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OENO One
VINE AND WINE OPEN ACCESS JOURNAL
By IVES International Viticulture & Enology Society

DOI: https://doi.org/10.20870/oeno-one.2022.56.3.4911

OPEN ACCESS

ORIGINAL RESEARCH ARTICLE

A grapevine by-product extract enriched in oligomerised stilbenes to control downy mildews: focus on its modes of action towards *Plasmopara viticola*

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Associate editor:
Annette Reineke

Received:
18 November 2021

Accepted:
14 June 2022

Published:
4 July 2022

ABSTRACT

Natural extracts, and particularly plant by-product extracts, can represent an eco-friendly alternative to synthetic pesticides to control crop diseases. We produced an extract from a mix of grapevine trunk and roots that contain a high amount of complex stilbenes (grapevine phytoalexins), especially ε- and r-viniferins. This extract conferred protection to grapevines against *Plasmopara viticola* (the causal agent of downy mildew) by displaying antimicrobial activities towards zoospore mobility and sporulation and by stimulating plant defences. From the perspective of its application in the field, a formulated preparation of the extract was proposed using polysorbate and sophorolipids. The formulated extract reduced the development of various oomycetes impacting grapevine, potato, tomato and melon in semi-controlled conditions. Thus, such grapevine extract constitutes a promising strategy to protect major cultivated plants against downy mildews.

KEYWORDS: resveratrol oligomers, crop protection, *Vitis vinifera*, antimicrobial activities, defence stimulation

ABBREVIATIONS :

ACT: actin
BM: Bordeaux mixture
CHIT: chitinase
CHS: chalcone synthase
DAD: diode array detector
DPI: days post-inoculation
DPT: days post-treatment
DW: dry weight
EF1: γ-chain of elongation factor 1
GAPDH: glyceraldehyde-3-phosphate dehydrogenase
HPLC: high-performance liquid chromatography
IC_{50}: the concentration of 50 % of downy mildew inhibition
MS: mass spectrometer
NQ: non-quantifiable
PAL: phenylalanine ammonia-lyase
PDS: plant defence stimulator
PR: pathogenesis-related proteins
ROMT: resveratrol-*O*-methyltransferase
RT-PCR: reverse transcriptase-polymerase chain reaction
STS: stilbene synthase
THIORYLS8: catalytic thioredoxin-like protein 4A
UHPLC: ultra-high-performance liquid chromatography

INTRODUCTION

Oomycetes are responsible for severe diseases affecting major crops. For instance, *Phytophthora infestans* is the causal agent of tomato and potato late blights, and *Pseudoperonospora cubensis* is associated with melon downy mildew. *Plasmopara viticola* affects grapevines, especially European cultivars, and represents one of the most destructive diseases in all vineyards worldwide (Spring *et al.*, 2019). The control of these fungal-like eukaryotes is largely ensured by using synthetic and/or copper-based fungicides (Sharma *et al.*, 2015; Corio-Costet, 2012). However, the intensive use of such pesticides is associated with environmental and health issues, plus the risk of the pathogen developing resistance. Considering copper-based fungicides, their long-term, repeated, and excessive applications result in their accumulation in vineyard soils with negative side effects (such as yield reduction or impacts on soil biota) (Lamichhane *et al.*, 2018). To reduce reliance on chemical fungicides, grape growers need alternative methods for disease management, and, in consequence, the development of eco-friendly control solutions has to be promoted. Amongst these alternatives, the use of microorganisms acting as plant pathogen antagonists, such as *Trichoderma harzianum* T39, has been proposed (Perazzolli *et al.*, 2011). Another promising strategy is based on the use of plant defence stimulators (PDS). For instance, benzothiadiazole has been shown to trigger grapevine protection against *Erysiphe necator* (powdery mildew), *P. viticola* (downy mildew) and *Botrytis cinerea* (grey mould) (Dufour *et al.*, 2013). This enhanced resistance results from the induction of plant defence reactions such as the accumulation of pathogenesis-related (PR) proteins (e.g., chitinases (CHIT) and β-1,3-glucanase) and the production of phytoalexins (stilbenes). Nevertheless, both biocontrol agents and PDS have been reported to lack constancy and efficacy in fields (Aveline *et al.*, 2019). Additional sustainable strategies have to be offered as the use of natural antimicrobial molecules. Some plant extracts, such as extracts of forest or herbaceous species, displayed oomycidal activities (Krzyzaniak *et al.*, 2018; Mulholland *et al.*, 2017; Thuerig *et al.*, 2016). The capacity of grapevine cane extracts to reduce downy mildew development has been reported both in greenhouse and vineyard conditions (Richard *et al.*, 2016). Stilbenes, the compounds present in high content in cane extracts, were proposed to be responsible for such protection as they display antimicrobial properties (Alonso-Villaverde *et al.*, 2011). They share a common backbone stilbene structure, exist as monomers and oligomers and differ in the type and position of the substituents (Pawlus *et al.*, 2012). Their biosynthesis occurs through the phenylpropanoid pathway with the involvement of two key enzymes, phenylalanine ammonia-lyase (*PAL*), which ensures the conversion of phenylalanine to cinnamate, and stilbene synthase (*STS*), which converts cinnamoyl-coenzyme A in stilbenoids (Chong *et al.*, 2009). Resveratrol, the basic unit, shows moderate antimicrobial activity. Besides, its derivatives, such as pterostilbene, a methylated form, and viniferins, isohopeaphenol and hopeaphenol, oligomerised compounds can be highly effective molecules against downy mildew (Pezet *et al.*, 2004; Gabaston *et al.*, 2017a; Schnee *et al.*, 2013). In addition, stilbenes have been reported to act as PDS (De Bona *et al.*, 2019). Regarding oligomerised stilbenes, they are present in grapevine canes and in higher contents in trunks and roots (Gabaston *et al.*, 2017a; Schnee *et al.*, 2013). These two latter perennial organs represent a huge amount of biomass (e.g., in France, around 0.4 million tons per year) due to the grubbing up of vines (France Agrimer, 2015; Ademe, 2009). This valuable biomass is still today poorly recovered despite its high potential value (Schnee *et al.*, 2013). To benefit from the ability of stilbenes to control diseases, preparations with solvents, emulsifiers, safeners and adjuvants etc., are required to allow these active compounds to counteract adverse climatic conditions (Martinez *et al.*, 1989; Hazra and Purkait, 2019). Eco-friendly formulations have to be developed as the ones based on lipid-related products (e.g., resins or vegetable oils) (Demanèche *et al.*, 2020; Erdogan *et al.*, 2019). Surfactants such as Tween 20 can help encapsulate hydrophobic compounds by forming emulsions with ethanol (Baranauskaite *et al.*, 2019). Amongst adjuvants of natural origin, sophorolipids represent promising candidates (Vaughn *et al.*, 2014; Giessler-Blank *et al.*, 2011).

In this study, we produced an extract enriched in oligomerised stilbenes from a mix of grapevine trunk and roots to study its protective capacities against different oomycetes with a focus on *P. viticola.* A formulated extract was proposed. We evaluate the antimicrobial properties of the oligomerised stilbenes extract (OSE) formulated or not, and in addition, we followed up on its effect on grapevine defence responses. Experiments were done under laboratory and/or semi-controlled conditions.

MATERIALS AND METHODS

1. Chemicals and Standards

For stilbenes extraction and their solubilisation, the solvents (ethyl acetate, methanol and ethanol) were purchased from Fisher Scientific (Loughborough, UK). For analysis, HPLC-grade acetonitrile and methanol, trifluoroacetic acid (TFA) (Sigma-Aldrich, St. Louis, MO), LC-MS-grade acetonitrile (VWR, Fontenay-Sous-Bois, France) and formic acid (Fisher Scientific, Loughborough, UK) were purchased. Water was purified by an Elga water purification system (High Wycombe, UK).

The purification of standard stilbenes (*E*-piceid, *E*-resveratrol, *E*-piceatannol, pallidol, ampelopsin A, parthenocissin, *E*-miyabenol C, hopeaphenol, isohopeaphenol, *E*-ε-, *E*-ω-, r2- and r- viniferins) from *Vitis vinifera* roots and cane extract was performed by preparative high-performance liquid chromatography (HPLC) as described by Gabaston and co-workers (Gabaston *et al.*, 2017a). Compounds were analysed by ultrahigh-performance liquid chromatography-ultraviolet-diode array detector coupled to a mass spectrometer (UHPLC-UV-DAD-MS) to determine identity and purity.

The purity of compounds was estimated ≥90 %. The percentage of purity of each standard was estimated based on their HPLC peak area regardless of the total mass of powder obtained after their isolation by preparative HPLC.

The Bordeaux mixture (BM) was a commercial preparation (KB Bouillie bordelaise RSR Dispersss Jardin, Scotts, France) containing 20 % copper sulphate.

The co-formulants were Tween 20 (polysorbate 20, Radiasurf 7137, Oléon, Compiègne, France), Tween 80 (polysorbate 80, Radiasurf 7157, Oléon, Compiègne, France) and sophorolipids (Rewoferm SL One, Evonik, Essen, Germany).

2. Preparation of the oligomerised stilbene-enriched extracts

Trunk and roots were collected from grapevines of *V. vinifera* cv. Merlot and Riparia Gloire de Montpellier as rootstock. This vineyard was located in the S outh-West region of France (Pessac-Léognan, Gironde). The vines were 32 years old and the vineyard was managed in a conventional way. After their collection, trunks and roots were dried at ambient temperature for 8 months until their moisture content was under 10 %. A mix of trunk and roots (70/30, w/w) was crushed to powder of small particles of 4 mm. Two successive extractions with ethyl acetate were performed on grapevine powder at 60 °C for 2 h. Four hundred grams of powder was firstly extracted with 4000 mL of pure ethyl acetate under stirring. After filtration on a cotton layer, the supernatant was collected and a second extraction was done on the plant powder with an ethyl acetate–water mixture (85/15, v/v). This latter filtrate was collected and pooled with the first one. Finally, to enrich the final extract in stilbenes, a water/ethyl acetate (50/50, v/v) liquid–liquid extraction was done. The organic phase was removed with a rotary evaporator at 38 °C and the concentrated extract was evaporated to dryness in a laboratory freeze dryer. The final product, a brown powder, was the oligomerised stilbenes enriched (OSE) extract.

The formulated OSE (F-OSE) extract consisted of a solution containing the OSE extract (300 mg/L) and a mixture of co-formulants (9 g/L). In this latter mixture, the co-formulants and their final concentration were: Tween 20 at 0.8 g/mL, Tween 80 at 0.15 g/mL and sophorolipids at 0.05 g/mL. Tween 20 and Tween 80 act as surfactants and allow a homogeneous distribution of the extract when sprayed on the leaves. Sophorolipids offer a good solvation of the extract.

3. Identification and characterisation of stilbenes by UHPLC-MS analysis

Analyses were carried out on an Agilent 1290 LC system series from Agilent Technologies (Santa Clara, CA, USA). The system is made of a binary pump, a vacuum degasser, an autosampler, a thermostatted column compartment and outfitted with an ultraviolet-visible diode-array detector (UV–vis DAD) (Agilent G4212B). Samples, prepared at 1 mg/mL, were solubilised in the methanol–water mixture (50/50, v/v) by ultrasonication before being filtrated with a PTFE membrane filter (0.2 µm, Millex-LG, Japan). One µL was injected into the system. The mobile phase consisted of water and pure acetonitrile (solvent A and B, respectively), both prepared in formic acid 1 %. The gradient used was the same as described in the work of Gabaston *et al.* (2017a). Analysis was performed at 30 °C. The mass spectrometer coupled to the UHPLC was an Esquire 6000 ion trap mass spectrometer with an ESI source (Bruker-Daltonics, Billerica, MA, USA). An Agilent Zorbax SB-C18 (100 mm × 2.1 mm × 1.8 µm) column was used for analysis. Three independent samples were analysed, and, in addition, technical triplicates were performed for each independent sample. Mass analysis conditions were performed as previously mentioned (Gabaston *et al.*, 2017a). Compounds were identified by UV spectrum and retention time from standards. Data were compared with those obtained previously in the laboratory (Gabaston *et al.*, 2017a). Each stilbene was quantified based on its respective standard calibration curve (with standards concentrations from 1, 5, 20, 50 and 100 µg/mL) at its maximum wavelength to not misestimate the quantity.

4. Plant and fungal material

4.1. Grapevine

For bioassays on foliar discs and analyses of defence responses (gene expression and stilbenes), woody cuttings of *V. vinifera* cv. Cabernet Sauvignon were used. They were provided by UMR SAVE (INRAE, Villenave d'Ornon, France). Canes were potted in sandy soil and developed plants were grown in a greenhouse under controlled conditions at 25/20 °C day/night temperature with 75 % relative humidity and a 15/9 h light/dark photoperiod.

V. vinifera cv. Merlot was used for measuring pest incidence and pest severity in semi-controlled conditions (average temperature of 23 °C, relative humidity of 50 % and a 15/9 h light/dark photoperiod). They were produced by Science Agro Atlantique (Saint Germain du Puch, France).

4.2. Other crops

Potato (*Solanum tuberosum*) variety Bintje, tomato (*Solanum lycopersicum*) variety Saint-Pierre and melon (*Cucumis melo*) variety Maltese F1 were cultivated under semi-controlled conditions at 25/20 °C day/night temperature with 75 % relative humidity and a 15/9 h light/dark photoperiod for respectively one month (6 leaves), 40 days (3–4 leaves) and one month (6 leaves) before the application of the first treatments.

4.3. Pathogens

For laboratory conditions, bioassays on grapevine *P. viticola* isolates (ANN-01) were collected in 2015 in a commercial vineyard of *V. vinifera* cv. Ugni-blanc in Charente (France) were used. The maintenance of *P. viticola* was done as previously described (Corio-Costet *et al.*, 2011).

For the semi-controlled assays on grapevines, tomato and melon, respective pathogens isolates used were *P. viticola*, *Phytophthora infestans* and *Pseudoperonospora cubensis*; all provided by BIOtransfer (Montreuil, France).

Suspensions were prepared at 48,000 spores/mL for *P. infestans* in the potato assay and 40,000 spores/mL for *P. infestans* and *P. cubensis* in the tomato and melon assays, respectively.

5. Antimicrobial assays

5.1. On *Plasmopara viticola*

To evaluate the direct oomycidal effect of the OSE extract on zoospores, we collected *P. viticola* (ANN-01) sporangia and placed them in sterile distilled water to obtain a solution at 15,000 sporangia/mL. After 1h in the dark, we added the OSE extract at different concentrations (5, 10, 25, 50 and 100 mg/L) to the sporangia solution and immediately performed microscope observation at 400-fold magnification. The inhibition of zoospores mobility was estimated in comparison to the 1 % ethanol control solution (0 % inhibition) after 5 min of treatment. At least 10 representative fields in triplicate were observed and 3 independent experiments were performed.

The ability of the OSE extract to damage the zoospores was also evaluated by an indirect assay. To do that, a solution of sporangia obtained as described before and treated for 5 min with the OSE extract at 5, 10, 25, 50 or 100 mg/L was used to inoculate grapevine foliar discs. The downy mildew development was estimated at six days after inoculation. Details about downy mildew inoculation and assessment of the oomycete development are described below (sub-part 5.2).

5.2. In laboratory conditions

The third and the fourth leaves below the apex of two-months-old grapevine cv. Cabernet Sauvignon cuttings were collected. Foliar discs (25 mm wide) were produced and randomly placed, the abaxial side upwards, into Petri dishes containing humidified Whatman paper. The OSE extract was prepared at different concentrations (from 25 to 300 mg/L) in sterile water with 1 % ethanol (v/v). The control contained only 1 % ethanol in distilled water. After being sprayed with the solutions (86.5 mL/m^2) and kept one night in the dark, the foliar discs were inoculated with 3 drops of 15 µL of a *P. viticola* sporangia suspension (15,000 sporangia/mL). Then they were incubated at 22 °C with a photoperiod of 16/8 h day/night for 7 days. Six foliar discs per dish, three dishes per condition and three independent experiments were performed. The density of sporulation was estimated by visual scoring to assess disease development. The control was set at 100 % sporulation and the values obtained for extracts conditions were compared to this control and estimated in the percentage of inhibition (Corio-Costet *et al.*, 2011). A dose–response curve was established and the concentration of 50 % of downy mildew inhibition (IC_{50}) was calculated as described by Corio-Costet *et al.* (2011).

To estimate the level of protection conferred by the OSE extract that resulted from defence stimulating properties, the following experiments were conducted. In the greenhouse, all leaves of the grapevine cuttings were treated by a spray of the OSE extract at 300 mg/L. The third and the fourth leaves were collected 1, 2, 3 and 6 days post-treatment (dpt) and washed carefully with distilled water. Foliar discs were taken and the remaining part of the leaves was freeze-dried for further phytoalexin analysis. *P. viticola* inoculation was performed on foliar discs as described above. Seven days after, disease development was estimated. Five independent biological replicates were used for each timing sample and condition and two independent experiments were done.

5.3. In semi-controlled conditions

For *P. viticola* assays, solutions of OSE extract at 300 mg/L, formulated or not, were tested as described above. BM was applied at 4.2 g/L. An untreated condition was done. Three independent experiments were done and the schedule of the first experiment was provided as an example in Figure 1. The modalities were evenly distributed by blocks in the greenhouse with 4 blocks of 4 plants by modality. The design of the experiments is presented in the supplementary Figure S1. In brief, the protocol was as follows. Every 8–10 days, the solutions were sprayed on all grapevine leaves for a total of three treatments (T_1, T_2, T_3). Three inoculations were performed: a first and artificial inoculation (primary infection, PI) and two secondary inoculations (SI_1 and SI_2), mimicking natural infections by misting water. To carry out the natural inoculations, a blowing fog was generated and dispersed into the air, thus allowing homogeneous distribution of the zoospores in the greenhouse. PI was carried out one day after T_1 and SI_1 was done as soon as the first symptoms appeared (2–3 days after T_2) and SI_2 9–10 days after T_3. Three assessments (A_1, A_2, A_3) were performed at least 7 days after PI, SI_1 and SI_2, respectively. The disease development was evaluated by visual observation. It was estimated as pest incidence and pest severity that represent the number of infected leaves and the leaf surface covered by sporulation, respectively, and expressed in the percentage of inhibition compared to the untreated control.

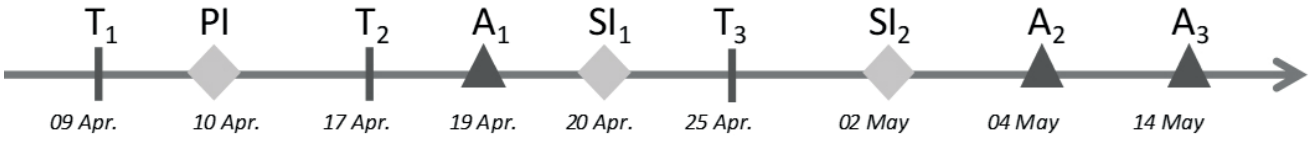

FIGURE 1. Treatments schedule of the greenhouse experiments on grapevine cuttings.

A total of three treatments (T1 to T3) were performed. PI means primary downy mildew inoculation (artificial), SI1 and SI2 correspond to secondary inoculations ("natural") and A1 to A3 refer to the three assessments. The dates of one out of three experiments were indicated.

For oomycetes affecting tomato (*Solanum lycopersicum*), melon (*Cucumis melo*) and potato (Solanum tuberosum), greenhouse trials were conducted with the formulated OSE extract at 1 g/L in the presence of co-formulants at 9 g/L. An untreated condition was done. The treatment was performed on 7-leaf stage plants for potato and 3-leaf stage plants for tomato and melon. The experimental designs for all pathosystems were similar to the one used for the *P. viticola* assay (Supplemental Figure S1). Potato and melon were artificially inoculated with *Phytophthora infestans* and *Pseudoperonospora cubensis*, respectively, 1 day after treatment, and tomato was inoculated with *P. infestans* 2 days after treatment. Pest incidence and severity were estimated as described before. Two assessments were performed at 7 and 14 days post-inoculation (dpi) for *P. infestans*/potato, 4 and 7 dpi for *P. infestans*/tomato and 6 and 12 dpi for *P. cubensis*/melon.

6. Real-time quantitative PCR analysis

For gene expression analysis, two conditions were considered: control (1 % ethanol) and OSE extract (300 mg/L). The solutions were sprayed on all leaves of Cabernet Sauvignon cuttings. The third and fourth leaves from the apex were collected at 0, 1, 2, 3 and 6 dpt. Five plants were used for each time of sampling and condition. Three independent experiments were done. After harvest, leaves were immediately frozen in liquid nitrogen and kept at –80°C until use. RNAs were obtained from 150 mg of grounded leaves with Spectrum™ Plant Total RNA kit (Merck, Germany) and treated with RNase-free DNase I kit (Promega Corp., USA). Reverse transcription was conducted on 2 µg of RNA using the GoScript™ Reverse Transcriptase (Promega Corp., USA). Real-time quantitative PCR analysis (qPCR) was carried out to determine the mRNA copy number of genes of interest. The selected genes and corresponding primer sets used were presented in Table 1. PCR reactions were performed in triplicates with the GoTaq® qPCR Master Mix (Promega Corp., USA) in 96-well plates (20 µL per well), according to supplier instructions. Melting curves were done to verify primer specificity. *THIORYLS8*, coding a catalytic thioredoxin-like protein 4A, was chosen as a housekeeping gene as it was the most stable according to BestKeeper© (Pfaffl *et al.*, 2004) software out of four housekeeping genes tested (glyceraldehyde-3-phosphate dehydrogenase, γ-chain of elongation factor 1, *THIORYLS8* and actin) (Dufour *et al.*, 2016). PCR efficiency amplification was evaluated. The slope of the linear regression of the log-transformed cDNA concentration was plotted against the respective CTs and according to the following formula $E = 10^{-\text{slope}}$. As efficiencies for each primer pair were not equal to 2, the ratio of expression was calculated according to the following formula (Pfaffl *et al.*, 2004).

7. Stilbenes extraction from grapevine leaves and analysis

We quantified the stilbene content of leaves treated with the OSE extract from the leaves that were also used for antimicrobial assays in laboratory conditions (Section 5.1). After the production of foliar discs for antimicrobial assays, the remaining part of the leaves was washed with tap water, dried gently with paper and immediately freeze-dried and put in powder. Then, two successive methanol (100 %) extractions followed by a third with a methanol-water mixture (90/10, v/v) were carried out on 60 mg of leaf powder, each for 1h30 under stirring and at room temperature. All the organic phases were pooled and evaporated, and the remaining extract was resuspended in a methanol–water mixture (30/70, v/v). Purification on a Supelclean® LC18 column (Supelco®, Bellefonte, USA) was performed. The resulting extract was resuspended in a methanol-water mixture (50/50, v/v) and analysed by UHPLC-MS as described above.

8. Statistical analyses

Statistical analyses were performed using R software. For variance homogeneity, a one-way analysis of variance (ANOVA) was applied ($p < 0.05$) and the mean values were separated by the Tukey test post-hoc statistic.

RESULTS

1. Chemical characterisation of the oligomerised stilbene-enriched extract

The stilbenes present in the OSE extract that was produced from the grapevine trunk and roots (70/30, w/w) were characterised by UHPLC-MS. Twelve molecules were identified and quantified: 3 monomers (*E*-piceid, *E*-piceatannol and *E*-resveratrol), 5 dimers (ampelopsin A, pallidol, parthenocissin, *E*-ε-viniferin and *E*-ω-viniferin) and 4 tetramers (hopeaphenol, isohopeaphenol, r- and r2-viniferins) (Table 2).

TABLE 1. Defence-related genes and corresponding primer sets used for qPCR.

Names	GenBank accession number	Forward primer (5′–3′)	Reverse primer (5′–3′)
THIORYLS8	XM_002283586	TCCCAATCGTGGCCGAACCG	TCACTCTGGATGGGCCGTCG
PAL	X75967	ACAACAATGGACTGCCATCA	CACTTTCGACATGGTTGGTG
CHS	X75969	CCAACAATGGTGTCAGTTGC	CTCGGTCATGTGCTCACTGT
STS	X76892	ATCGAAGATCACCCACCTTG	CTTAGGCGGTTCGAAGGACAG
ROMT	FM178870	TGAGCTCCCAGTCAACCCAGAGA	CGCATGAGACGGTACACGCATT
PR3	VVU97521	TATCCATGTGTCTCCGGTCA	TGAATCCAATGCTGTTTCCA
PR10	AJ291705	GCTCAAAGTGGTGGCTTCTC	CTCTACATCGCCCTTGGTGT

Names, Genbank accession number, forward and reverse primers.

The limits of detection and quantification, the accuracy and the linearity were determined. Another stilbene, the *E*-miyabenol C trimer, was identified, but it was under the limit of quantification.

Mass values of each stilbene were in accordance with those obtained previously in the laboratory (Table S1) (Gabaston *et al.*, 2017a). The compounds present in a relatively high amount in the OSE extract (in mg/g of DW extract) were *E*-resveratrol (95.41), *E*-ε-viniferin (94.02), r-viniferin (73.74), isohopeaphenol (63.67) and hopeaphenol (47.07) (Table 2). Stilbenes present in minor quantity were ampelopsin A (23.08), r2-viniferin (21.77), pallidol (18.52), *E*-ω-viniferin (12.75), *E*-piceatannol (11.07) and parthenocissin A (6.47). The presence of *E*-piceid was also noted (2.38).

2. Protection assays in vitro against grapevine downy mildew

2.1. Antimicrobial assays on zoospores

First, we evaluated the potential of the OSE extract at different concentrations (5 to 100 mg/L OSE) to reduce *P. viticola* zoospore mobility. The mobility of zoospores (Figure 2) was not impaired by the extract at 5 mg/L. At 10 mg/L, we noted a reduction of zoospores mobility by 50 % after 5 min of treatment. From 25 mg/L, during the first 2 min after stilbenes addition, the spores were unaffected (Figure 3). Then, the zoospores were unable to move, seemed to vibrate and finally burst after 3-4 min. Second, we performed an inoculation assay to confirm the ability of the OSE extract to damage the zoospores. For that, *P. viticola* spores that were treated for 5 min with different concentrations of the OSE extract were used to inoculate grapevine foliar discs. After 7 days, the downy mildew development was estimated by assessing the sporulation level. Regarding foliar discs inoculated by spores pre-treated with the OSE extract at 5 mg/L, no difference was noted compared to the control (non-treated spores) (Figure 2). For the foliar discs inoculated by spores that have been placed in contact with the OSE extract at 10 mg/L, a 24 % inhibition of *P. viticola* growth was observed and a total inhibition was noted for the 25 mg/L OSE extract condition.

2.2. Protection assays on grapevine foliar discs

The capacity of the OSE extract to reduce the development of *P. viticola* was evaluated in laboratory conditions using a foliar disc assay. For that, the OSE extract was sprayed at different concentrations (25 to 300 mg/L) on grapevine discs 24 h prior to their inoculation. Control (1 % ethanol) did not inhibit sporulation (Figure 4A). Regarding the OSE extract, for the lowest concentration (25 mg/L), the level of sporulation was similar to the control. From 50 to 300 mg/L, concentration-dependent inhibition of the sporulation was noted: at 50 mg/L, a 30 % sporulation reduction was achieved, at 200 mg/L, a strong inhibition (88 %) was obtained and, at 300 mg/L, the extract totally prevented the *P. viticola* development. The determined IC_{50} was equal to 70 mg/L.

TABLE 2. Stilbenes content in the OSE extract. Limits of Detection (LOD in µg/mL), Limits of Quantification (LOQ in µg/mL) and Calibration Curve Coefficients (R^2) of standard stilbenes. Stilbene content is expressed in mg/g DW as the means ± S.D. (in italics) of three independent samples analysed in triplicate and the percentage of presence in the total extract. NQ means detected but not quantified because of low levels.

Compound	LOD	LOQ	R^2	Content	
				(mg/g OSE extract)	(%)
E-piceid	0.6	1.8	1	2.38 ± *0.07*	0.2
E-piceatannol	2.6	7.9	0.997	11.07 ±0.24	1.1
E-resveratrol	1.4	4.2	0.999	95.41 ±1.68	9.5
Total monomers				108.86 ±10.18	10.9
ampelopsin A	0.6	1.7	1	23.08±0.52	2.3
pallidol	1.4	4.2	0.999	18.52 ±0.24	1.9
parthenocissin A	2.1	6.3	0.999	6.47 ±0.29	0.6
E-ε-viniferin	0.8	2.6	1	94.02 ±1.48	9.4
E-ω-viniferin	2.7	8.0	0.997	12.75 ±0.75	1.3
Total dimers				154.83 ±17.05	15.5
E-miyabenol C	2.9	8.7	0.999	NQ (8.69 ± *1.21*)	NQ
Total trimer				NQ	NQ
hopeaphenol	1.5	4.7	1	47.07 ±0.32	4.7
isohopeaphenol	1.8	5.5	1	63.67 ±0.43	6.4
r-viniferin	1.6	4.9	0.999	73.74 ±1.51	7.4
r2-viniferin	1.7	5.2	0.999	21.77 ±0.40	2.2
Total tetramers				206.26 ±23.53	20.6
Total				469.95 ±46.83	47.0

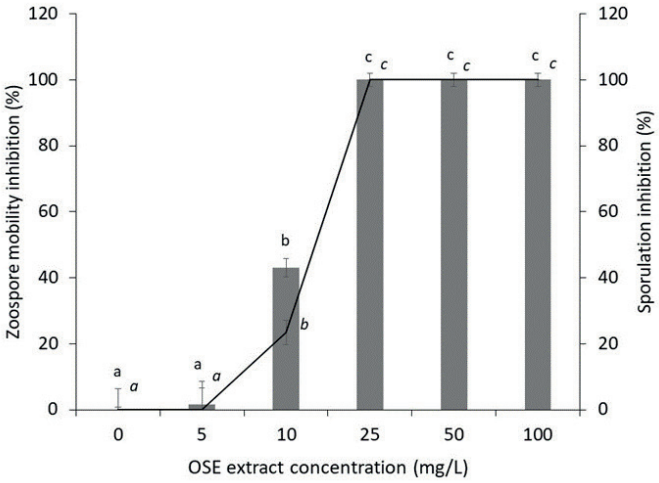

FIGURE 2. Effect of the OSE extract on *Plasmopara viticola* zoospores.

Control corresponds to 1 % ethanol condition. The percentage of inhibition of zoospores mobility (bars) and the percentage of inhibition of sporulation (curve) were indicated. The significant difference between each treatment was set at $p < 0.05$, letters represent significance (straight and italic letters apply to the mobility and sporulation assessment).

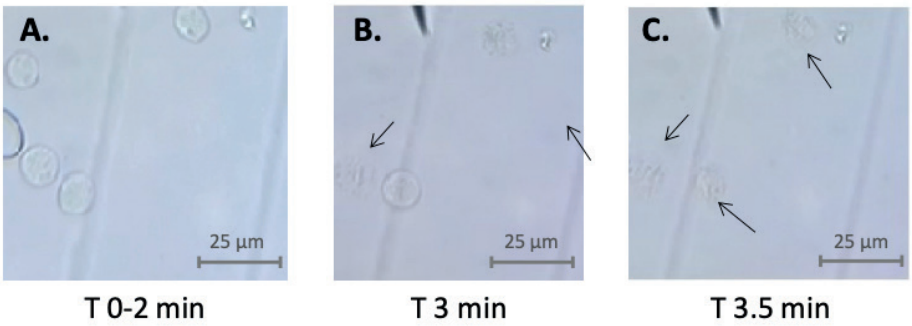

FIGURE 3. Microscopic observation of *Plasmopara viticola* zoospores in the presence of OSE extract.

The mobility of zoospores was followed during 3.5 min post-OSE extract treatment at 25 mg/L: 0—2 min (A), 3 min after treatment (B) and after 3.5 min (C). Degraded zoospores are indicated by dark arrows. Pictures are taken using a 400× optical microscope.

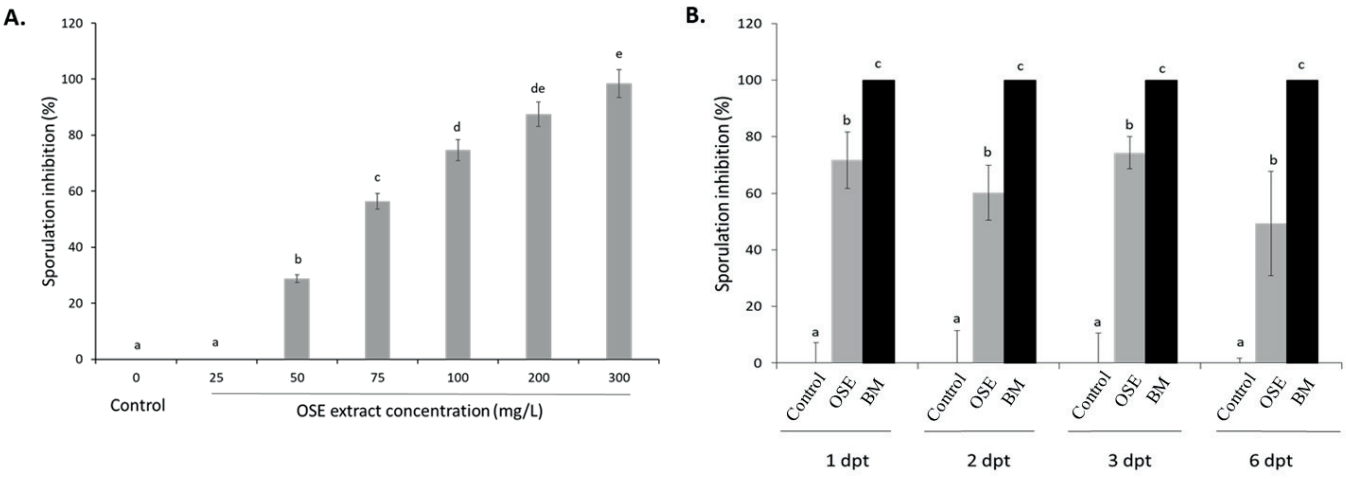

FIGURE 4. Protection level on grapevine treated by the OSE extract against *Plasmopara viticola* evaluated by foliar disc assay.

Sporulation inhibition was evaluated post-inoculation on foliar discs treated by 1 % ethanol (Control), OSE extract at different concentrations (A), and on foliar discs obtained from leaves of greenhouse plants pre-treated by 1 % ethanol (Control), OSE extract at 300 mg/L (light grey) or Bordeaux Mixture (BM, black) at 1, 2, 3 and 6 dpt (days post-treatment) (B). Results are expressed as means ± SEM of a percentage of *P. viticola* sporulation inhibition compared to the control (100 % infected, 0 % inhibition). The significant difference between each condition was set at $p < 0.05$, the letters represent significance.

To estimate the duration of the protective effect of the OSE extract, experiments were performed on grapevine cuttings in a greenhouse. The OSE extract was sprayed at its IC_{100} (300 mg/L) on the leaves and the Bordeaux mixture (BM at 4.2 g/L) was applied as a positive control.

At 1, 2, 3 and 6 dpt, leaves were collected, leaf disks generated and inoculated with *P. viticola* spores. Whatever the day of sampling, the OSE extract triggered a similar level of protection (Figure 4 B). The protection was relatively high, up to 72 % at 3 dpt, and it tended to decrease over time (46 % of protection at 6 dpt). Leaves treated by BM presented no disease development.

3. Protection assays in planta against downy mildews

3.1. On grapevine cuttings

We carried out assays in semi-controlled conditions to estimate the protective effect of the OSE extract in planta. Moreover, to be as close as possible to the final mode of application of the OSE extract in the field, we tested a formulated OSE extract. It consisted of the OSE extract with 9 g/L of a mixture of 80 % Tween 20, 15 % Tween 80 and 5 % sophorolipids. Thus, four modalities were considered: non-formulated OSE extract (OSE) at 300 mg/L, formulated OSE (F-OSE) extract at 300 mg/L, copper sulphate (BM for "Bordeaux Mixture" at 4.2 g/L) and untreated condition (NT). Whatever the treatment, no phytotoxicity was observed for the duration of the experiment.

At the date of the first assessment (A_1, after the primary inoculation (PI)), the pest incidence was similar regardless of the treatment done with no reduction (Figure 5A). Concerning pest severity, the OSE extract cannot trigger protection against this artificial infection (Figure 5B). However, the F-OSE extract displayed a high protection efficiency (70 % pest severity reduction), higher than BM (51 % reduction).

At A_2 (after the secondary inoculations (SI)), the protection level related to pest incidence for the OSE extract was 6 % and was not significantly different from the control. F-OSE extract and BM inhibited pest incidence with a respective reduction of 23 and 63 %. Based on the third assessment (A_3), we noticed that both F-OSE and OSE extracts could not reduce the pest incidence and only BM significantly moderated it (30 % of reduction). Regarding pest severity at A_2 and A_3 (the two "natural" inoculations (SI)), the OSE extracts, formulated or not, significantly diminished it by approximately 35 to 50 % (Figure 5B). BM, the fungicide reference, strongly limited the level of pest severity (83 %).

3.2. On tomato, potato and melon

All these experiments were carried out in a greenhouse. To proceed as close to its final way of use as possible, only the F-OSE extract was considered. We determined its activity towards oomycetes that are responsible for late blight on popular fresh market vegetable crops by using the following pathosystems: *P. infestans*/potato, *P. infestans*/tomato and *P. cubensis*/melon. As grapevine leaves were not fully protected against *P. viticola* by F-OSE when used at 300 mg/L in greenhouse assays (Figure 4) and we lacked hindsight on the effectiveness of such extract against other oomycetes, a higher concentration of F-OSE extract of 1 g/L was used for potato, tomato and melon.

Regarding potato late blight, we observed a strong inhibition of both pest incidence (68 %) and pest severity (approx. 90 %) at A_1 and A_2 (Table 3). For tomato late blight, F-OSE did not significantly reduce pest incidence (approx. 15 %);

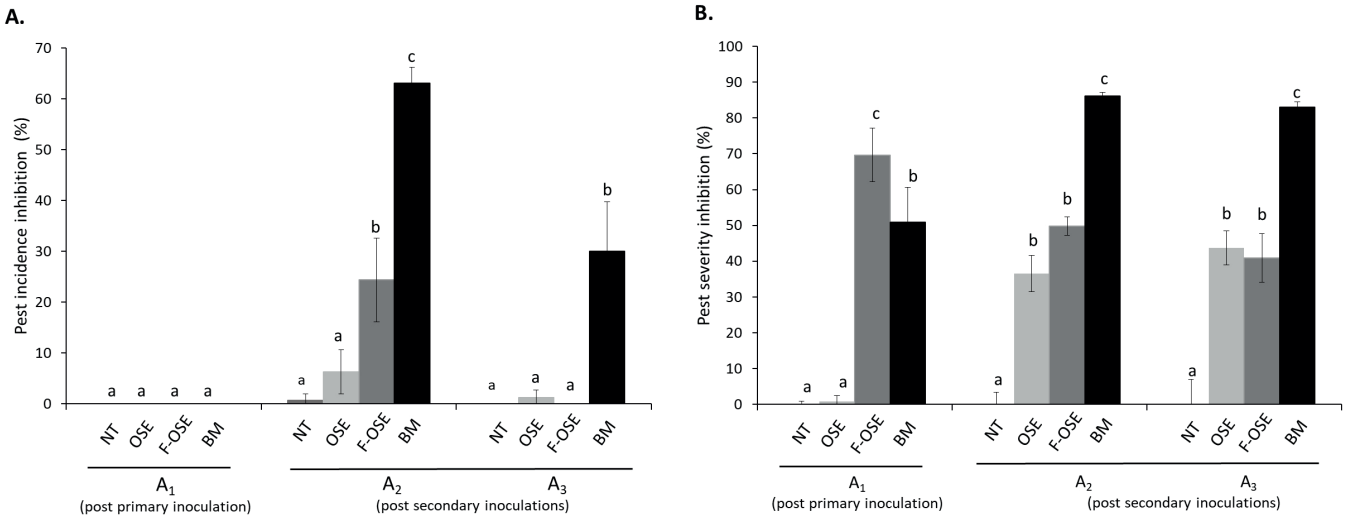

FIGURE 5. Protection level of grapevine treated by OSE extracts against *Plasmopara viticola* in the greenhouse.

Pest development was expressed in the percentage of pest incidence (A) and pest severity (B). The protection level was evaluated in planta by performing 4 modalities: untreated condition (NT, pale grey), non-formulated OSE extract (OSE) at 300 mg/L (light grey), formulated OSE (F-OSE) at 300 mg/L (dark grey) and "Bordeaux Mixture" (BM) at 4.2 g/L (black). A1, A2 and A3 correspond to the date of the 3 assessments (A1, after the primary inoculation and 2 treatments; A2 and A3, after the secondary inoculations and 3 treatments). Values are means of triplicate data of one representative experiment out of three. A significant difference between each extract was set at $p < 0.05$, letters represent significance.

TABLE 3. Pest incidence and pest severity inhibition of the formulated OSE extract (F-OSE) extract in different pathosystems.

Pathosystem	Pest incidence inhibition (%)		Pest severity inhibition (%)	
	A_1	A_2	A_1	A_2
P. infestans/potato	68.02 ± 14.51*	68.51 ± 7.52*	86.85 ± 5.62*	88.28 ± 6.85*
P. infestans/tomato	15.0 ± 8.40	13.35 ± 12.18	77.29 ± 4.17*	70.59 ± 14.27*
P. cubensis/melon	6.25 ± 4.79	0	75.63 ± 11.66*	33.64 ± 9.24*

The experiments were performed in a greenhouse. Two assessments (A1 and A2) were carried out. Results are expressed in percentage of inhibition as means ± SEM compared to the untreated control. The significant difference between each condition and untreated control was set at *$p < 0.05$.

however, it strongly limited pest severity (approx. 75 % inhibition). Similar results regarding pest severity were obtained for *P. cubensis* at A_1; nevertheless, the reduction was slight at A_2 (from 75 to 34 % at A_1 and A_2, respectively). The inhibition of pest incidence for melon late blight was 6 % at A_1 and null at A_2.

4. Grapevine defence responses and resistance induced against downy mildew by the OSE extract

To investigate the capacity of the OSE extract to modulate defence responses in grapevine leaves, we used the stilbenes extract at 300 mg/L, not formulated. F-OSE was not selected to avoid the potential co-formulants effect. The control consisted of 1 % ethanol. Treated leaves were harvested at 1, 2, 3 and 6 dpt.

4.1. Defence gene expression

We studied the expression of six defence-related genes: two genes encoding pathogenesis-related (PR) proteins (a chitinase (*PR3*) and a *PR10* protein) and four genes encoding enzymes involved in polyphenol biosynthesis (phenylalanine ammonia-lyase (*PAL*), chalcone synthase (*CHS*), stilbene synthase (*STS*) and resveratrol-*O*-methyl transferase (*ROMT*)).

One day after OSE application, five genes out of the six were slightly but significantly modulated: *PAL, ROMT, CHS* and *PR3* were up-regulated, whereas *STS* was inhibited (Figure 6). Two days post-treatment, only *PR3* was still significantly induced. Three days after treatment, no genes were modulated. At 6 dpt, the expression of *PR3* was highly induced, while strong repression of *STS* was observed. *PR10* expression was not modified throughout the time of the study.

4.2. Phytoalexin production

To determine the effect of the OSE extract on stilbenes production, leaves were collected at 0, 1, 2, 3 and 6 dpt, and, after their harvest, they were washed thoroughly with water to remove sprayed solutions. Whatever the time-point, no stilbenes from the OSE extract can be detected in the leaves (data not shown). Only *trans*-piceid, the glycosylated form of resveratrol, was detected in a sufficient amount to be quantified (Figure 7). No differences in its content were observed after OSE extract treatment (approx. 1.5 µg/mg DW), except at 1 dpt with a significant accumulation of this stilbene (2.3 µg/mg DW) in the leaves, with a value 1.5 times higher than that of the control.

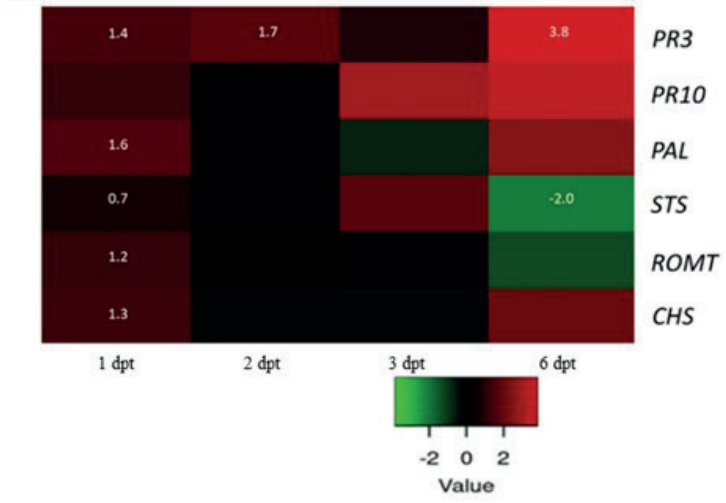

FIGURE 6. The pattern of relative expression of defence genes in grapevine leaves treated by OSE extract (300 mg/L).

Columns represent the sampling date in days post-treatment (dpt) and line the genes. A three-coloured scale was used to show log-2 transformed fold induction of each gene. Up-regulated genes appear in shades of red, with expression levels higher than 2 in bright red. Down-regulated genes appear in shades of green, with intensity lower than −2 in bright green. Numbers in boxes represent the significant changes in gene expression ($p \leq 0.05$) in treated leaves compared to untreated ones (control). Values are means of triplicate data of one representative experiment out of three.

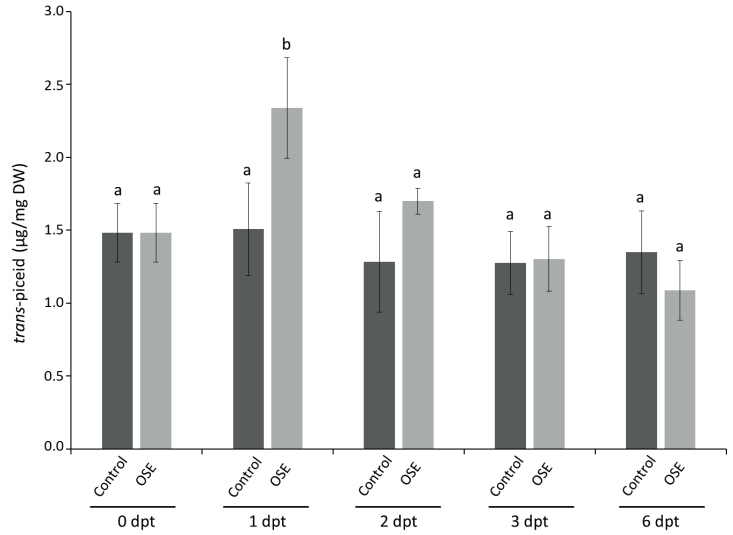

FIGURE 7. Piceid content in leaves treated by the OSE extract.

The *trans*-piceid content was evaluated in leaves treated with 1 % ethanol (Control, dark grey) or OSE extract at 300 mg/L (light grey) at 0, 1, 2, 3 and 6 dpt (days post-treatment). Results are expressed as means ± SEM. The significant difference between each condition was set at $p < 0.05$, the letters represent significance. Three independent experiments were done.

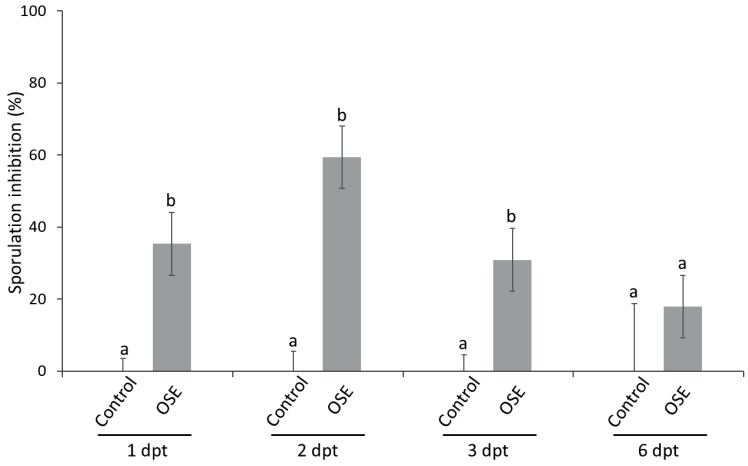

FIGURE 8. Conferred protection of the OSE extract against *Plasmopara viticola* evaluated by a foliar disc assay.

The OSE extract or ethanol 1 % (Control) were sprayed on the leaves of greenhouse plants. After 1, 2, 3 and 6 dpt (days post-treatment), F3 and F4 leaves were collected, washed and foliar disks generated and inoculated. Results are expressed as means ± SEM of the percentage of *P. viticola* sporulation inhibition in comparison to the control plants (100 % infected, 0 % inhibition). The significant difference between each condition was set at $p < 0.05$, letters represent significance.

4.3. Evaluation of the protection conferred by the OSE extract

To consider the conferred protection of OSE extract against *P. viticola* due to its defence stimulating activities and not to its antimicrobial capacity, leaves were treated with the OSE not formulated at 300 mg/L or ethanol 1 % (Control) and collected at 1, 2, 3 and 6 days post-treatment. After harvesting, leaves were washed and foliar discs were generated and inoculated.

We noted that the percentage of sporulation was reduced at 1 to 3 dpt in the OSE extract condition (Figure 8). At 1 dpt, the protection level conferred by OSE was 36 %. The highest inhibition was observed 2 dpt with values reaching 59 %. Then the protection decreased at 3 dpt (31 %) until it was no longer effective at 6 dpt.

DISCUSSION

In this study, we evaluated the protective effect of a grapevine extract obtained from a mix of grapevine trunk and roots which are vineyard co-products. This extract, named the OSE extract, was particularly enriched in stilbenes as it contains 470 mg/g DW of stilbenes. This value is in accordance and even higher than the ones of grapevine canes and wood and root extracts, which presented a stilbene content of 340, 351 and 224 mg/g, respectively (Gabaston *et al.*, 2017a). Furthermore, the OSE extract displayed a higher stilbene content in comparison to other extracts of plant species containing stilbenes as *Pinus pinaster* (knot) and *Picea abies* (bark) that presented 5 and 18.5 mg/g of stilbenes, respectively (Gabaston *et al.,* 2017b; Gabaston *et al.,* 2017c).

This discrepancy can be relative to the plant species, the plant part and/or the extraction methods. Concerning individual stilbenes, the OSE extract contained resveratrol, dimers and oligomerised stilbenes in higher quantity than other grapevine extracts (Gabaston *et al.,* 2017a). For instance, it was richer in hopeaphenol and isohopeaphenol than a grapevine root extract and richer in hopeaphenol in comparison to a wood extract (Gabaston *et al.,* 2017a). The quantity in total and individual stilbenes that we have extracted reflected their content in the plant organs. Indeed, it is in accordance with the mean values of stilbenes quantified in wood and roots of different grapevine cultivars that were presented in several studies and reviewed by Goufo and his colleagues (Goufo *et al.,* 2020).

The data reported in the presented work showed that the OSE extract could impair *P. viticola* development thanks to different modes of action. This is in accordance with the publication of De Bona *et al.,* who reported a dual mode of action for a grapevine cane extract (De Bona *et al.*, 2019). Firstly, we showed that the OSE extract displayed a direct antimicrobial action. Indeed, it strongly impaired *P. viticola* zoospores: it reduced their mobility, a basic function to initiate infection (Spring *et al.*, 2019) and impacted their viability as zoospores burst in its presence. Such OSE pre-treated zoospores, when inoculated on grapevine foliar discs, had probably a reduced ability in their infection process as the oomycete growth was diminished. We also noticed that the OSE extract, if sprayed on foliar discs prior to their inoculation, resulted in a protective effect against *P. viticola* with an IC_{50} of 70 mg/L in accordance with the IC_{50} previously reported for wood and roots extracts (60 and 120 mg/L, respectively) (Gabaston *et al.*, 2017a). The IC_{100} of the OSE extract was determined at 300 mg/L based on foliar disc assays. However, if sprayed at this concentration on whole leaves of plants placed in a greenhouse, the protection was incomplete (average of 64 %). This discrepancy can result from differences in the repartition of the OSE extract at the leaf surface and/or in the environmental conditions. The direct antimicrobial capacity of the OSE extract probably resulted from its high content in stilbenes representing 47 % of the extract and particularly in its richness in complex forms (Gabaston *et al.,* 2017a; Schnee *et al.,* 2013). Indeed, stilbenes, especially oligomerised forms (hopeaphenol, ε- and r-viniferins) and *trans*-resveratrol, have been shown to affect spore mobility and display toxicity on them (Gabaston *et al.*, 2017a; De Bona *et al.*, 2019). This lethal activity can result partly from the inhibition of cellular respiration and/or from the degradation of plasma membranes (Fröbel and Zyprian, 2019; Adrian and Jeandet, 2012; Koh *et al.*, 2016). Considering the environmental conditions that could impact the OSE extract activity when applied in the greenhouse, it has been reported, for instance, that UV triggers isomerisation of stilbenes (Martinez *et al.*, 1989), thus potentially modifying their oomycidal activity.

Secondly, in addition to its direct antimicrobial mode of action, the OSE extract can confer grapevine resistance toward *P. viticola* by activating plant defence mechanisms. Indeed, even after its removal from the leaves (by washing) prior to inoculation, the OSE extract treatment triggered sporulation inhibition up to 60 % at 2 dpt. This conferred resistance resulted in its ability to modulate the expression of some defence-related genes and the production of polyphenols. For instance, *PR3* was induced at 6 dpt. *PR3* could be involved in oomycete resistance as several oomycetes possess chitin-synthesising enzymes and their timing of expression suggests their role in the prevention of host colonisation (Hinkel and Ospina-Giraldo, 2017; Yan *et al.*, 2017). To our knowledge, the expression of this gene was never assessed after treatment with an extract enriched in stilbenes. Nevertheless, a maple leaf extract containing a high content of polyphenols was shown to trigger the overexpression of *PR3* in tobacco and a grape marc extract enhanced chitinase activity in grapevine (Peghaire *et al.*, 2020; Filippi *et al.*, 2019). *PR10* was not affected by the OSE extract treatment. De Bona and its colleagues mentioned that the expression of this gene was unaffected after a grapevine cane extract treatment, whereas they reported a strong induction of *PR5* and *PR6* and repression of *PR1* and *PR14* (De Bona *et al.*, 2019). The OSE extract also modulated, even very slightly, at 1 dpt, four genes involved in polyphenol synthesis: *PAL, ROMT, CHS* and *STS.* The study done by De Bona indicated that the application of a cane stilbene extract inhibits the expression of *STS* (De Bona *et al.*, 2019). Our data were quite consistent with this result as *STS* was repressed after the OSE application at 6 dpt. This effect could be attributed to the presence of resveratrol in these extracts as this latter was reported to negatively regulate *Vv*MYB14, a transcription factor of *STS* (Jeandet *et al.*, 2019; Gindro *et al.*, 2017). Moreover, *ROMT,* a gene encoding a protein involved in the biosynthesis of pterostilbene, a highly active compound against *P. viticola*, tended to be repressed at 6 dpt. Regarding *PAL*, the key enzyme in the phenol synthesis pathway, its gene was slightly up-regulated at 1 dpt. Nevertheless, none of the analysed antimicrobial stilbenes, like resveratrol and pterostilbene, seemed to be newly synthesised. However, piceid accumulated, which is the glycosylated form of resveratrol that does not exhibit any antimicrobial capacity. The production of this compound may indicate the occurrence of a general plant defence response (Figura *et al.*, 2012; Lambert *et al.*, 2012; Jeandet *et al.*, 2002). In addition to *PAL* induction at 1 dpt, *CHS* was up-regulated. Thus, the assumption of an up-regulation of the flavonoid pathway can be proposed. Flavonoids have been previously shown to play a part in resistance to *P. viticola* (Agati *et al.*, 2008) and it will be relevant to perform further experiments to unveil the role of these compounds in the tolerance conferred by the OSE extract treatment.

The protective effect of the OSE extract treatment resulting from its different modes of action was noted on grapevine cuttings in semi-controlled conditions. Indeed, treatment with the OSE extract at 300 mg/L reduced the pest severity by up to 40 to 50 % due to *P. viticola* in the context of assessments done after secondary infections (the ones mimicking a "natural infection").

These results were in accordance with the reduction of attack frequency from 59 to 69 % and disease reduction from 83 to 88 % obtained after a cane extract treatment (Richard *et al.*, 2016). However, this latter cane extract was applied at 5 g/L, a relatively high concentration, and no protective effect was obtained if used at 1 g/L. We remarked that the OSE extract treatment, and also BM, cannot inhibit neither the pest incidence nor the pest severity after the first inoculation (the "artificial" one). This probably resulted in the too high level of disease pressure of this type of inoculation.

In this study, we have also performed the treatment with a formulated OSE extract (F-OSE) to evaluate its protective effect under end-use conditions. Eco-friendly co-formulants (Tween® 20, Tween® 80 and sophorolipids) were used to optimise the long-term solubility of the OSE extract compounds, particularly the one of stilbenes, and to obtain a good wettability of the target surface and a resistance to leaching to develop its use in the vineyard. All the assays were performed in semi-controlled conditions and with various crops (grapevine, melon, tomato and potato). Considering grapevine, the F-OSE treatment at 300 mg/L resulted in a protection efficiency of 40 to 50 % against *P. viticola* regarding pest severity, a level of protection similar to the one obtained for the OSE extract after secondary infections ("natural infection"). In addition, F-OSE inhibited pest severity after a primary inoculation and in a strong way (approx. 70 %). The presence of co-formulants may result in this enhanced protection, compared to OSE alone, thanks to better solubilisation of the OSE extract active compounds and/or their effective spread at the leaf surface. The co-formulants may have also formed a protective layer at the leaf surface, thus avoiding penetration of *P. viticola* zoospores. In addition, they can directly inhibit the pathogen as they possess antimicrobial activities (Figura *et al.*, 2012; Haque *et al.*, 2019; Sen *et al.*, 2017). Regarding *P. infestans* on potato and tomato and *P. cubensis* on melon, the F-OSE extract (at 1 g/L) effectively reduced pest incidence (6 to 68 %) and pest severity (33 to 88 %) of these other oomycetes. The level of reduction mainly differed according to the pathosystem considered, with the highest protection obtained for potato and the lowest for melon. These discrepancies might result from the ability of each downy mildew species to bypass the toxicity of stilbenes and/or other compounds present in the F-OSE extract. Other plant extracts have been reported to reduce the development of mildew species and late blight (Goufo *et al.*, 2010; Forrer *et al.*, 2017). For instance, an extract of rhubarb, a stilbene-producing plant, was effective against the potato late blight (Jeandet *et al.*, 2019). The oomycidal properties of pure stilbene were also reported; as for pterostilbene, the methylated form of resveratrol, which inhibited *P. infestans* (Ward *et al.*, 1975). To our knowledge, this is the first time that a stilbene extract obtained from grapevine by-products was reported for its action against different mildews, especially in greenhouse conditions.

CONCLUSION

Taken together, the present findings showed that a grapevine extract obtained from trunks and roots and enriched in oligomerised stilbenes displayed a protective effect on various mildews affecting different crops (grapevine, melon, tomato and potato). This ability to protect plants against oomycetes was conferred by direct inhibition properties and by the stimulation of some plant defences (stilbenes and pathogenesis-related proteins expression). These results can help the future development of active extracts based on stilbenoids for crop protection and, more particularly, for vineyard disease control.

ACKNOWLEDGEMENTS

The authors gratefully thank StilNov Laboratory (ANR LabCom Project ANR-14-LAB5-0005-01) and Château Rochemorin (Martillac, France) for providing grapevine material and for the purification of stilbene standards. We are also grateful to M-F. Corio-Costet and S. Gambier for providing the plants and the grapevine downy mildew strain (INRAE, UMR SAVE).

Notes

The authors declare no competing financial interest.

Funding

This work was supported by the Comité Interprofessionnel des Vins de Bordeaux (CIVB). We are thankful to the Bordeaux Metabolome Facility and MetaboHUB (ANR-11-INBS-0010 project) for their financial support.

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