JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

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# Three Types of Elicitors Induce Grapevine Resistance against Downy Mildew via Common and Specific Immune Responses

Aleksandra Burdziej, Anthony Bellée, Enora Bodin, Josep Valls Fonayet, Noël Magnin, Anna Szakiel, Tristan Richard, Stéphanie Cluzet,* and Marie-France Corio-Costet*

Cite This: *J. Agric. Food Chem.* 2021, 69, 1781−1795 Read Online

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**ABSTRACT:** Three recognized plant defense stimulators (PDS), methyl jasmonate (MeJA), benzothiadiazole (BTH) and phosphonates (PHOS), were sprayed on grapevine *Vitis vinifera* cuttings and conferred resistance to the biotrophic pathogen *Plasmopara viticola*. The effects on molecular defense-related genes and polyphenol content (stilbenes and flavanols) were revealed at 6 and 8 days post-elicitation. The transcript accumulation was consistent with the signaling pathway specific to the elicitor, salicylic acid for BTH, and jasmonic acid for MeJA, with some cross-talks. PHOS tended to modulate the defense responses like BTH. Moreover, in response to a downy mildew inoculation, the leaves pre-treated with PHOS and BTH overproduced pterostilbene, and after MeJA treatment, piceids and ε-viniferin, compared to uninoculated elicitor-treated leaves. These results provide evidence of the different modes of action of PDS and their role in sustainable viticulture.

**KEYWORDS:** *BTH, MeJA, phosphonates, Vitis vinifera, PDS, polyphenols, Plasmopara viticola, gene expression*



## INTRODUCTION

The causal agent of downy mildew, *Plasmopara viticola* (Berk. & M. A. Curtis) Berl. & De Toni, in the grapevine (*Vitis vinifera*) contributes heavily to yield and quality losses and is a worldwide factor in the economics of agriculture. Its control is generally achieved by repetitive use of synthetic fungicides, which can lead to treatment resistance and threatens the environment and human health.[1] Copper-based fungicides used in organic farming can also be ecologically harmful.[2] For sustainable viticulture, alternative or complementary strategies of protection have been developed, such as the activation of plant-induced resistance by the use of biodegradable, non-toxic substances called elicitors or plant defense stimulators (PDS).[3] Most of them are able to mobilize the plant to produce an enhanced and faster initiation of defense reactions in case of a subsequent infection (priming).[3]

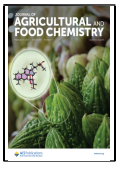
Major plant defense reactions include cell wall reinforcement and accumulation of defense compounds; i.e., pathogenesis-related proteins (PR) and phytoalexins (antimicrobial secondary metabolites).[4,5] The most important phytoalexins in Vitaceae are stilbene polyphenols.[6,7] For example, *trans*-resveratrol and its derivatives, ε- and δ-viniferins, pterostilbene, isohopeaphenol, and miyabenol C accumulated in grapevine leaves infected with *P. viticola* and displayed anti-mildew activities.[8−11]

Various elicitors are reported as inducers of defense-related responses in different experimental models of grapevine. They act commonly through phytohormone signaling pathways mediated by either salicylic acid (SA), jasmonic acid (JA), and/or ethylene. SA-dependent signaling is often established for the defense against biotrophic and hemi-biotrophic pathogens,[12] resulting in grapevine protection against *P. viticola* and *Erysiphe necator* (the causal agent of powdery mildew).[8,13,14] However, JA signaling in the resistance of grapevine to these pathogens was also suggested.[15,16] Furthermore, SA and JA pathways have been reported to be activated simultaneously,[12] which can promote resistance against pathogens using different mechanisms, e.g., necrotrophic fungi such as *Botrytis cinerea* (the causal agent of gray mold).[17]

Methyl jasmonate (MeJA) and benzothiadiazole (BTH) are two grapevine elicitors that have frequently been studied because of their plant protection properties. MeJA, acting through JA signaling, stimulates the downstream production of stilbenes in leaves and berries coupled with the up-regulation of *PAL* (phenylalanine ammonia lyase) and *STS* (stilbene synthase) gene expression and, thus, enhances resistance against powdery mildew.[16] Treatment of grapevine leaves and berries with BTH an analogue of SA triggers efficient protection, both locally and systemically, against *P. viticola*, *E. necator*, and *B. cinerea*[8,13,18] through the accumulation of specific polyphenols, PR-protein gene overexpression, and cell wall reinforcement.[17,19] Phosphonates induce local and systemic protection against mildews, through both the induction of defense responses and direct antifungal activity;[8,20,21] however, their mode of action on a specific immune signaling pathway remains unclear.

Elicitors do not constitute a universal approach for the replacement of conventional pesticides. Indeed, their

Received: September 23, 2020
Revised: January 8, 2021
Accepted: January 13, 2021
Published: February 2, 2021







https://dx.doi.org/10.1021/acs.jafc.0c06103
*J. Agric. Food Chem.* 2021, 69, 1781−1795

effectiveness can vary according to the pathosystem, the cultivar, or environmental conditions.[13,22] Consequently, to develop an efficient and reliable elicitor-based strategy for vineyard protection, more knowledge about their mechanisms of action and improved application methods are needed. The aim of this study was to examine common and specific grapevine defense responses to downy mildew after elicitation with three different elicitors (BTH, MeJA, and phosphonates) using approaches based on biological, molecular, and chemical measurements.[23] Polyphenols (stilbenes and flavanols) content and defense-related gene expression were assessed in *V. vinifera* cv. Cabernet Sauvignon leaves subjected to elicitation (6 days post-treatment, 6 dpt) and subsequent inoculation with *P. viticola* (8 dpt) or 2 days post-inoculation (8 dpt-2 dpi). Simultaneously, the efficacy of protection of the elicitor treatments toward *P. viticola* was evaluated. These experiments provide insight into the persistence of the PDS effect on grapevine leaves at relatively long periods of time after treatment.

## MATERIALS AND METHODS

**Plant and Pathogen Material.** *Vitis vinifera.* Grapevine plants (*V. vinifera* cv. Cabernet Sauvignon) were propagated from wood cuttings (Château Couhins, Gironde, France) in the greenhouse. After 3 weeks, rooted cuttings were potted in sandy soil and grown with a 16 h photoperiod (350 $\mu$mol/m$^2$/s). Two month-old plants with 10−12 leaves were used for the experiments.

*Plasmopara viticola.* One isolate (ORG) from the laboratory collection, collected from a vineyard (Gironde, France) in 2014, was used. It was propagated on grapevine leaves in a growth chamber (22 ± 2 °C) with a daily photoperiod of 16 h light/8 h dark. Inoculation was performed according to the procedure previously described,[24] with a spore suspension at 8500 sporangia/mL.

**Chemicals.** Three elicitors were studied. Two crop protection products were tested: a synthetic analogue of SA, BTH (acibenzolar-*S*-methyl (ASM) or *S*-methyl benzo[1,2,3]thiadiazole-7-carthioate, CAS No. 135158-54-2, Bion 50WG, Syngenta, Switzerland), and phosphonates (PHOS) (mono- and dipotassium salts of phosphorous acid, CAS No. 13977-65-6 and 13492-26-7, LBG-01F34, De Sangosse, France). The latter is a registered product against downy mildew on grapevine as a fungicide and an elicitor. Methyl jasmonate (MeJA, 95%, CAS No. 1211-29-6) and its wetting agent, Triton X-100 (Triton, >95%, CAS No. 9002-93-1), were purchased from Sigma (USA). Methanol (>99.8%, CAS No. 67-56-1) was supplied by Prolabo (France). Water was purified using an Elga water purification system (ElgaLabWater, USA) with a resistivity of no less than 18 MΩ·cm.

**Plant Treatments and Inoculation with *P. viticola*.** *Elicitation.* Foliar cuttings (70) were treated separately with the following elicitors with 14 plants per condition: MeJA at a final concentration of 5 mM (1.09 g/L) in the presence of Triton at 0.1%, BTH at 2 g/L, and PHOS at 1.5 g/L. MeJA was dissolved in 1% EtOH and added to an aqueous solution containing Triton, which functioned as a co-formulator. The control plants were treated with distilled water or Triton (0.1%). All solutions were sprayed on grapevine leaves using a micro-diffuser (Ecospray).

Two leaves below the apex (the third and the fourth, L3 and L4, respectively) were harvested per plant after 6 days post-treatment (6 dpt). From eight collected leaves (L3 and L4) of four cuttings sampled per condition, foliar discs (25 mm-wide) were excised for *P. viticola* biological assays. The remaining parts of leaves (6 dpt) were cut in half and frozen at −80 °C for gene expression and polyphenol analysis.

Twenty whole leaves (L3 and L4) of 10 cuttings per condition were thoroughly rinsed with water, inoculated, or not, with downy mildew, and collected after 48 h (8 dpt, uninoculated, or 8 dpt-2 dpi, inoculated, leaves) for gene expression and polyphenol analyses. Foliar discs were removed on the day of the harvest from these 20 leaves for estimation of *P. viticola* disease intensity.

*Inoculation.* Inoculation with *P. viticola* was done according to the method described previously.[24] Each of the detached leaves, per condition, was placed with the abaxial side uppermost on moist Whatman paper in Petri dishes. The leaves were inoculated with 15 droplets of 15 $\mu$L of spore suspension (8500 sporangia/mL) or were not inoculated (controls) and were incubated overnight at 22 °C in darkness. Twenty-four hours after inoculation, the droplets were removed, and all the leaves were placed at 22 °C under a 16 h day/8 h night photoperiod (25 $\mu$E/m$^2$/s). At 48 h post-inoculation, all the leaves were sampled, cut in half, and frozen at −80 °C for gene expression and polyphenol analysis.

*Disease Intensity Measurement.* Disease intensity was estimated by measuring growth and degree of sporulation of *P. viticola* on each drop, as described previously.[24] From all detached leaves, one leaf disc was collected and used to follow disease development. Leaf discs were placed in Petri dishes containing a moist Whatman paper and inoculated as described above, with 3 droplets of spores/disc. Then, leaf discs were incubated for 7 days. The contamination level was assessed with a visual scale from 0 to 100% of sporulation, calculated as previously described.[8,14] The mean values for sporulation inhibition for each product were subjected to statistical analyses by a nonparametric test (Kruskal−Wallis) using R x64 3.0.3 software, and significant differences were determined by Tukey's test at the 5% significance level.

**Extraction of Polyphenols.** The leaves were frozen in liquid nitrogen and stored at −80 °C. Polyphenols were extracted overnight from freeze-dried, finely powdered leaves (50 mg) by agitation with 2.5 mL of methanol (MeOH). After centrifugation (3500 rpm, 10 min), the procedure was repeated twice over 1.5 h with new portions of MeOH (2.5 mL). The supernatants of each sample were pooled and concentrated using a Speed Vac (Savant, USA). Extracts (1 mL) were then purified on a Sep-Pak C18 cartridge (Sigma-Aldrich) in order to remove chlorophylls. After elution of polyphenols with 90% MeOH, the extracts were evaporated to dryness and then dissolved in 1 mL of 50% MeOH. Samples were filtered through 0.45 $\mu$m polytetrafluoroethylene (PTFE) membrane filters. During preparation, samples were protected from light.

**Analysis of Polyphenols.** Polyphenol analysis was performed as previously described.[25] Quantities of analyzed compounds were determined from calibration curves of pure standards (*trans*-resveratrol, ε-viniferin, pterostilbene, *cis*- and *trans*-piceids, catechin, and epicatechin) at concentrations ranging from 0.004 to 20 mg/mL. Concentrations were expressed in $\mu$g/mg of pure phenolic compound. The linearity of the response of the standard molecules was checked by plotting the peak area versus the concentration of the compounds. All standard polyphenols were produced and purified in laboratory conditions (UR Oenology, Villenave d'Ornon, France). For all experiments, samples were analyzed in triplicate. One-way analysis of variance (ANOVA) and the Tukey's multiple comparisons test were applied ($p \leq 0.05$) to investigate the differences in polyphenol content between corresponding control and elicited and/or inoculated leaves (R software version 3.4.3).

**Gene Expression Analysis by RT-qPCR.** Total RNA was extracted using the Spectrum Plant Total RNA kit (Sigma) with some modifications as previously described previously.[17] All leaves were ground to a fine powder in liquid nitrogen. Leaf powder (200 mg) was added to 2 mL of extraction buffer preheated to 56 °C, and a chloroform:isoamyl alcohol (24:1, v/v) purification was performed. The next steps were conducted using the Spectrum Plant Total RNA kit (Sigma) according to the manufacturer's instructions and digested with DNase I.[17] RNA quantitation was carried out with a Denovix DS-11 spectrophotometer. Reverse-transcription using 10 $\mu$g of total RNA was performed using 2 $\mu$M oligo-d(T)$_{15}$, ribonuclease inhibitor, and M-MLV reverse transcriptase (Invitrogen) according to the manufacturer's instructions. The cDNA was kept at −20 °C. High-throughput gene expression was quantified using microfluidic dynamic array (Fluidigm) technology. We used


https://dx.doi.org/10.1021/acs.jafc.0c06103
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specific primer sets of the "NeoViGen96" chip that were designed previously.[26] Details of genes are listed in Table S1. Five different genes (*VvEF1γ*, *VvGAPDH*, *VvTHIORYLS8*, *VvTIP41*, and *VvTUA*) were used as references to normalize the cDNA template at the beginning of the reaction. Pre-amplification of cDNA, qPCR analysis with Fluidigm technology, data processing, and statistical analyses were performed as described previously.[17]

## RESULTS

**Grapevine Protection.** The elicitors applied on the grapevine leaves 6 days before inoculation (6 dpt) conferred different levels of resistance toward *P. viticola* (Figure 1).

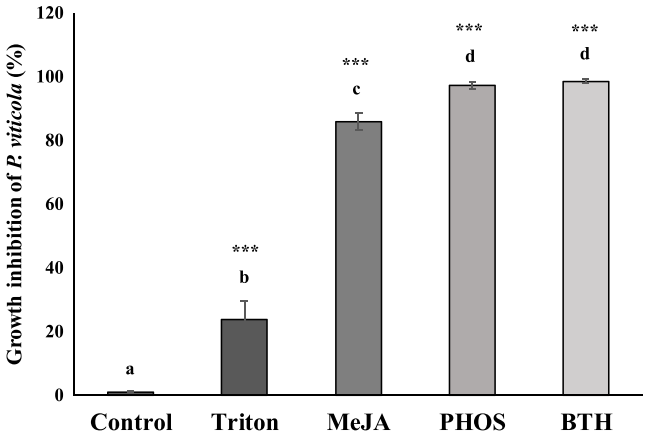


**Figure 1.** Growth inhibition of *Plasmopara viticola* on grapevine leaves that were treated 6 days before inoculation with Triton, MeJA, PHOS, or BTH. Data were expressed in percentage of inhibition relative to control. Different letters above the columns show significant differences at $p \leq 0.05$. Asterisks (***) denote the significance level (0 to 0.001) as compared to the control sample.

BTH and PHOS treatment provided the strongest protection, with an inhibitory effect on the pathogen's growth, of 98.5 ± 0.6 and 97.3 ± 1.1%, respectively, in comparison to the control. Lesser, but high-level, inhibition was noted for MeJA treatment (85.8 ± 2.7%) as well as for its co-formulant, Triton, which induced weak, but significant, protection (23.8 ± 5.8% in *P. viticola* growth reduction). No phytotoxic effect was observed for the plants, regardless of the treatment.

**Gene Expression.** *Gene Expression at 6 days Post-treatment.* At 6 dpt (Figure 2A), gene overexpression occurred mainly after BTH and Triton treatments (22 and 15 genes induced, respectively) but was lower in the leaves treated with MeJA and PHOS (8 and 6 up-regulated genes, respectively). Conversely, BTH and Triton treatments significantly repressed only a few genes (2 and 1, respectively), while MeJA and PHOS treatments led to a significant repression of 50% or more of all modulated genes.

All elicitors triggered the overexpression of two PR protein genes: *VvPR5* (thaumatin-like protein) and, to a lesser extent, *VvPR4* (chitinase) (Figures 3 and 4). Leaves treated with Triton or BTH shared some additional up-regulated PR genes, such as *VvPR2* (glucanase), *VvPR3*, and *VvPR8* (chitinases class I and III, respectively), and BTH specifically modulated the genes *VvPR11* and *VvPR12*. In addition, PHOS and Triton induced the genes *VvPR5* and *VvPR6* (serine protease inhibitor). The overexpression of *VvPR9* (lignin-forming peroxidase) and *VvPR14* (lipid transfer protein) and the repression of *VvPR6* (serine protease) were specific for MeJA treatment. Among the other PR protein genes modulated by MeJA, *VvPR7* (subtilisin-like endoprotease) and *VvPR11* (chitinase I) were similarly repressed by PHOS. Triton treatment also resulted in the overexpression of the genes *VvPR10* and *VvPR4*.

Concerning secondary metabolites (Figures 3 and 4), transcripts of phenylpropanoid pathway genes accumulated significantly in Triton-treated leaves and even more in BTH-treated ones. These genes were involved either in resveratrol biosynthesis (*VvPAL* and *VvSTS*) or in the flavonoid pathway (e.g., chalcone synthase, *VvCHS*, and anthocyanidin synthase, *VvLDOX*).

The genes involved in the redox system and the oxylipin pathway were more discriminating, since any gene shared the same pathway for all treatments, and only few genes were common in two treatments. Only BTH led to a significant overexpression of several glutathione *S*-transferases (*VvGTS2*, *VvGST3*, and *VvGST4*). Among the oxylipin pathway, lipoxygenase 13 (*VvLOX13*), involved in jasmonate biosynthesis, was overexpressed by Triton and, more strongly, by MeJA. As expected, BTH significantly induced the expression of lipoxygenase 9 (*VvLOX9*).

For cell wall reinforcement, only PHOS treatment led to the overexpression of several genes directly involved in the biosynthesis of callose (*VvCAL*) and the modification of the cell wall with pectin methyl esterase (*VvPECT*) and cinnamoyl-CoA reductase (*VvCAD*) (Figures 3 and 4). The latter was also slightly overexpressed after MeJA treatment.

Indole pathway genes were poorly modulated, except by PHOS, which significantly down-regulated anthranilate synthase (*VvANTS*) and chorismate mutase (*VvCHORM*), and by BTH, which up-regulated *VvCHORM* and *VvICS* (isochorismate synthase). It should be noted that MeJA treatment repressed the *VvICS* gene, which is also involved in the salicylic acid biosynthesis pathway.

Finally, among the genes of the hormone-signaling class, the SA pathway genes were overexpressed in BTH-treated leaves with the *VvPAL*, *VvICS*, and, especially, *VvSAMT* genes, the latter coding for SA methylase. In contrast, MeJA treatment led to a repression of the *VvSAMT* gene and an overexpression of a key gene of the ethylene pathway (*VvACO1*, 1-aminocyclopropane-1-carboxylic acid oxidase). Phosphonates conjointly led to the repression of the genes *VvSAMT* and *VvGH3-6* (JA-Ile-synthase), suggesting a repression of SA and JA biosynthesis. Only the transcription factor *VvWRKY2* was commonly up-regulated by Triton and MeJA (Figures 3 and 4).

*Gene Expression at 8 days Post-treatment and 2 days after P. viticola Inoculation.* Globally, at 8 dpt, all treatments showed a slight overexpression of genes, except for PHOS, which repressed 2 genes. Five and 6 genes were up-regulated in Triton- and BTH-treated leaves, respectively, and 12 genes were up-regulated in MeJA-treated leaves. After inoculation (8 dpt-2 dpi), the general effect on treated leaves led to contrasting profiles. Noticeably, Triton and MeJA treatments modulated several genes (33 and 35, respectively), mainly repressing them (63.6 and 57.14%, respectively). Conversely, BTH and PHOS treatments overexpressed genes (e.g., 94.5% in BTH treated leaves), with a significant difference in the total number of genes modulated (36 and 9, respectively). More precisely, in untreated inoculated leaves (8 dpt-2 dpi), all genes were down-regulated compared to uninoculated leaves (8 dpt), particularly those encoding PR


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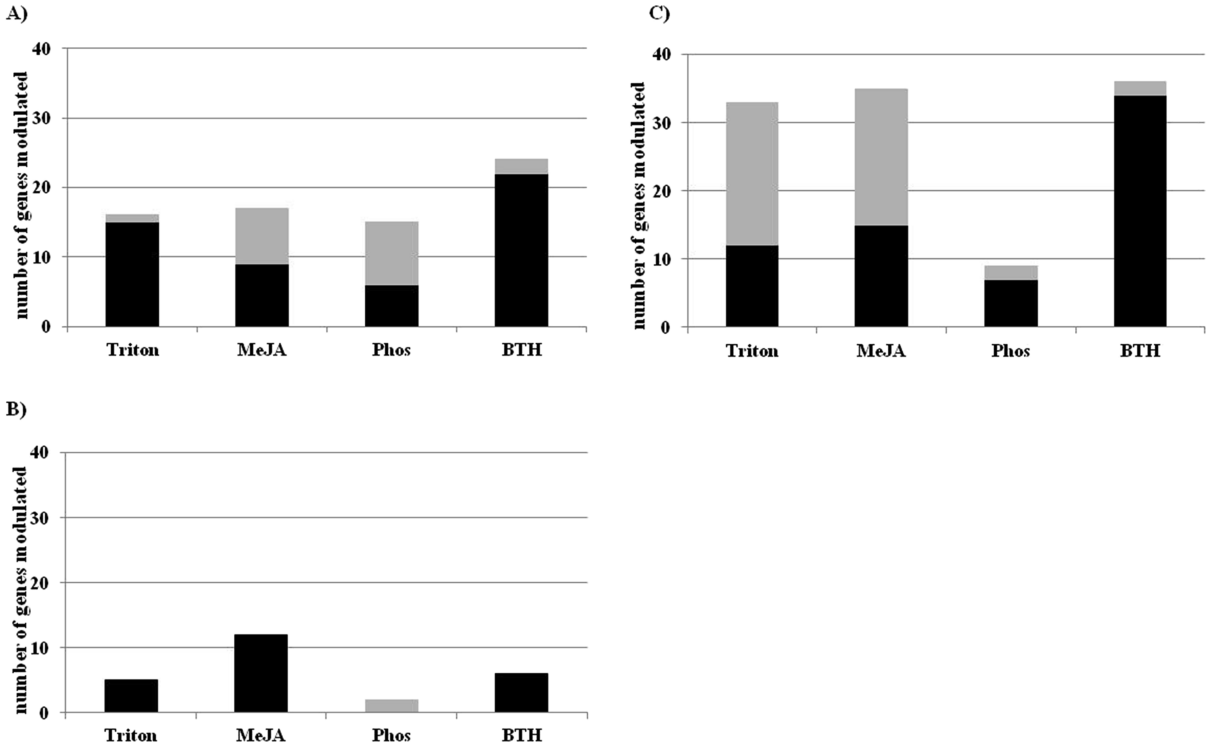


**Figure 2.** Numbers of defense genes significantly up-regulated or down-regulated at 6 days after treatment (A), 8 days after treatment and uninoculated (B), and 8 days after treatment and 2 days after inoculation (C). Overexpressed genes are represented in black and down-related genes in gray.

proteins (except *VvPR1*) and genes coding for enzymes involved in phenylpropanoid biosynthesis.

In treated leaves, after inoculation, major differences were revealed depending on the elicitor pretreatment. Triton and MeJA triggered nearly the same gene expression changes (Figures 5 and 6), and their effects were characterized by a significant repression of most of the PR protein genes (*VvPR5*, *VvPR6*, *VvPR7*, *VVvPR8*, *VvPR10*, *VvPR11*, *VvPR12*, and *VvPR15*). Only the genes *VvPR1* and *VvPR14* (lipid transfer protein) were overexpressed and specific to these two treatments. While PHOS treatment had nearly no effect on PR proteins, BTH induced a widespread overexpression of PR proteins, with the exception of *VvPR14*. One gene, *VvPR2*, coding for a glucanase, was commonly overexpressed in MeJA-, PHOS-, and BTH-treated leaves after inoculation. The modulation of genes involved in secondary metabolism exhibited also different profiles between MeJA and Triton and between PHOS and BTH treatments (Figures 5 and 6). As prior to inoculation (at 6 dpt), BTH treatment overexpressed mainly genes coding for stilbene and flavonoid biosynthesis, which, albeit to a lesser extent, was also achieved with PHOS treatment, stimulating the flavonoid pathway genes. In contrast, MeJA and Triton repressed the majority of genes, except chalcone synthase (*VvCHI*) and farnesyl diphosphate synthase (*VvFPPS*).

Regarding the genes involved in parietal reinforcement, only Triton and MeJA treatments overexpressed the callose synthase gene (*VvCAL*). While PHOS treatment did not induce any gene modulation, BTH favored the overexpression of the genes *VvAlli*, *VvPECT*, and *VvCAD*. The latter (*VvCAD*) was also induced by MeJA (Figures 5 and 6). After inoculation, the genes of the chorismate pathway, *VvCHORS* and *VvCHORM*, were overexpressed in the PHOS- and MeJA-treated leaves, respectively.

Concerning the redox status and oxylipins, inoculation led to an almost general overexpression of the glutathione *S*-transferase *VvGST5* in all treated leaves, except in BTH leaves. However, BTH treatment induced all the other GST genes (*VvGST2*, *VvGST3*, and *VvGST4*). Lipoxygenase 9 (*VvLOX9*) was up-regulated by BTH-treated leaves, and lipoxygenase 13 (*VvLOX13*) was overexpressed in both BTH- and MeJA-treated leaves. Regarding the hormone signaling pathway, the ascorbate peroxidase gene (*VvAPX*) was overexpressed only in leaves treated with MeJA or Triton (Figures 5 and 6). More interesting was the overexpression of *VvEDS1* in MeJA- and Triton-treated leaves and, by contrast, the repression of this gene by PHOS and BTH treatment. The same trend was observed for *VvGH3-6* and *VvSAMT* genes repressed in MeJA- and Triton-treated leaves and overexpressed in BTH-treated leaves and, conversely, for the *VvACC* gene overexpressed in MeJA and Triton leaves and repressed in BTH leaves.

**Polyphenol Analysis. Polyphenol Content at 6 days Post-treatment.** The main grapevine foliar stilbenes (*trans*-resveratrol, pterostilbene, *cis*- and *trans*-piceids, *ε*-viniferin) and two flavanols (catechin, epicatechin) were quantified. At 6 dpt (Table 1 and Figure 7A) in control leaves, the total amount of polyphenols reached 5.81 ± 0.26 μg/mg DW with 5.58 and 0.23 μg/mg DW of stilbenes and flavanols, respectively. In leaves treated with Triton or MeJA, total stilbene and flavanol content increased significantly, 2- to 5-fold more than in control leaves. In contrast, after PHOS or BTH treatments, stilbenes decreased approximately 4-fold. The amount of flavanols increased 3-fold only after BTH treatment. The enhancement of total stilbene content by


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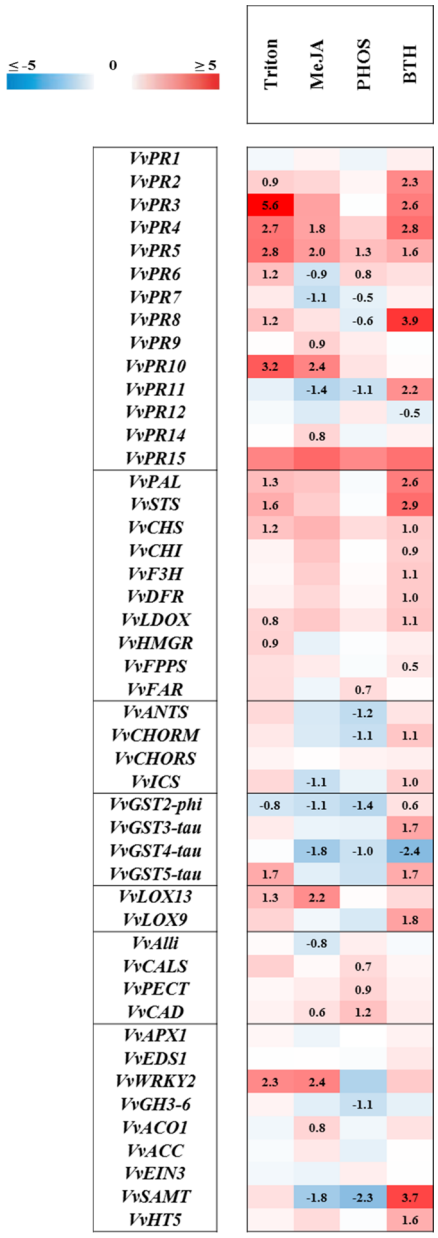


**Figure 3.** Pattern of relative expression of defense genes in grapevine leaves 6 days after treatment with Triton, MeJA, PHOS, or BTH. Expression data were given after log2 transformation. Gene expression of untreated leaves was used as a reference to calculate the relative expression. Each column represents a treatment modality, and each line corresponds to one gene represented by a single row of boxes. The color scale bars represent the ratio values corresponding to the mean of three independent experiments. Genes up-regulated appear in shades of red, with an expression level higher than 5 in bright red, while those down-regulated appear in shades of blue, with an intensity lower than −5 in dark blue. Numbers in boxes represented the significant changes in gene expression ($p \leq 0.05$) in treated-leaves compared to untreated ones (control).

MeJA or Triton treatment compared to the control was explained by the accumulation of *trans*-resveratrol, piceids (*cis* and *trans*), and ε-viniferin, mainly in the case of MeJA treatment. The content of pterostilbene was unchanged under all experimental conditions. Regarding flavanols, in leaves treated with MeJA, Triton, or BTH, the content of catechin was 5.1-, 3.1-, and 2.6-fold higher, respectively, in regard to the controls, while the amount of epicatechin increased significantly in MeJA- or BTH-treated leaves (11.0- and 7.5-fold, respectively).

**Polyphenol Content 8 days Post-treatments and 2 days after Inoculation.** At 8 dpt (Table 2 and Figure 7B) in the control, uninoculated leaves, the total content of polyphenols increased to 36.06 ± 3.02 μg/mg DW, with stilbenes and flavanols reaching 11.64 and 24.41 μg/mg DW, respectively. After inoculation, the control leaves exhibited no significant change in polyphenol content, even if we noted a tendency to an increase. MeJA, Triton and BTH treatments affected the content of total polyphenols in both uninoculated and inoculated leaves, resulting from stilbenes, in the case of MeJA and Triton treatments, and from flavanols for BTH treatment. The quantity of total stilbenes was 3.8- and 5.1-fold higher, respectively, in Triton- and MeJA-treated leaves (8 dpt) in comparison to the control (8 dpt), with, at 8 dpt-2 dpi, an increase in total stilbene content of 2.3- and 6.9-fold for Triton and MeJA, respectively. The inoculated MeJA-pretreated leaves triggered a significant enhancement of total stilbenes (from 59.71 ± 2.07 μg/mg DW in MeJA at 8 dpt to 113.71 ± 2.95 μg/mg DW in MeJA 8 dpt-2 dpi, i.e., 1.9-fold). Flavanol content significantly increased in both uninoculated and inoculated BTH-treated leaves and resulted in 9.6- and 7.4-fold higher levels compared to the respective controls. A slight increase in flavanols was also noted in Triton 8 dpt and MeJA 8 dpt-2 dpi experiments (4.1- and 3.8-fold, respectively) in comparison to their respective controls. The content of individual compounds within stilbenes and flavanols indicated their contribution in the main profiles (Table 2 and Figure 8B). At 8 dpt, both MeJA and Triton treatments markedly enhanced the biosynthesis of piceids compared to the control (5.7- and 4.0-fold, respectively), representing approximately 88 and 84%, respectively, of total stilbenes. Moreover, a 1.8-fold and a 7.2-fold higher content of piceids was noted in MeJA-treated inoculated leaves, compared to MeJA-treated leaves and the control inoculated leaves, respectively. ε-Viniferin was the second major stilbene in both MeJA- and Triton-treated leaves, and its content was significantly higher than in the control at 8 dpi (8.5- and 5.2-fold more, respectively). As in the case of piceids, the amount of ε-viniferin was also greater after inoculation of MeJA-treated leaves (2.8-fold). The content of *trans*-resveratrol varied between 0.88 and 2.69 μg/mg DW, but not significantly, regardless of treatment, except for MeJA-treated, 8 dpt and 8 dpt-2 dpi leaves, where *trans*-resveratrol was undetectable. The amount of pterostilbene increased both in PHOS-treated, 8 dpt and 8 dpt-2 dpi leaves in comparison to their respective controls (2.4-fold in both samples). Interestingly, pterostilbene content increased 2.7-fold higher after inoculation of BTH-treated leaves compared to BTH-treated leaves at 8 dpt. The content of catechin increased notably in BTH-treated leaves at 8 dpt (8.4-fold), as well as after inoculation (6.7-fold), in comparison to the controls. However, the content of epicatechin was 20.9- and 14.0-fold higher in BTH-treated 8 dpt and BTH-treated 8 dpt-2 dpi, respectively, compared to their controls. The amount of epicatechin was also higher in MeJA-treated 8 dpt-2 dpi, compared to the control 8 dpt-2 dpi (9.5-fold). However, inoculation did not significantly modify the quantity of flavanols, whatever the treatment, even in BTH-pretreated leaves.


https://dx.doi.org/10.1021/acs.jafc.0c06103
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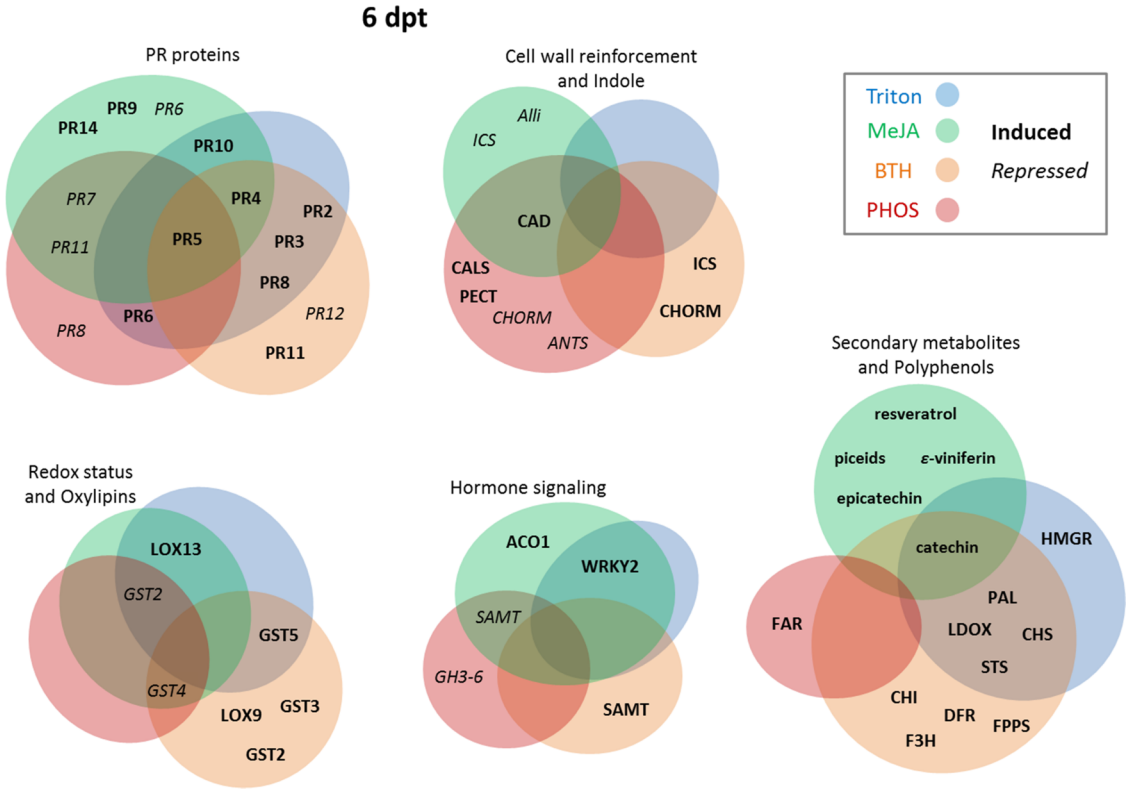


**Figure 4.** Venn diagram showing the significantly modulated genes in grapevine leaves 6 days after treatment with Triton, MeJA, PHOS, or BTH. Induced genes are represented in bold and inhibited ones in italic. Each treatment modality is represented by an ellipse with the following color: blue (Triton), green (MeJA), orange (BTH), and red (PHOS).

**Combined Analysis of Grapevine Responses to Elicitations, Inoculation, and Protection against *P. viticola*.** Principal component analysis (PCA) was used to summarize the data and highlight the potential links between the protection conferred by a treatment and the plant defense response (biochemical and transcriptomic analyses) (Figure 9). Most of the variance was contained in the first two principal components, which captured 79.12% of the total variability. Axis 1, explaining 65.89% of the total variability, discriminated Triton and MeJA treatments versus the control, and PHOS and BTH treatments, mainly related to the modulation of some genes involved in the SA signaling, genes related to the ET pathway (*VvWRKY*, *VvEDS1*, *VvACC*, *VvICS*, and *VvEIN3*), and *VvPR14*, combined with piceid and ε-viniferin content. In the same way, PHOS and BTH treatments were well discriminated from the controls and from the Triton and MeJA treatments, essentially on the basis of the modulation of many genes, combined with flavanol and pterostilbene production. The *P. viticola* growth was positively correlated to a high *trans*-resveratrol content but was negatively correlated to epicatechin production and *VvPR3* and *VvCAD* gene regulation. The second axis explained only 13.23% of the variability; however, it provided an explanation for the difference between MeJA- and Triton-treated leaves. The weak inhibition of downy mildew growth in Triton-treated leaves compared to the strong inhibition obtained in MeJA-treated leaves (Figure 1) could be explained by the low level of ε-viniferin in leaves, post-Triton application.

## DISCUSSION

In the context of limiting pesticide inputs in vineyards, some encouraging results in induced resistance of grapevine against downy mildew by PDS use have been reported.[13,26,27] The effectiveness of different elicitors varies[13,22] but could be optimized with a better understanding of their mechanism of action and by providing reliable biomarkers of the protection that they confer to the plant. In this study, we adopted the BioMolChem method[23] in order to assess the effect of treatments of grapevine leaves with MeJA (and its co-formulant, Triton), BTH, and PHOS at 6 dpt and inoculated with *P. viticola* at 8 dpt and 8 dpt-2 dpi to evaluate a potential priming phenomenon.

**Grapevine Defense Responses in Leaves at 6 days Post-treatment.** Polyphenol accumulation is a characteristic response in grapevine subjected to elicitation and/or inoculation with downy mildew; however, it may differ among cultivars, organs, experimental conditions, or time of incubation.[6,10,28] Enhancement of stilbene production and of transcripts encoding enzymes implicated in their biosynthesis that are triggered by MeJA treatment on grapevine leaves is well known in the literature.[16,29,30] In our study, in MeJA-treated leaves, an increased content of piceids, ε-viniferin, and resveratrol at 6 dpt was noted but not the accumulation of *VvPAL* and *VvSTS* transcripts, which may be explained by the relatively late time point of sample collection compared to previous studies, including ours.[16] The co-formulant of MeJA, Triton, induced both the accumulation of stilbenes and the induction of *VvPAL* and *VvSTS* transcripts. The effects may result from the surfactant activity of this substance, creating tissue injury, which liberates cutin monomers acting as


https://dx.doi.org/10.1021/acs.jafc.0c06103
J. Agric. Food Chem. 2021, 69, 1781−1795

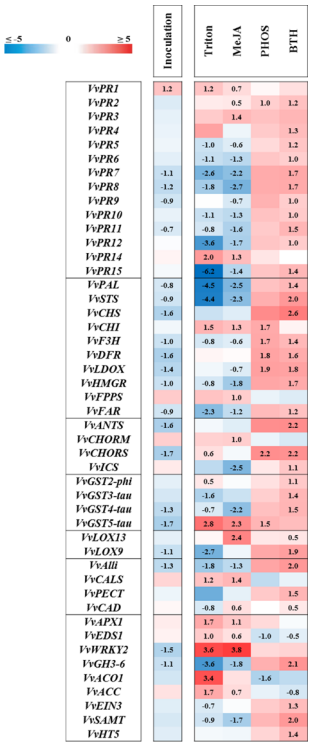


**Figure 5.** Pattern of relative expression of defense genes in grapevine leaves 8 days after treatment and 2 days after *Plasmopara viticola* inoculation. The first column represents the relative defense gene expression in untreated leaves 2 days after inoculation, with untreated and uninoculated leaves as a reference to calculated relative expression. The other columns represent the effects of treatment (Triton, MeJA, PHOS, or BTH), with untreated and inoculated leaves as a reference to calculate relative expression. The color scale bars represent the ratio values corresponding to the mean of three independent experiments. Genes up-regulated appear in shades of red, with an expression level higher than 5 in bright red, while those down-regulated appear in shades of blue, with an intensity lower than −5 in dark blue. For the first column, numbers in boxes represented the significant changes in gene expression in untreated and inoculated leaves ($p \leq 0.05$) compared to untreated and inoculated leaves (control). For the remaining columns, numbers in boxes represented the significant changes in gene expression in treated and inoculated leaves ($p \leq 0.05$) compared to untreated and inoculated leaves (control).

damage-associated molecular patterns, eliciting plant immune responses.[31] Moreover, similar gene transcription profiles were reported upon wounding and after JA elicitation;[31] hence, the similarity in the expression of defense genes between Triton and MeJA treatments in our study.

Unexpectedly, BTH and PHOS treatments reduced the content of resveratrol, piceids, and ε-viniferin, and, to a lesser extent, pterostilbene. However, the inducing effect of these elicitors reported in the literature occurred after relatively short periods of time (from 48 to 72 hpt) in comparison to the time of collection used in our study (6 dpt). On the other hand, BTH significantly up-regulated *VvPAL* and *VvSTS* gene expression, as was previously reported by other authors.[8,13,17] Thus, either post-transcriptional regulations could limit the production of stilbenes, or the newly synthesized monomeric and dimeric molecules were rapidly converted to larger oligomers or conjugated molecules not identified in our study. Upon PHOS treatment, stilbene content was lower than the control, but no change in the gene expression of *VvSTS* and *VvPAL* occurred.

All treatments, except PHOS, triggered an accumulation of flavanols in comparison to the controls. Additionally, BTH induced the expression of *VvCHS* and *VvF3H*, while Triton induced the expression of *VvCHS* and *VvLDOX*, genes that are implicated in the biosynthesis of flavanols. The positive impact of SA and JA and/or their analogs on the production of grapevine flavanols has been already described.[32]

BTH treatment up-regulated many PR protein genes, particularly those encoding β-1,3-glucanases and chitinases (*VvPR2*, *VvPR3*, *VvPR4*, *VvPR7*, *VvPR8*, and *VvPR11*). Such effect was previously reported in different plants species, including grapevine.[8,17,26] In contrast, few PR protein genes were modulated in Triton- and MeJA-treated leaves and also very few in PHOS-treated leaves. Previous works reported the ability of MeJA and potassium phosphite to induce PR protein genes in grapevine and other plants but at relatively early times of leaf collection after elicitation by the chemicals.[16,21,26,33]

GST genes were nearly specifically overexpressed in BTH-treated leaves. It is well known that BTH, as a SA analog, increases reactive oxygen species (ROS) in cells,[34] and GSTs are a family of enzymes that detoxify cytotoxic compounds by conjugation of reduced glutathione to a wide range of substrates and are involved in the transport of secondary metabolites (phytoalexins, anthocyanins).[35]

As expected, BTH induced *VvLOX9*, a gene of the oxylipin pathway, as described previously,[8] while MeJA and Triton treatments resulted in the overexpression of the *VvLOX13* gene coding for a protein involved in the biosynthesis of jasmonate.[36] The specificity of SA and JA signals was also exhibited for the gene *VvSAMT*, encoding salicylic acid carboxyl methyltransferase, which was repressed in MeJA-treated leaves and up-regulated in BTH-leaves along with *VvICS* transcripts.

In contrast to *VsSAMT* and *VvICS*, *VvWRKY2* was strongly induced by both Triton and MeJA, but not by BTH. The WRKY gene family plays a key role in modulating gene expression upon biotic and abiotic stresses, and their expression can be activated by pathogen infection or elicitor treatment.[37] In grapevine, *VvWRKY2* is induced by wounding or after infection with *P. viticola*.[38] Moreover, a functional analysis of *VvWRKY2* in tobacco showed that its over-expression reduced the susceptibility of plants to various fungi.[38] The positive effect of Triton treatment on *VvWRKY2* expression strongly suggested its involvement in grapevine stress responses.

The overall effect of PHOS treatment included a specific repression of the indole pathway and GST genes, accompanied by overexpression of genes involved in parietal reinforcement (*VvCALS*, *VvPECT*, and *VvCAD*). The up-regulations of the latter genes would be in agreement with the prime callose deposition described in *Arabidopsis*.[39]

Finally, cross-talk among phytohormones such as JA and SA is crucial in order to modulate plant resistance.[12] Since polyphenol levels and gene expression profiles differ in MeJA- and BTH-elicited leaves, we suggest a lack of overlap of these two pathways at 6 dpt.

**Grapevine Leaf Protection against *P. viticola* Conferred by Elicitors in Relation to Defense Responses at 8 dpt-2 dpi.** Varied levels of protection against *P. viticola* were observed according to the PDS used.


https://dx.doi.org/10.1021/acs.jafc.0c06103
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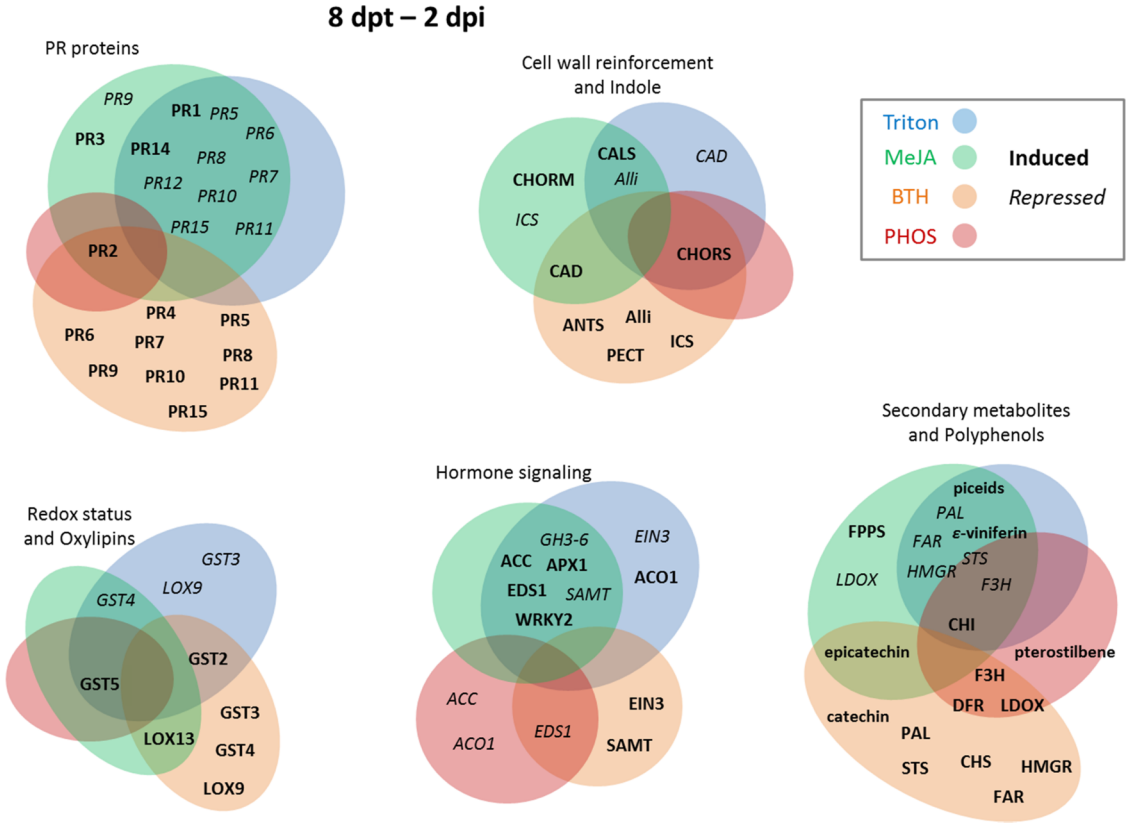


**Figure 6.** Venn diagram showing the significantly modulated genes in grapevine leaves 8 days after Triton, MeJA, PHOS, or BTH treatment and 2 days after *Plasmopara viticola* inoculation. Induced genes are represented in bold and inhibited ones in italic. Each treatment modality is represented by an ellipse with the following color: blue (Triton), green (MeJA), orange (BTH), and red (PHOS).

**Table 1. Content of Polyphenols (μg/mg DW) in Grapevine Leaves 6 days after Treatment (6 dpt)[a]**

| compound | control | Triton | MeJA | PHOS | BTH |
|---|---|---|---|---|---|
| | | | Stilbenes | | |
| *trans*-resveratrol | 0.27 ± *0.15* | 0.52 ± *0.10* | 0.83 ± *0.09** | 0.04 ± *0.01* | 0.07 ± *0.02* |
| piceids (sum) | 2.63 ± *1.56* | 5.50 ± *0.09* | 8.05 ± *0.20** | 0.30 ± *0.03* | 0.55 ± *0.16* |
| pterostilbene | 1.06 ± *0.52* | 1.51 ± *0.42* | 0.78 ± *0.12* | 0.82 ± *0.35* | 0.59 ± *0.15* |
| *ε*-viniferin | 1.63 ± *1.15* | 4.26 ± *0.88* | 9.65 ± *0.74*** | 0.26 ± *0.10* | 0.13 ± *0.03* |
| *total stilbenes* | 5.58 ± *0.35* | 11.79 ± *0.66**** | 19.33 ± *1.85**** | 1.41 ± *0.48*** | 1.33 ± *0.01*** |
| | | | Flavanols | | |
| catechin | 0.21 ± *0.01* | 0.66 ± *0.04*** | 1.07 ± *0.09**** | 0.28 ± *0.05* | 0.54 ± *0.07*** |
| epicatechin | 0.02 ± *0.00* | 0.09 ± *0.04* | 0.22 ± *0.03** | 0.03 ± *0.02* | 0.15 ± *0.06*• |
| *total flavanols* | 0.23 ± *0.01* | 0.75 ± *0.00** | 1.29 ± *0.12*** | 0.31 ± *0.07* | 0.69 ± *0.13** |
| | | | Total Polyphenols | | |
| total polyphenols | 5.81 ± *0.36* | 12.53 ± *0.65**** | 20.62 ± *1.97**** | 1.73 ± *0.55*** | 2.02 ± *0.14*** |

[a]Control: untreated leaves, Triton: leaves treated with Triton X-100, MeJA: leaves elicited with methyl jasmonate, PHOS: leaves elicited with phosphonates (LBG-01F34), BTH: leaves elicited with benzothiadiazole. Results are referenced to leaf dry weight and expressed in μg/mg DW as the means ± S.D. (in italics) of three independent samples analyzed in triplicate. Asterisks denote the significance levels as compared to control samples: 0 to 0.001 (***), 0.001 to 0.01 (**), 0.01 to 0.05 (*), 0.05 to 0.1 (•), $p \geq 0.05$ (no symbol) as a result of one-way analysis of variance (ANOVA). n.d.: not determined.

BTH and PHOS treatments resulted in fairly similar responses that differ from the responses triggered by MeJA and Triton. Accumulation of more or less specific antimicrobial molecules was responsible for these differences, as demonstrated by PCA. We also confirmed that *trans*-resveratrol was not a good marker of resistance, as was described previously.[8]

Stilbene content in MeJA- and Triton-treated leaves was higher than in the control leaves at 8 dpt and particularly at 8 dpt-2 dpi. Accumulation of *ε*-viniferin was a consequence of MeJA application and inoculation by *P. viticola* at 8 dpt-2 dpi. Viniferins are considered as important biomarkers of grapevine resistance to *P. viticola*.[10] In addition, *trans*- and *cis*-piceids, the glycosylated forms of resveratrol, were found in higher quantities in MeJA-treated leaves, inoculated or not, compared to controls. *ε*-Viniferin and piceid accumulation was reported following both biotic stresses and elicitors, such as MeJA, in grapevine leaves.[16] Thus, we confirmed that biosynthesis of stilbenes is mediated by jasmonates, as suggested by the fairly strong repression of *VvPAL* and


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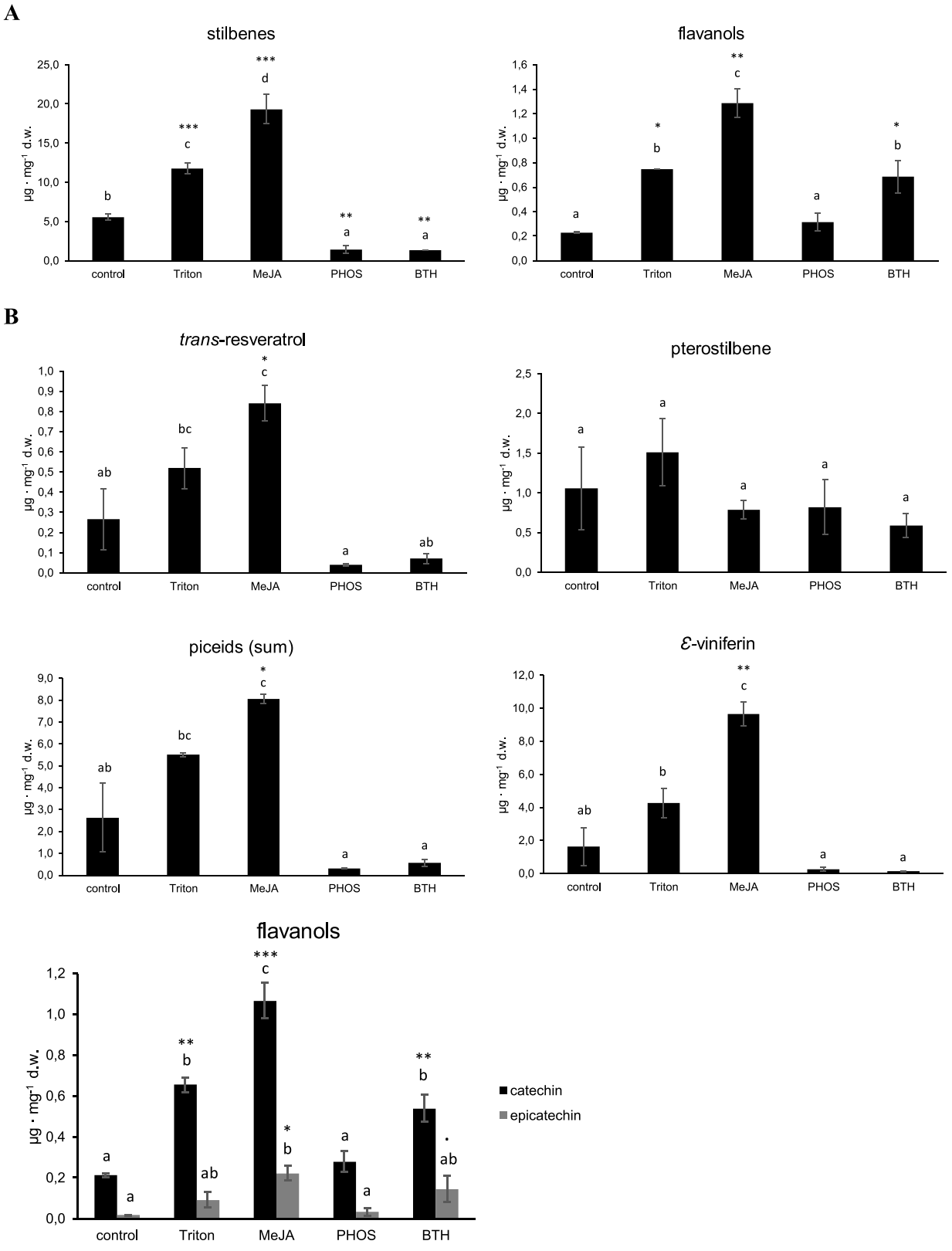


**Figure 7.** Quantification of stilbenes and flavanols in grapevine control leaves and in leaves 6 days after treatment with Triton, MeJA, PHOS, or BTH. Asterisks denote the significance levels as compared to control samples: 0 to 0.001 (***), 0.001 to 0.01 (**), 0.01 to 0.05 (*), 0.05 to 0.1 (.), $p \geq 0.05$ (ns). Lowercase letters indicate statistical differences between treatment modalities ($p \leq 0.05$). (A) Total of stilbenes or flavanols. (B) Individual compounds.

*VvSTS* genes at 8 dpt-2 dpi. Triton treatment triggered a similar stilbene profile as MeJA, but with a content of total stilbenes that was significantly lower.

Despite a generally low amount of pterostilbene (a methoxylated derivative of resveratrol), even upon microbial challenge, it is one of the most toxic stilbenes toward fungi,



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**Table 2. Content of Polyphenols (μg/mg DW) in Grapevine Leaves 8 days after Treatment and 48 h after Inoculation (8 dpt/8 dpt 2 dpi)[a]**

| | control | | Triton | | MeJA | | PHOS | | BTH | |
|---|---|---|---|---|---|---|---|---|---|---|
| compound | 8 dpt | 8 dpt 2 dpi | 8 dpt | 8 dpt 2 dpi | 8 dpt | 8 dpt 2 dpi | 8 dpt | 8 dpt 2 dpi | 8 dpt | 8 dpt 2 dpi |
| | | | | | Stilbenes | | | | | |
| *trans*-resveratrol | 1.33 ± *0.16* | 1.69 ± *0.54* | 2.36 ± *0.37* | 2.69 ± *0.76* | n.d.* | n.d.*** | 0.88 ± *0.50* | 1.00 ± *0.14* | 1.46 ± *0.13* | 0.89 ± *0.08* |
| piceids (sum) | 9.16 ± *0.48* | 12.91 ± *1.27* | 36.94 ± *1.05**** | 30.07 ± *0.31**** | 52.39 ± *2.54**** | 94.14 ± *3.31**** | 13.44 ± *0.90*• | 14.70 ± *0.56* | 13.24 ± *0.04* | 13.64 ± *0.56* |
| pterostilbene | 0.35 ± *0.23* | 0.52 ± *0.27* | 0.66 ± *0.01* | 0.80 ± *0.10* | 0.50 ± *0.21* | 0.26 ± *0.05* | 0.83 ± *0.12*• | 1.26 ± *0.17*** | 0.28 ± *0.09* | 0.76 ± *0.13* |
| ε-viniferin | 0.80 ± *0.10* | 1.29 ± *0.25* | 4.16 ± *1.14*** | 4.75 ± *0.64**** | 6.82 ± *0.18**** | 19.3 ± *2.07**** | 0.73 ± *0.06* | 0.96 ± *0.12* | 0.86 ± *0.12* | 0.71 ± *0.06* |
| *total stilbenes* | *11.64 ± 0.42* | *16.41 ± 1.28* | *44.12 ± 1.80**** | *38.32 ± 0.70**** | *59.71 ± 2.07**** | *113.71 ± 2.95**** | *15.88 ± 0.31* | *17.92 ± 0.33* | *15.85 ± 0.14* | *16.00 ± 0.19* |
| | | | | | Flavanols | | | | | |
| catechin | 22.18 ± *5.16* | 22.97 ± *2.68* | 83.19 ± *19.48*• | 56.82 ± *13.34* | 59.79 ± *0.22* | 72.79 ± *4.27* | 38.03 ± *10.35* | 58.58 ± *9.87* | 186.81 ± *42.81**** | 154.47 ± *13.30**** |
| epicatechin | 2.23 ± *0.54* | 2.52 ± *0.32* | 15.71 ± *4.54* | 10.22 ± *2.53* | 13.15 ± *0.59* | 23.99 ± *1.63*** | 5.56 ± *1.75* | 6.71 ± *1.22* | 46.69 ± *8.83**** | 35.38 ± *4.65**** |
| *total flavanols* | *24.41 ± 3.29* | *25.49 ± 1.73* | *98.90 ± 24.02*• | *67.04 ± 15.87* | *72.94 ± 0.81* | *96.78 ± 3.37*• | *43.59 ± 6.99* | *65.29 ± 6.35* | *233.51 ± 51.64**** | *189.85 ± 17.90**** |
| | | | | | Total Polyphenols | | | | | |
| total polyphenols | 36.06 ± *5.23* | 41.90 ± *5.01* | 143.02 ± *36.51*** | 105.36 ± *23.43* | 132.65 ± *1.78** | 210.49 ± *4.03**** | 59.47 ± *11.91* | 83.21 ± *10.68* | 249.35 ± *73.23**** | 205.85 ± *30.86*** |

[a]Control: untreated leaves, Triton: leaves treated with Triton X-100, MeJA: leaves elicited with methyl jasmonate, PHOS: leaves elicited with phosphonates (LBG-01F34), BTH: leaves elicited with benzothiadiazole. Results are referenced to leaf dry weight and expressed in μg/mg DW as the means ± S.D. (in italics) of three independent samples analyzed in triplicate. Asterisks denote the significance levels as compared to control samples: 0 to 0.001 (***), 0.001 to 0.01 (**), 0.01 to 0.05 (*), 0.05 to 0.1 (•), $p \geq 0.05$ (no symbol) as a result of one-way analysis of variance (ANOVA). n.d.: not determined.

including *P. viticola*.[10] In this study, the content of pterostilbene increased after PHOS treatment and especially after inoculation. However, as in the case of MeJA and Triton treatments, we did not observe a correlation between the level of *VvPAL* and *VvSTS* expression and production of stilbenes. This result underscores the importance of the time of sampling. Molecules can be produced early, in a biphasic profile, late, or synthesized in different forms from those quantified (e.g., larger oligomers, glyco-conjugates, or other forms).

Catechin and epicatechin also contributed to the protective effect of BTH-pretreated leaves since their accumulation, along with the overexpression of genes involved in their biosynthesis (*VvCHS* and *VvDFR*), was observed. Flavanols have been shown to have a beneficial effect against *P. viticola*.[40] Furthermore, the SA pathway promotes the increase in catechins and pro-anthocyanidins.[41]

Almost all PR protein genes studied were overexpressed in BTH-treated leaves at 8 dpt-2 dpi, as partly reported in previous studies.[8,17,26] In MeJA-, PHOS-, and Triton-treated leaves very few PR protein genes were up-regulated after inoculation, suggesting a major role of stilbenes in protection. What is more, two TAU type GST-type (*GST3* and *GST4*) and one PHI-type (*GST2*) genes were strongly overexpressed in BTH-treated and inoculated leaves. We suggest that phytoalexins in BTH-treated leaves are synthesized and potentially conjugated and/or transported using these GST genes. In Triton-, MeJA-, and PHOS-treated and inoculated leaves, the up-regulated *VvGST5* gene seemed to be more specifically involved.

The induction of expression of LOX genes is generally thought to occur under the JA signaling pathway. Thus, their induction both by MeJA and BTH in this study suggests cross-talk between the jasmonate and SA pathways. The induction of *VvLOX9* has been reported in vineyard leaves treated with BTH.[17] In BTH-treated leaves at 8 dpt-2 dpi, the SA pathway was prevalent with the up-regulation of *VvSAMT*, which was, in contrast, repressed in MeJA-treated leaves. Similar to what was observed for *VvSAMT*, and the implication of induction of the SA signaling pathway after BTH application, genes of the chorismate pathway (*VvICS*, *VvANTS*, *VvCHORM*, and *VvCHORS*) were up-regulated by BTH but poorly or even negatively regulated by Triton or MeJA treatment. *VvG3H-6* was overexpressed in BTH-treated and repressed in MeJA-treated leaves. This is consistent with the function of these proteins that conjugate amino acids to jasmonate or auxin and lead to activation, inactivation, or degradation of these molecules.[42]

The up-regulation of *VvWRKY2* observed in MeJA-treated leaves at 8 dpt-2 dpi could potentially be the result of the surfactant effect of its co-formulant, Triton, on the cell wall, similar to 77% of the genes commonly modulated by MeJA and Triton. The question arises as to whether Triton stimulates the jasmonate pathway or whether the presence of Triton in the MeJA solution leads to the gene modulations observed in the MeJA-treated leaves. The first hypothesis is the more plausible as Triton can create tissue damage, more or less mimicking wounding.

Among the genes modulated after infection, ascorbate peroxidase (APX) regulates ROS levels in different subcellular compartments and prevents potential $H_2O_2$-derived cellular damage.[43] In this study, *VvAPX1* was up-regulated in Triton- and MeJA-treated and inoculated leaves but remained unaffected in PHOS- and BTH-treated leaves, which could be in agreement with a previous work[44] describing APX as an enzyme somewhat inhibited by SA induction.



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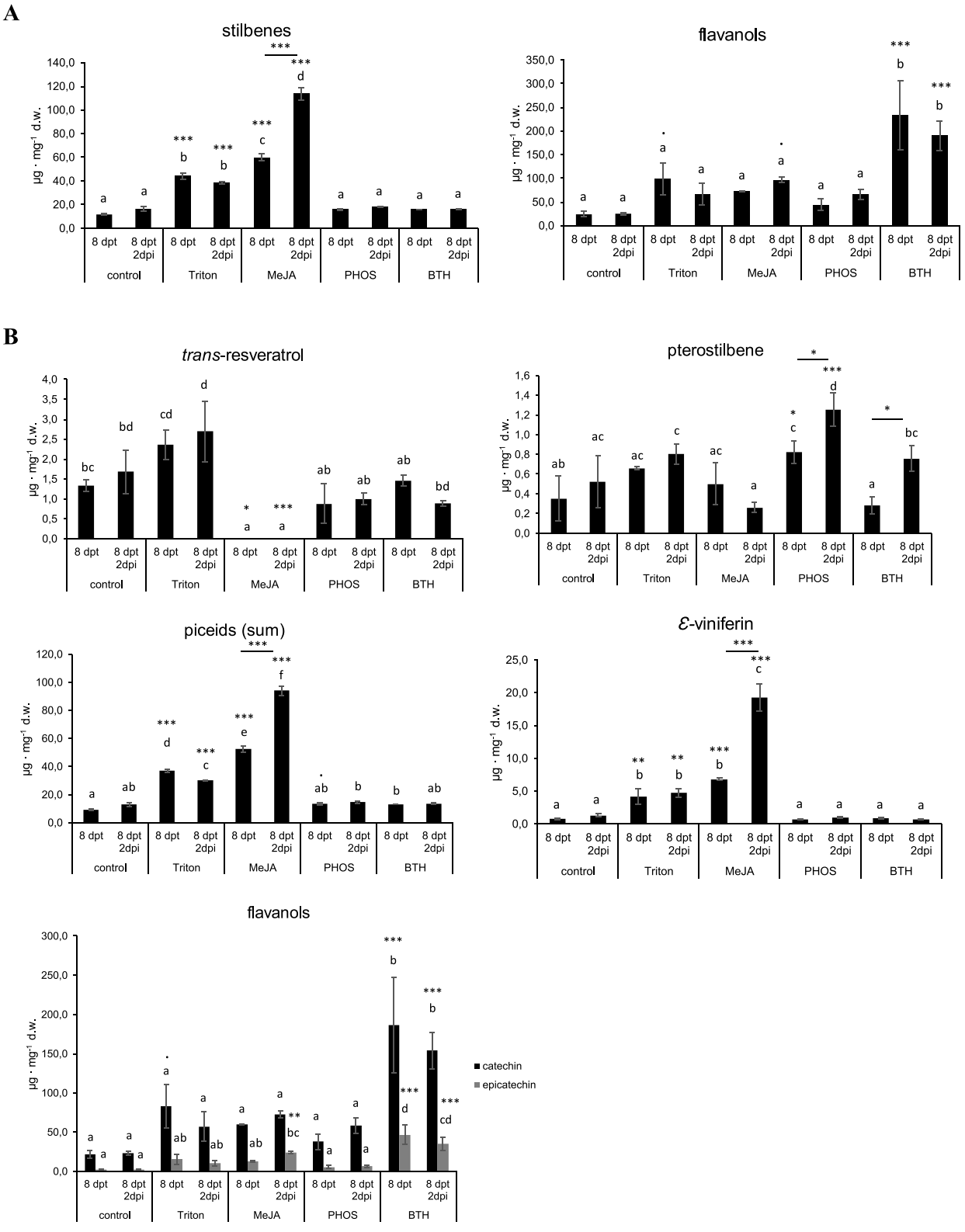


**Figure 8.** Quantification of stilbenes and flavanols in grapevine leaves 8 days after elicitation and 2 days post-inoculation with *Plasmopara viticola* (8 dpt, 8 dpt-2 dpi). Asterisks denote the significance levels as compared to control samples: 0 to 0.001 (***), 0.001 to 0.01 (**), 0.01 to 0.05 (*), 0.05 to 0.1 (.), $p \geq 0.05$ (ns). Lowercase letters indicate statistical differences between treatment modalities ($p \leq 0.05$). (A) Total of stilbenes or flavanols. (B) Individual compounds.

After inoculation, *VvEDS1* was overexpressed in Triton- and MeJA-treated leaves, but was repressed by PHOS and BTH. This gene has been shown to be stimulated by SA treatment and inoculation with *P. viticola* in *Vitis vinifera* over short periods of time;[45] thus, once again, the time of sampling may explain the difference compared to our results. Furthermore, EDS1 and SA signaling were found to act redundantly on downstream resistance genes.[46] We suggest



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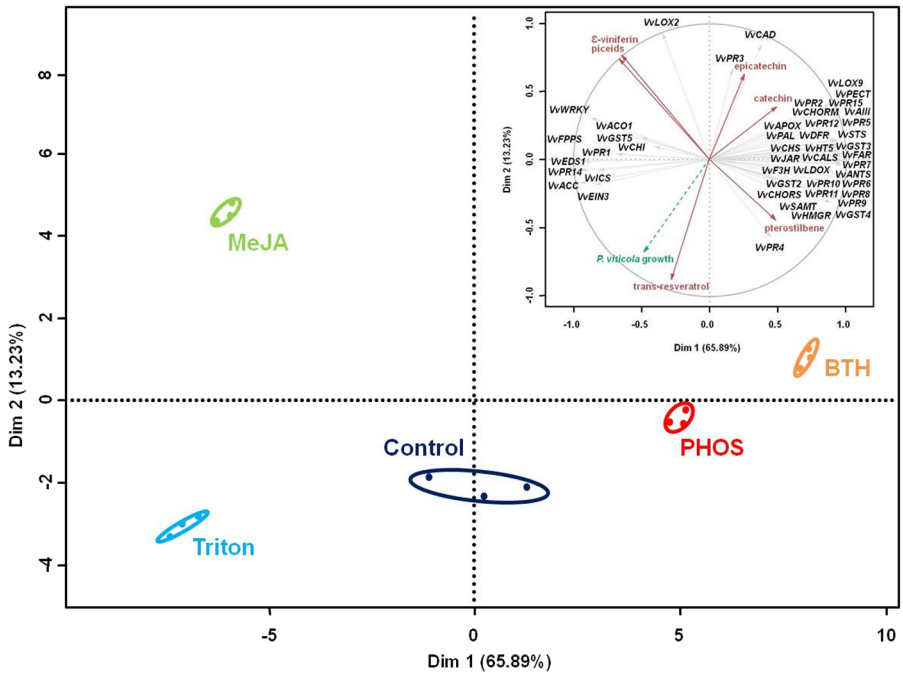


**Figure 9.** Multiple factor analysis (MFA) and cluster of genes differentially expressed, *Plasmopara viticola* growth, and stilbenes/flavanols concentration obtained 8 days after treatment and 2 days after inoculation (8 dpt-2 dpi). Projections on standard unit circle of quantitative variables and quantitative illustrative variables were represented. Distribution of plant responses and severity data on principal planes defined by two axes obtained by PCA of gene expression profiles, evaluation of *P. viticola* growth, and polyphenols analysis, using all treatment modalities data. Ellipses representing the 95% confidence intervals were calculated for each modality.

that, at 8 dpt-2 dpi, the actions of MeJA and SA on the *EDS1* gene would be reversed from what was observed 24 or 48 h after treatment.

Genes involved in the ET pathway (*VvACC* and *VvACO*) were up-regulated at 8 dpt-2 dpi in leaves pretreated with Triton and MeJA and down-regulated by PHOS and BTH. Perhaps, the ET pathway had been induced precociously upon BTH treatment, as described in other studies.[17,26,47] This point may be supported by the overexpression of *VvEIN3*, a key positive factor of ET signaling, which affects many hormonal pathways, e.g., SA biosynthesis gene *SID2*, or cytokinin signaling.[48] The up-regulation of *VvEIN3* could be related either to a priming effect on the ET pathway or to more complex cross-talk regulation between the different hormonal pathways.

Finally, the PDS studied here conceivably activated the corresponding signaling pathways, i.e., BTH and MeJA mediated the responses via SA and JA signaling, respectively.[49,50] Moreover, we confirmed the employment of the SA pathway by PHOS since the PCA positioned this treatment close to BTH and opposite of MeJA. Indeed, the direct and indirect effect of phosphites on oomycetes[14,20] appears to be dose-dependent and would be implicated in the SA pathway at a concentration of 10 mM.[21] The gene modulations observed (flavonoid biosynthesis genes and *VvPR2*) may be related to an indirect protection effect of PHOS treatment. In addition, we identified the complexity of the interactions, the interdependence between different signaling pathways, and the establishment of effective defenses against pathogens of different lifestyles.[15]

To conclude, a comparison of the effects of MeJA, BTH, and PHOS treatment on long-lasting defense reactions in grapevine leaves was shown for the first time. Even if other studies, such as RNA sequencing or proteomics, should be proposed to deepen the plant components activated due to elicitation, the combined approach based on biological, molecular and chemical measurements that we used revealed the establishment of resistance against downy mildew via more or less specific modulation of resistance molecular and metabolic biomarkers. Our results could contribute to a better understanding of the mechanisms of action of the PDS studied here, leading to their improved application in the vineyard.

## ■ ASSOCIATED CONTENT

### Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.jafc.0c06103.

Selected genes and corresponding primer sets used for *Vitis vinifera* leaves with "NeoVigen96" chip that were analyzed in the Biomark HD system (PDF)

## ■ AUTHOR INFORMATION

### Corresponding Authors

**Stéphanie Cluzet** − *University of Bordeaux, Faculté des Sciences Pharmaceutiques, Unité de recherche Œnologie, EA 4577, USC 1366 INRAE, Equipe Molécules d'Intérêt Biologique (GESVAB), ISVV, 33882 Villenave d'Ornon cedex, France;* orcid.org/0000-0002-1653-4539; Phone: +33(0)557575954; Email: stephanie.cluzet@u-bordeaux.fr

**Marie-France Corio-Costet** − *INRAE, UMR Santé et Agroécologie du Vignoble (1065), ISVV, Labex Cote, CS 20032, 33882 Villenave d'Ornon, France;* orcid.org/0000-0003-2206-9482; Phone: +33(0)557122625; Email: marie-france.corio-costet@inrae.fr



https://dx.doi.org/10.1021/acs.jafc.0c06103
J. Agric. Food Chem. 2021, 69, 1781−1795

### Authors

**Aleksandra Burdziej** − *University of Bordeaux, Faculté des Sciences Pharmaceutiques, Unité de recherche Œnologie, EA 4577, USC 1366 INRAE, Equipe Molécules d'Intérêt Biologique (GESVAB), ISVV, 33882 Villenave d'Ornon cedex, France; Department of Plant Biochemistry, Faculty of Biology, University of Warsaw, 02-096 Warszawa, Poland*

**Anthony Bellée** − *INRAE, UMR Santé et Agroécologie du Vignoble (1065), ISVV, Labex Cote, CS 20032, 33882 Villenave d'Ornon, France*

**Enora Bodin** − *INRAE, UMR Santé et Agroécologie du Vignoble (1065), ISVV, Labex Cote, CS 20032, 33882 Villenave d'Ornon, France*

**Josep Valls Fonayet** − *University of Bordeaux, Faculté des Sciences Pharmaceutiques, Unité de recherche Œnologie, EA 4577, USC 1366 INRAE, Equipe Molécules d'Intérêt Biologique (GESVAB), ISVV, 33882 Villenave d'Ornon cedex, France;* orcid.org/0000-0002-1359-4114

**Noël Magnin** − *INRAE, UMR Santé et Agroécologie du Vignoble (1065), ISVV, Labex Cote, CS 20032, 33882 Villenave d'Ornon, France*

**Anna Szakiel** − *Department of Plant Biochemistry, Faculty of Biology, University of Warsaw, 02-096 Warszawa, Poland*

**Tristan Richard** − *University of Bordeaux, Faculté des Sciences Pharmaceutiques, Unité de recherche Œnologie, EA 4577, USC 1366 INRAE, Equipe Molécules d'Intérêt Biologique (GESVAB), ISVV, 33882 Villenave d'Ornon cedex, France;* orcid.org/0000-0002-5308-8697

Complete contact information is available at: https://pubs.acs.org/10.1021/acs.jafc.0c06103



### Funding

This work was supported by CIVB (Interprofessional Committee of Bordeaux Vines) and Institute National de la Recherche Agronomique pour l'agriculture, l'alimentation et l'environnement (INRAE) Bordeaux Aquitaine. The Bordeaux Metabolome Facility and MetaboHUB (ANR-11-INBS-0010 project) have also provided support. We are thankful to the French Government and the French Embassy in Poland for their financial support.


### Notes

The authors declare no competing financial interest.

## ■ ACKNOWLEDGMENTS


We are grateful to S. Gambier for plant productions, L. De Bastard from Syngenta for supplying acibenzolar-*S*-methyl (BTH), O. André from De Sangosse for supplying LBG. The study was carried out in the framework of the Cluster of Excellence COTE.


## ■ ABBREVIATIONS

ANOVA, analysis of variance; BTH, benzothiadiazole; cDNA, complementary deoxyribonucleic acid; dpi, days post-inoculation; dpt, days post-treatment; DW, dry weight; ET, ethylene; GST, glutathione *S*-transferase; JA, jasmonic acid; LOX, lipoxygenase; MeJA, methyl jasmonate; M-MLV reverse transcriptase, Moloney murine leukemia virus reverse transcriptase; PAL, phenylalanine ammonia lyase; PCA, principal component analysis; PDS, plant defense stimulators; PHOS, phosphonates; PR, pathogenesis-related; qPCR, quantitative polymerase chain reaction; RNA, ribonucleic acid; SA, salicylic acid; STS, stilbene synthase; *VvACC*, 1-amino-cyclopropane-1-carboxylic acid oxidase; *VvACO1*, 1-amino-cyclopropane-1-carboxylic acid oxidase; *VvAlli*, alliinase; *VvANTS*, anthranilate synthase; *VvAPOX*, ascorbate peroxidase; *VvAPX1*, ascorbate peroxidase; *VvCAD*, cinnamoyl-CoA reductase; *VvCALS*, callose synthase; *VvCHI*, chalcone isomerase; *VvCHORM*, chorismate mutase; *VvCHORS*, chorismate synthase; *VvCHS*, chalcone synthase; *VvDFR*, dihydroflavonol reductase; *VvEDS1*, lipase 3/enhanced disease susceptibility gene; *VvEF1γ*, elongation factor eEF1 gamma chain (eEF1-gamma); *VvEIN3*, ethylene-insensitive 3-binding F box protein 1; *VvF3H*, flavanone-3-hydroxylase; *VvFAR*, ent-kaurene synthase; *VvFPPS*, farnesyl pyrophosphate synthase; *VvGAPDH*, glyceraldehyde 3-phosphate dehydrogenase; *VvGH3-6*, JA-Ile-synthase; *VvGST2-phi*, glutathione *S*-transferase class-phi; *VvGST3, 4, 5-tau*, glutathione *S*-transferase Tau class; *VvHMGR*, 3-hydroxy-3-methylglutaryl coenzyme A reductase; *VvHT5*, hexose transporter; *VvICS*, isochorismate synthase; *VvLDOX*, leucoanthocyanidin synthase; *VvPECT*, pectin methyl esterase; *VvPR1*, pathogen-related protein 1 gene; *VvPR10*, ribonuclease; *VvPR11*, chitinase class V; *VvPR12*, defensin-like protein-oxalate oxidase; *VvPR14*, lipid transfer protein; *VvPR15*, germin-like protein-oxalate oxidase; *VvPR2*, beta-1,3-glucanase; *VvPR3*, endochitinase class; *VvPR4*, chitinase class IV; *VvPR5*, thaumatin-like protein; *VvPR6*, serine protease inhibitor; *VvPR7*, subtilisin-like endoprotease; *VvPR8*, acidic endochitinase-like; *VvPR9*, cationic peroxidase 1; *VvSAMT*, salicylic acid methyl transferase; *VvTHIORYLS8*, catalytic thioredoxin-like protein 4A; *VvTIP41*, TIP41-like protein; *VvTUA*, tubulin alpha; WRKY, WRKY transcription factor

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*J. Agric. Food Chem.* 2021, 69, 1781−1795