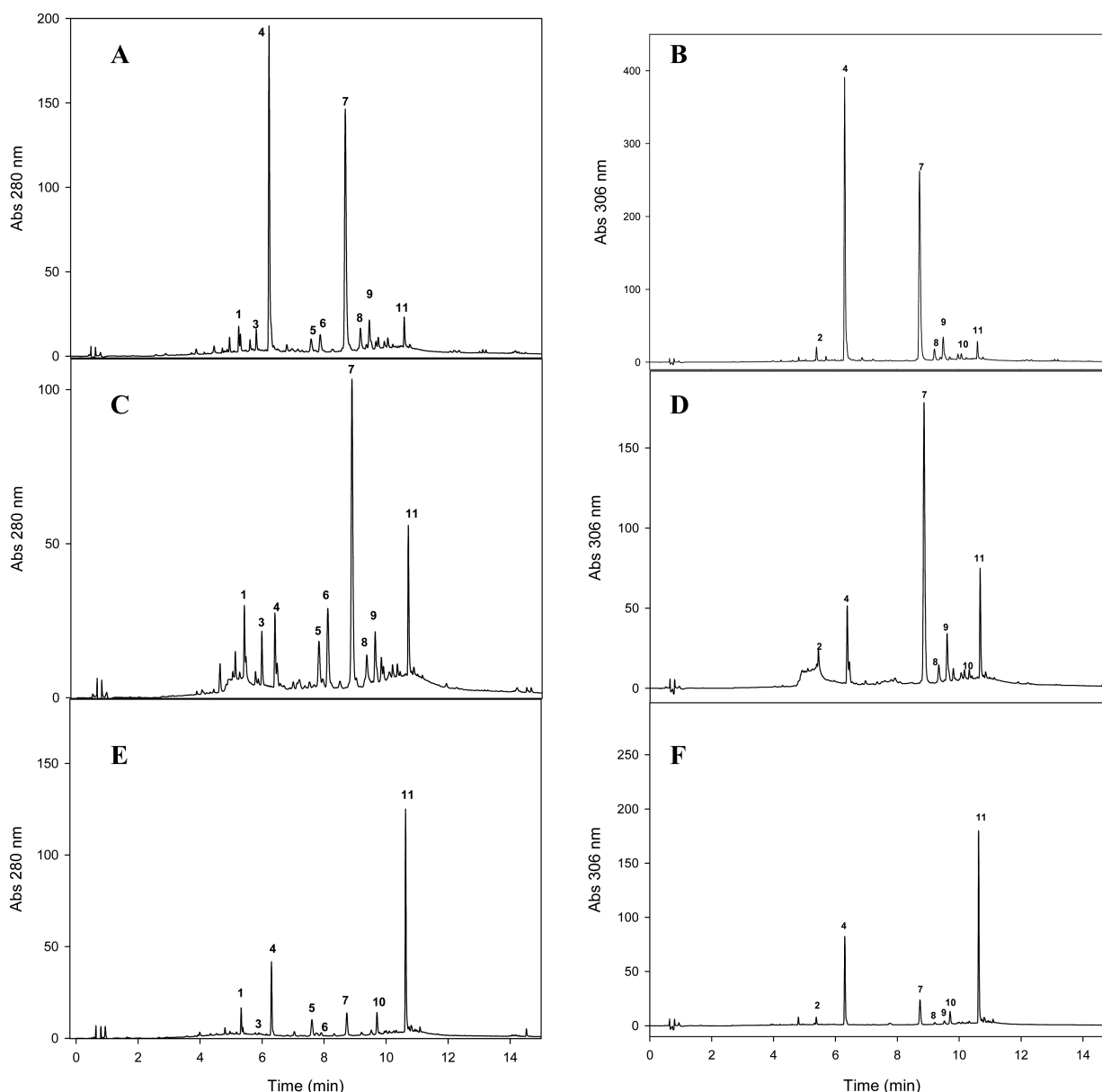


Figure 1. UHPLC-DAD chromatogram of cane extract at 280 nm (A) and 306 nm (B), wood extract at 280 nm (C) and 306 nm (D), and root extract at 280 nm (E) and 306 nm (F). (1) ampelopsin A, (2) (*E*)-piceatannol, (3) pallidol, (4) (*E*)-resveratrol, (5) hopeaphenol, (6) isohopeaphenol, (7) (*E*)-*ε*-viniferin, (8) (*E*)-miyabenol C, (9) *E*-*ω*-viniferin, (10) *r*2-viniferin, and (11) *r*-viniferin.

detection. Extracts were solubilized at $40 \text{ mg}\cdot\text{mL}^{-1}$ in methanol–water (50/50, v/v) and filtered on PTFE $0.45 \mu\text{m}$. The solvent system consisted of water acidified with 0.025% TFA as solvent A and acetonitrile acidified with 0.025% TFA as solvent B. Elution was performed at a flow rate of $20 \text{ mL}\cdot\text{min}^{-1}$ with the following gradient: 40% B (from 0 to 5 min), 40–55% B (from 5 to 25 min), 55–100% B (from 25 to 26 min), 100% B (from 26 to 31 min), 100–40% B (from 31 to 32 min), and 40% B (from 32 to 35 min).

NMR Experiments. NMR analyses were conducted on a Bruker Ultrashield 600 MHz NMR spectrometer equipped with a cryogenic TXI probe head. NMR spectra were obtained in 3 mm tubes with d_4 -methanol or acetone- d_6 as solvent. Bruker Topspin software version 3.2 was used for data analysis.²⁴

Antifungal Tests against Downy Mildew. Sporangia were obtained as described above, harvested, and suspended in sterile water at $4 \text{ }^\circ\text{C}$ before inoculation on the abaxial face of leaves with 20 droplets of $15 \mu\text{L}$. Grapevine leaves were kept one night in the dark for stomata opening and zoospore penetration. Using a vacuum pump, the residual droplets were aspirated and leaves were incubated for 7 days

under controlled conditions ($22 \text{ }^\circ\text{C}$) with a 16 h light day ($35 \mu\text{m}^{-2}\cdot\text{s}^{-1}$).²¹ For antifungal assays, 25-mm-wide leaf discs were generated with a pastry cutter and randomly disposed on humidified filter paper in Petri dishes. Eight repetitions were performed for each concentration. The different extracts were prepared in sterile water with 1% of ethanol with a range of seven concentrations (0, 50, 100, 200, 300, 500, and 800 mg/L). In the same conditions, stilbenes were solubilized in sterile water with 1% ethanol according to the procedure described by Pezet et al.⁹ and Schnee et al.¹¹ Stilbene was prepared at eight concentrations (0, 10, 20, 50, 100, 200, 500, and 1000 μM). Total solubility was obtained for ampelopsin A, hopeaphenol, isohopeaphenol, (*E*)-*ε*-viniferin, (*E*)-miyabenol C, and *r*2-viniferin, while a colloidal solution was formed for (*E*)-resveratrol and *r*-viniferin at the highest concentration. Dilutions were sprayed with a microdiffuser (0.7 mm) having a pressure reserve (Ecospray) on the leaf discs. Controls were done by spraying sterile water with 1% of ethanol onto leaf discs. One day later, the leaf surfaces were dried and 3 droplets of $15 \mu\text{L}$ per disc were deposited from a spore suspension at 15×10^3 sporangia per mL. As described above, inoculated leaf discs

Table 1. UHPLC-DAD-MS/MS Data of Stilbenes Determined in Hydroalcoholic Extracts

stilbene	peak	t_R	λ_{max} (nm)	pseudomolecular ion $[M-H]^-$	product ions
ampelopsin A	1	5.4	283	469	451, 375, 363, 347, 335, 281, 257, 227
(<i>E</i>)-piceatannol	2	5.5	290, 304, 325	243	225, 215, 201, 181, 175, 157, 141, 135, 107
pallidol	3	5.9	284	453	435, 406, 391, 369, 359, 346, 273, 265
(<i>E</i>)-resveratrol	4	6.4	306, 319	227	185, 143
hopeaphenol	5	7.8	283	905	811, 717, 611, 451, 357, 299
isohopeaphenol	6	8.1	283	905	811, 717, 675, 611, 451, 358, 265
(<i>E</i>)- ϵ -viniferin	7	8.9	285, 308, 323	453	435, 411, 369, 359, 347, 253, 225
(<i>E</i>)-miyabenol C	8	9.4	323	679	661, 637, 611, 573, 479, 451, 409, 345, 273, 228
(<i>E</i>)- ω -viniferin	9	9.7	322	453	435, 411, 395, 369, 333, 307, 285, 251, 225
<i>r</i> 2-viniferin	10	9.8	285, 328	905	811, 799, 693, 545, 451, 359, 265
<i>r</i> -viniferin	11	10.7	286, 321	905	887, 811, 799, 717, 545, 451, 359, 317

were cultivated for 7 days. Three independent experiments were carried out. Downy mildew development was measured according to the density of mycelium and number of sporulation sites. By comparison with the control discs, a conversion to a percentage of inhibition was performed. The relation between inhibition values and \log_{10} of the concentration allowed dose–response curves to be obtained. The \log_{10} dose to inhibit 50% of downy mildew (CI_{50}) was calculated from the regression equation of the linear part of the sigmoid curve.^{21,25,26}

Statistical Analyses. Three independent experiments of eight repetitions were carried out for each stilbene extract. Data are shown as means \pm SEM. The statistical analysis was performed on the three extracts at each specific concentration. One-way ANOVA followed by Tukey's multiple comparison post hoc tests was carried out by using R software. Significant differences between each extract was set at *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$, and $\bullet p < 0.1$.

RESULTS AND DISCUSSION

Chemical Characterization of Stilbene Extracts. Extracts from grapevine wood and root as well as reference grapevine cane extract were characterized. Eleven stilbenes were identified by LC-MS and by comparison with standards in the extracts as follows: ampelopsin A, (*E*)-piceatannol, pallidol, (*E*)-resveratrol, hopeaphenol, isohopeaphenol, (*E*)- ϵ -viniferin, (*E*)-miyabenol C, (*E*)- ω -viniferin, *r*2-viniferin, and *r*-viniferin (Figure 1, Supporting Information). Mass spectral data for these compounds are shown in Table 1. Mass data for (*E*)-piceatannol, pallidol, (*E*)-resveratrol, hopeaphenol, isohopeaphenol, (*E*)- ϵ -viniferin, (*E*)-miyabenol C, and (*E*)- ω -viniferin were in agreement with those described by other authors with minor changes in product ions owing to different fragmentation conditions.^{27–30} However, differences were found in the mass spectral data for ampelopsin A, *r*2-viniferin, and *r*-viniferin. Gorena et al.²⁸ reported different product ions for the above-mentioned compounds, probably owing to different conditions in the LC-MS method and the sample preparation (water/ acetonitrile 85:15 v/v). In addition to mass spectrometry data, UV–vis data based on λ_{max} (Table 1) and UV spectrum (data not shown) corroborated those in the literature. Ampelopsin A, hopeaphenol, isohopeaphenol and pallidol showed, respectively, λ_{max} at 283, 283, 281, and 284 nm, in agreement with the absence of conjugation between phenol units.^{31,14,32} In agreement with the literature, (*E*)-resveratrol, (*E*)-piceatannol, (*E*)- ϵ -viniferin, (*E*)- ω -viniferin, (*E*)-miyabenol C, *r*2-viniferin, and *r*-viniferin were characterized by λ_{max} at 306, 324, 324, 322, 323, 328, and 321 nm, respectively, meaning the presence of one double bond in trans-configuration in their structure.^{33–35,14,36,37} In addition to UV–vis and mass spectral data, stilbenes were also analyzed by NMR. All ¹H NMR spectra of stilbenes matched with data reported in the literature,

thereby confirming unambiguously the nature of each stilbene (Supporting Information). Furthermore, vitisinol C and δ -viniferin were also detected in all the extracts but under the LOQ (data not shown).

Stilbene concentrations varied widely depending on the nature of the extracts. Of note, each compound was quantified with its own calibration curve at the maximum wavelength (Table 1) so the amount was not underestimated. The main compounds were (*E*)- ϵ -viniferin (126.12 mg/g of dw extract) and (*E*)-resveratrol (61.15 mg/g of dw extract) in cane extract; (*E*)- ϵ -viniferin (79.39 mg/g of dw extract), isohopeaphenol (79.13 mg/g of dw extract), and *r*-viniferin (56.91 mg/g of dw extract) in wood extract; *r*-viniferin (128.29 mg/g of dw extract), ampelopsin A (21.78 mg/g of dw extract), and *r*2-viniferin (20.93 mg/g of dw extract) in root extract (Table 2). In light of the pattern described from the same tissue (cane, wood, and root) by other authors, the results can be somewhat generalized. Indeed, it appears that grapevine cane usually has (*E*)- ϵ -viniferin and (*E*)-resveratrol as its main compounds,^{38–40} grapevine wood has more (*E*)- ϵ -viniferin and isohopeaphenol,⁴¹ while grapevine root is generally rich in *r*-viniferin, ampelopsin A, and *r*2-viniferin.^{37,31,42} Furthermore, (*E*)-piceatannol had low concentrations in the three extracts while *r*2-viniferin was low in cane extract (7.17 mg/g of dw extract), (*E*)-resveratrol was low in wood extract (7.26 mg/g of dw extract), and pallidol was low in root extract (0.73 mg/g of dw extract). Cane and wood extracts showed similar total stilbene concentrations (Table 2), ~350 mg/g of extract, while root extract showed a somewhat lower concentration in stilbenes (223.72 mg/g of extract).

The relative abundance of each stilbene was different among the extracts. (*E*)- ϵ -Viniferin was the most abundant in cane and wood extracts with 12.6% and 7.9%, respectively, while it was low in root extract (1.3%). The opposite was found for *r*-viniferin, with a richness of 12.8% in root extract but only 5.6% and 2.0% in wood and cane extracts, respectively. These results show that the degree of oligomerization of stilbene increased from the aerial organs of the grapevine to the root system. Indeed, monomeric stilbenes ((*E*)-resveratrol and (*E*)-piceatannol) were found in higher quantities in grapevine cane (6.4%) than in wood and root (1.1% and 1.2%, respectively). Dimeric stilbenes ((*E*)- ϵ -viniferin, (*E*)- ω -viniferin, ampelopsin A, and pallidol) were predominant in grapevine cane and wood (17.3% and 15.1%, respectively), while the content was low in roots (3.6%). Finally, tetrameric stilbenes (*r*-viniferin, *r*2-viniferin, isohopeaphenol, and hopeaphenol) were abundant in grapevine wood and roots (17.2% in both of

Table 2. Limits of Detection (LOD) ($\mu\text{g}/\text{mL}$), Limits of Quantification (LOQ) ($\mu\text{g}/\text{mL}$), and Calibration Curve Coefficients (R^2) of Standard Stilbenes; Stilbene Content in Hydroalcoholic Extracts (mg/g of dw Extract), with Percentage between Brackets

	ampelopsin A	(E)-piceatannol	pallidol	(E)-resveratrol	hopeaphenol	isohopeaphenol	(E)- ϵ -viniferin	(E)-miyabenol C	(E)- ω -viniferin	r2-viniferin	r-viniferin	total stilbenes
LOD	0.23	0.04	0.17	0.03	0.16	0.30	0.07	0.22	0.07	0.25	0.10	
LOQ	0.69	0.13	0.53	0.08	0.48	0.92	0.21	0.68	0.20	0.75	0.31	
R^2	0.998	0.995	1	0.999	1	0.998	1	1	0.998	0.997	0.995	
cane	16.84 (1.7%)	3.35 (0.3%)	12.76 (1.3%)	61.15 (6.1%)	14.22 (1.4%)	35.21 (3.5%)	126.12 (12.6%)	21.08 (2.1%)	17.14 (1.71%)	7.17 (0.7%)	19.87 (2.0%)	339.99 (34%)
wood	36.84 (3.7%)	3.78 (0.4%)	19.29 (1.9%)	7.26 (0.7%)	26.64 (2.4%)	79.13 (7.9%)	79.39 (7.9%)	13.39 (1.3%)	15.54 (1.6%)	12.98 (1.3%)	56.91 (5.6%)	351.45 (35%)
root	21.78 (2.2%)	1.21 (0.1%)	0.73 (0.07%)	10.95 (1.1%)	18.14 (1.8%)	5.29 (0.5%)	13.01 (1.3%)	2.12 (0.2%)	1.27 (0.1%)	20.93 (2.1%)	128.29 (12.8%)	223.72 (22%)

them) contrary to cane, in which the content was the lowest (7.6%).

Thus, the three extracts (cane, wood, and root) showed total stilbene concentrations in the same range. However, different levels of bioactivity of the stilbene extracts could be expected owing to their different compositions.

Antifungal Activity of the Extracts and Stilbenes on *Plasmopara viticola*. Once the extracts had been characterized, antifungal assays were performed by adding the extracts on the leaves prior to inoculation in order to investigate the preventive efficacy of the extracts on *Plasmopara viticola*. Extracts were prepared in ethanol to ensure their dissolution, and the final ethanol concentration was 1% (v/v). One percent of ethanol in water does not affect zoospore mobility and disease development.⁹ The level of sporulation was determined by visual scoring at 7 days postinoculation as previously described.²¹

Grapevine cane extract (Vineatrol) has recently been tested in greenhouse and vineyard.¹² It showed a significant antimildew action in vivo comparable to the copper product traditionally used. From these data, we evaluated the activity of the grapevine wood and root extracts and compared them with this active cane extract.

The bioactivities of the three stilbene extracts were tested at different concentrations (Figure 2). A wide range of concentrations (50–800 mg/L) was assayed. No inhibition was observed in the control. Wood and root extracts totally inhibited the growth of *Plasmopara viticola* from 500 mg/L, as did cane extract from 800 mg/L (Figure 2). At 100 mg/L, sporulation was inhibited 3.1%, 32.0%, and 62.6% by cane, root, and wood extracts, respectively. Thus, the wood extract followed by root extract showed a better antimildew activity than the reference cane extract. Schnee et al.¹¹ tested various crude cane extracts at a set concentration against *Plasmopara viticola*, *Erisiphe necator*, and *Botrytis cinerea*. However, they classified the extracts as fungitoxic (+)/nonfungitoxic (–) but did not report the percentage of sporulation inhibited. *Leontodon filii* extract has also been found to be moderately efficient against *Plasmopara viticola*.⁴³

The IC_{50} , i.e., the concentration that inhibited 50% of disease development, was also determined for the extracts (cane, wood, and root) by using a dilution series. This allowed a more precise calculation of the IC_{50} values, as confirmed by the high correlation coefficient of each regression equation of the linear part of the sigmoid curve (Table 3). The lowest value was found for wood extract (60 mg/L), followed by root extract (120 mg/L) and cane extract (210 mg/L). Thus, wood extract showed the highest antifungal activity (Table 3), more than three times superior to cane extract, although their stilbene levels were similar (Table 2). Remarkably, the root extract with the lowest content of stilbenes had a huge antimildew activity, almost twice better than the cane extract.

IC_{50} was then calculated for the main stilbenes found in the extracts. To this end, a previous purification of the main compounds was carried out as described in the Materials and Methods section. For pure stilbenes, data for IC_{50} are expressed both in mg/L, for the purpose of comparison with the extract data, and in μM , so that results from other authors can be compared (Table 3).

The lowest IC_{50} , and thus the highest efficacy against *P. viticola*, was shown by *r*-viniferin (12 μM), followed by hopeaphenol (18 μM) and *r*2-viniferin (20 μM), in agreement with the findings of Schnee et al.,¹¹ who reported 12 and 26 μM

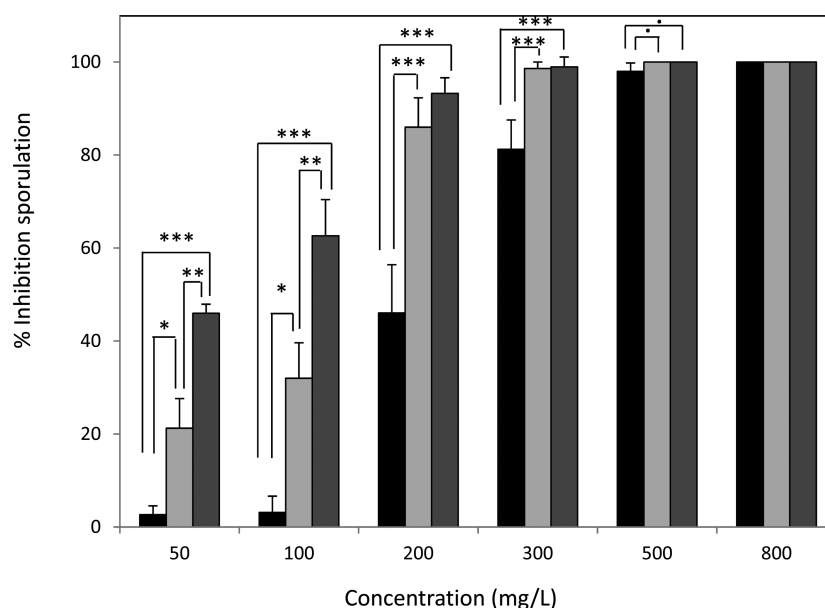


Figure 2. Effect of stilbene extracts (cane, root, and wood) on *Plasmopara viticola* sporulation in artificially inoculated leaf disc 7 days after inoculation. Results are expressed as means \pm SEM. Significant difference between each extract was set at *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$, and • $p < 0.1$. Black bar = cane extract, light gray bar = root extract, and dark gray bar = wood extract.

Table 3. Concentration (mg/L) Causing 50% Inhibition of Mildew Development Monitored by Sporulation of *Plasmopara viticola*

	IC ₅₀ ^a	R ²
cane extract	210	1
root extract	120	1
wood extract	60	0.9717
ampelopsin A	438 (934 ^b)	1
(<i>E</i>)-resveratrol	110 (484 ^b)	0.9812
hopeaphenol	16 (18 ^b)	1
isohopeaphenol	40 (45 ^b)	0.9784
(<i>E</i>)- ϵ -viniferin	70 (155 ^b)	1
(<i>E</i>)-miyabenol C	40 (103 ^b)	0.9983
<i>r</i> 2-viniferin	18 (20 ^b)	0.9966
<i>r</i> -viniferin	10 (12 ^b)	1

^aCalculated from dose–response curves + correlation coefficients.

^bData expressed in μ M.

for *r*-viniferin and hopeaphenol, respectively. However, they did not determine IC₅₀ for *r*2-viniferin. Therefore, *r*-viniferin is as toxic as pterostilbene (IC₅₀ 12.7 μ M) and δ -viniferin (IC₅₀ 14.7 μ M),⁹ the most potent toxic stilbenes.³² IC₅₀ values higher than those found for *r*-viniferin were found for isohopeaphenol (45 μ M), (*E*)-miyabenol C (103 μ M), and (*E*)- ϵ -viniferin (155 μ M), in increasing order. To our knowledge, this is the first time that IC₅₀ values are reported for *r*2-viniferin, isohopeaphenol, and (*E*)-miyabenol C. However, lower IC₅₀ values have been reported for (*E*)- ϵ -viniferin, e.g., 63 and 71.2 μ M.^{9,11}

The highest IC₅₀ values were found for (*E*)-resveratrol and ampelopsin A, 484 and 934 μ M, respectively (Table 3). In fact, (*E*)-resveratrol is considered to be a precursor of compounds of higher fungal toxicity rather than as an antifungal compound itself.⁴⁴ Our data are again in contrast with those of other authors. For example, Schnee et al.¹¹ reported 282 and 121 μ M for the IC₅₀ of ampelopsin A and (*E*)-resveratrol, respectively, while Pezet et al.⁹ found 145 μ M for the IC₅₀ of (*E*)-resveratrol. These differences in IC₅₀ values could be due to differences in

the antifungal bioassays used. While the above-mentioned authors prepared a mixture of zoospores and stilbenes and then added it on the leaves for curative purposes, stilbene solutions were added on the leaves in the current experiments and inoculated the zoospores 1 day later in order to test the preventive effect of the extracts on *Plasmopara viticola* infection. Furthermore, our results are in agreement with those of Malacarne et al.,⁴⁵ showing a negative correlation between the degree of stilbene oligomerization in *Vitis* hybrid leaves and the evolution of *P. viticola* infection.

To summarize, we demonstrate the ability of stilbene extracts obtained from *Vitis vinifera* waste to inhibit *Plasmopara viticola*. Wood extract had the highest antifungal bioactivity against downy mildew in vitro followed by root extract and finally the reference cane extract. Root extract contained the lowest stilbene concentration but it showed a high antifungal bioactivity against *Plasmopara viticola* in vitro. The high concentrations of *r*-viniferin and to a lesser extent of *r*2-viniferin in root, coupled with a potential synergistic effect that may occur between stilbenes, could contribute to this fact. We confirm that *r*-viniferin is highly active against *P. viticola*. Although wood extract and cane extract showed similar total stilbene concentrations, the concentrations of *r*2-viniferin, *r*-viniferin, hopeaphenol, and isohopeaphenol in wood extract were higher than those in cane extract, so this could account for the difference in their bioactivities. Moreover, the IC₅₀ data for *r*2-viniferin, isohopeaphenol, and (*E*)-miyabenol C are reported for the first time.

The present findings strongly suggest that grapevine waste such as wood and roots from *Vitis vinifera* may be preferentially used as cheap sources of bioactive stilbenes for developing natural fungicides. Indeed, wood and roots together contain the four most active compounds (*r*-viniferin, *r*2-viniferin, hopeaphenol, and isohopeaphenol) at high levels. The potential reduction in the use of toxic fungicides coupled with the exploitation of grapevine waste would represent a significant step forward in promoting sustainability in viticulture.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jafc.7b00241.

¹H NMR annotated spectrum of isolated stilbenes (PDF)

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Notes

The authors declare no competing financial interest.

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