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Comparison of the Impact of Two Molecules on Plant Defense and on Efficacy against *Botrytis cinerea* in the Vineyard: A Plant Defense Inducer (Benzothiadiazole) and a Fungicide (Pyrimethanil)

Anthony Bellée,*,† Stéphanie Cluzet,‡ Marie-Cécile Dufour,† Jean-Michel Mérillon,‡ and Marie-France Corio-Costet*,†

†UMR Santé et Agroécologie du Vignoble (1065), ISVV, Labex Cote, CS 20032, INRA, 33882 Villenave d'Ornon, France

‡Faculté des Sciences Pharmaceutiques, Unité de Recherche Œnologie EA 4577, USC 1366 INRA, Equipe Molécules d'Intérêt Biologique (GESVAB), Institut des Sciences de la Vigne et du Vin, CS 50008, Université de Bordeaux, 33882 Villenave d'Ornon, France

Supporting Information

ABSTRACT: Grapevine is subject to diseases that affect yield and wine quality caused by various pathogens including *Botrytis cinerea*. To limit the use of fungicides, an alternative is to use plant elicitors such as benzothiadiazole (BTH). We investigated the effect of a fungicide (Pyrimethanil) and an elicitor (benzothiadiazole) on plant defenses. Applications for two consecutive years in the vineyard significantly reduced gray mold. Two and seven days after treatments, the expressions of 48 genes involved in defenses showed differential modulation (up- or down-regulation) depending on treatment. Some genes were identified as potential markers of protection and were linked to an increase in total polyphenols (TP) in leaves. Surprisingly, the fungicide also induced the expression of defense genes and increased the polyphenol content. This suggests that BTH acts as an efficient elicitor in the vineyard and that Pyrimethanil may act, in part, as a defense-inducing agent on the vine.

KEYWORDS: *BTH, elicitor, quercetin-3-O-glucuronide, Pyrimethanil, gene expression, gray mold, polyphenol, Vitis vinifera*

INTRODUCTION

Although grapevine is one of the major fruit crops in the world, it is sensitive to a wide range of pathogens that can cause yield losses and affect harvest quality. Gray mold due to *Botrytis cinerea* is a ubiquitous necrotrophic ascomycete fungus that induces grape desiccation, rot, and biochemical changes in grapevine berries from veraison to harvest, thereby reducing wine quality.[1] The fungus remains latent and develops until the postveraison period, when berry defenses weaken and bunch rot appears.[2] Disease control is currently achieved by fungicides like Pyrimethanil (N-(4,6-dimethylpyrimidin-2-yl)aniline), which belongs to the pyrimidinamine family and inhibits methionine biosynthesis and laccase activity on *B. cinerea*.[3,4] Repeated treatments have led to the emergence of resistance within pathogen populations[5,6] and the presence of fungicide residues in berries and wine.[7] Thus, during the past decade, the quest has been to develop alternative protection strategies.[8−10]

An approach to prevent and limit pathogen attacks, and thus further development, consists of inducing plant defenses with plant defense stimulators (PDS) or elicitors that do not have any direct effect on the pathogen. Many compounds or microorganisms can act as elicitors such as plant or pathogen molecules like oligo-, poly saccharides, peptides, proteins and lipids, abiotic compounds, plant hormones, and microorganisms.[9,11] Signal perception leads to the release of reactive oxygen species (ROS) and to the modulation of gene expressions that induce reinforcement of the plant cell wall, the accumulation of antimicrobial compounds (phytoalexines), or hypersensitive cell death (HR).[12] Many studies have reported the efficiency of elicitors in controlled conditions.[9,13] For example, chitosan (chitin derivative) and ergosterol (fungus sterol) are very effective *in vitro* for stimulating grapevine defenses and thus inhibiting the development of *Botrytis cinerea*.[14−16] However, these promising results are often disappointing in the vineyard, where the complexity of the plant/pathogen/environment interaction is a considerable challenge to effective and repeatable disease control.[8] Thus, few elicitors are currently registered for controlling plant diseases and particularly *B. cinerea*.[9]

Acibenzolar-S-methyl [benzo(1,2,3)-thiadiazole-7 carbothiolic acid)] or benzothiadiazole (BTH), a salicylic acid (SA) analog, induces systemic immunity against different pathogens of several plant species.[17,18] Its actions are due to its properties as an SA analog. The SA signaling pathway is essential for the establishment of local and systemic-acquired resistance (SAR), in concert with ethylene and jasmonic acid.[19,20] From the infection site, signals mediated by SA lead to the systemic induction of plant defenses activated by various stimuli. This in turn affects the expression of genes or proteins, thereby inducing defense signals and strengthening cell walls.[21] Several studies in grapevine have demonstrated that BTH induces resistance to *Plasmopara viticola*, *Erysiphe necator*,[22,23] and *B. cinerea*[24,25] and that the resistance is linked to an increase in total polyphenols in berries and to PR-protein gene overexpression. Using SAR induction through the SA pathway

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as an alternative strategy to protect plants against a wide range of pathogens requires a biological inducer such as BTH.

The present study investigated the value of BTH as elicitor and its efficiency in the management of *B. cinerea* in the vineyard compared to Pyrimethanil over a period of two consecutive years. The objectives were to improve the understanding of the mode of action of BTH and Pyrimethanil and to compare their efficiency against *B. cinerea* in the vineyard under strong epidemic pressure. For two consecutive years, field assays were performed and plant responses after elicitor and fungicide treatments were monitored in leaves collected directly in the vineyard. First, total polyphenol content was measured as well as major phenolic compounds. Then transcripts were monitored in leaves by studying specific microfluidic dynamic arrays of 48 genes involved in grapevine defenses. The genes analyzed were associated with SAR cell responses with glutathione-S-transferase (5); PR-proteins (15); stilbenoid, flavonoid, and isoprenoid pathway (12); cell wall reinforcement (4); oxylipin pathway (3); and phytohormone (SA, JA, and ethylene) pathway (9). Harvest yield and must quality were analyzed, and the gene induction and the polyphenol content in leaves were correlated with the protection induced on the harvested grapes. The results provide new insights into the action of two types of plant protection products and provide potential markers of protection against *B. cinerea*.

MATERIALS AND METHODS

Chemicals. Formulated acibenzolar-S-methyl (S-methyl benzo-[1,2,3,]thiadiazole-7-carbothioate) or benzothiadiazole was used (BTH, Bion 50WG, Syngenta) as elicitor. Pyrimethanil (N-(4, 6-dimethylpyrimidin-2-yl) aniline) (Scala BASF agro, 40% of active substance) was used as reference fungicide.

Fungal Material. *Botrytis cinerea* strain CCB16 (Château Couhins, France) was obtained from naturally infected berries collected from the same plot of the vineyard experiment in 2016. The fungus was maintained on malt-agar medium (MA, 20 g/L of malt and 15 g/L of agar) at 4 °C. The inoculum was subcultured by transferring colonized agar plugs (5 mm diameter) to fresh medium (MA) with a photoperiod of 16 h light/8 h dark at 22 °C for 5 days.

Antifungal Activity. The effect of Pyrimethanil and BTH on the mycelial growth of *B. cinerea* was assessed *in vitro* on MA containing different concentrations of compound (0, 0.1, 1, 10, 100, 1000, and 5000 mg/L). Pyrimethanil and BTH dispersed in sterilized water were incorporated into the MA medium at 50 °C, and 15 mL was poured into Petri dishes. Mycelial plugs (5 mm) were placed at the center of the dishes, and eight replications per concentration for each product were carried out. Plates were covered and incubated at 22 °C with 16/8 h day/night photoperiod for 4 days. Radial growth was assessed by measuring the fungal development in two perpendicular diameters and calculating the mean diameter for each concentration. Means of the growth rates at each concentration 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.

Plant Material and Open Field Treatments. Experiments in the vineyard were conducted at Château Couhins, Bordeaux, in the southwest region of France, on *V. vinifera* cv Sémillon. The vine stocks were 10 years old at the beginning of the experiments and were grafted with Fercal rootstock. The plot was divided into blocks of 10 vines, with five blocks per row and each of the five blocks randomly distributed in the plot. Three treatment modalities (untreated, BTH and Pyrimethanil) were performed. Products were applied at 400 L/ha three times per year. The first application was at the full flowering stage 65 on the BBCH scale corresponding to May 21, 2014 and June 9, 2015. The second application was 2 weeks later (67–69 on the BBCH scale), corresponding to June 4, 2014 and June 23, 2015. The third and last application was performed at the beginning of bunch closure (stage 77 on the BBCH scale), that is, July 9, 2014 and July 7, 2015. An antibotrytis fungicide consisting of formulated Pyrimethanil was dissolved in water and applied at the dose of 1 kg/ha as recommended by the manufacturers. BTH was dissolved in water and applied at 800 g/ha, corresponding to 400 g/ha of active ingredient.

***B. cinerea* Assessment.** Notations were carried out late in the season. They began August 25, 2014 and August 19, 2015 and continued every week until the harvest. On each block, the gray mold was visually assessed as the percentage on 100 bunches randomly chosen on 10 vines from each plot and with each modality. Disease severity (percentage covering each bunch) and incidence (percentage of bunches exhibiting symptoms) were calculated for each plot of each treatment. Severity and incidence curves were drawn, and each point on the curves was subjected to statistical analyses by parametric tests (ANOVA) followed by pairwise comparisons using Tukey's post hoc test to determine differences between the different treatment modalities at the 5% significance level. Areas under the disease progress curve (AUDPC) were also calculated for the severity and the incidence of *B. cinerea* to assess the overall effectiveness of the product during the season. These areas were calculated using the formula: $\text{AUDPC} = \Sigma\ (X_i + X_{i+1})/2(t_{i+1} - t_i)$ where X_i corresponded to either disease severity or incidence (%) at assessment i, X_{i+1} corresponded to either the severity or incidence (%) at subsequent assessment $i + 1$, and $(t_{i+1} - t_i)$ corresponded to the number of days between the two consecutive assessments. Similar statistical analyses were performed on the means of the AUDPC for severity and incidence to determine differences between treatment modalities at the 5% significance level. Statistical analyses were carried out using R x64 3.0.3 software.

Physicochemical Composition of Grapes. When the grapes had reached maturity, they were harvested, that is, on September 25, 2014 and September 14, 2015. All clusters were harvested, counted, and weighed for each block. The average number of clusters per vine and the average weight of a cluster were calculated. For each modality, clusters were separated into three batches, which were squeezed to obtain must. The must was cleared by centrifugation, and the supernatant was analyzed by refractometry to determine its sugar content and the potential alcoholic strength. Then pH and treatable acidity were measured using an automatic titrator (Cogétude). For each data set obtained during the harvest, statistical analyses were performed on the means to determine significant differences between treatment modalities. Nonparametric (Kruskal and Wallis) or parametric tests (ANOVA) followed by pairwise comparisons using Tukey's post hoc test allowed us to determine significant differences at the 5% significance level. The choice of the parametric or nonparametric test depended upon the homogeneity of variances (Levene test). Statistical analyses were carried out using Rx64 3.03 software.

Polyphenol Spectrophotometric Detection and Quantification. Three grapevine leaves were sampled on each block (3 leaves × 5 blocks × 4 modalities) at different times 2 days and 7 days after the second treatment (June 4) in 2014. Phenolic compounds of freeze-dried leaves (100 mg) were extracted using 100% MeOH (8 mL) overnight at 4 °C. Following centrifugation at 3500*g* (10 min), supernatants were recovered (5 mL), evaporated to dryness in a vacuum rotary evaporator at 40 °C, and resuspended in 30% MeOH (1 mL). Leaf extracts were purified on a Supelclean LC-18 solid phase extraction (SPE) column (Supelco, USA) to remove chlorophylls. Elution was carried out with 90% MeOH; eluates were evaporated to dryness and dissolved in 50% MeOH (1 mL). Samples were then filtered through PTFE filters (0.45 μm) and kept at −20 °C until analysis. Total phenolic contents of dried leaves were measured with the Folin-Ciocalteu method adapted to 96-well plates.[26] Briefly, 20-fold diluted leaf extracts (20 μL) were mixed with Folin-Ciocalteu's reagent (100 μL) and incubated 2–3 min at room temperature. A solution of sodium carbonate at 75 g/L (80 μL) was then added to the mixture. After 1 h of incubation in the dark at room temperature, total polyphenols were determined by measuring the absorbance at 765 nm with a Fluostar Optima plate reader (BMG Labtech). Quantification was done with respect to a standard curve of gallic acid, and the results

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were expressed as milligrams of gallic acid equivalent (GAE) per gram of dry weight (DW) of leaf extract (mg GAE/g). All samples were analyzed in triplicate. Data were reported as means ± standard error mean (SEM). Concerning the antioxidant measurements, parametric tests were used to assess variance and correlation after a Kolmogorov−Smirnov test to confirm the normality of the data. Statistical comparisons were calculated by analysis of variance (one-way analysis of variance, ANOVA). Significant differences ($P < 0.05$), after a Tukey correction for multiple comparisons, were determined using GraphPad Prism 5.03 for Windows (GraphPad Software, San Diego, CA, USA).

Analysis of Major Phenolic Compounds by HPLC−Mass Spectrometry (LC−MS). All analyses were performed on a 1290 series UHPLC (Agilent Technologies, Santa Clara, CA, USA) chromatography apparatus, which included an autosampler module, a degasser, a binary pump, a column heater/selector and a UV−visible-DAD detector from the same provider. Chromatographic separation was performed on a Zorbax C18 column (2.1 μm, 100 mm × 2.1 mm), Agilent) and the column temperature was maintained at 25 °C. The flow rate was typically set at 0.4 mL/min. Acidified water (0.1% formic acid; v/v (solvent A) and acetonitrile (solvent B) were used as mobile phases. The following gradient was programmed: 0 min 83% A, 17% B; 0.4 min 83% A, 17% B; 4.4 min 70% A, 30% B; 7.4 min 62% A, 38% B, 9 min 50% A, 50% B; 10 min 0% A, 100% B; 11 min 0% A, 100% B; 11.2 min 83% A, 17% B.

The LC apparatus was connected to an Esquire LC−ESI−MS/MS from Bruker Daltonics (Billerica, MA, USA). The HPLC output was split 1:10 in the MS detector.

Total ion chromatograms were obtained using negative mode with a range of m/z 110−1500. The capillary voltage was −3700 V, the capillary end voltage 127.7 V, the skimmer voltage 40 V, and trap drive 68.7.

Nitrogen, the drying gas, was set as 5 L/min and 325 °C, and nebulizer pressure was set to 15 psi. The MS data were processed through Data Analysis 3.2 software (Bruker Daltonics, Bremen, Germany). Analyses were performed using DAD chromatograms obtained at 280 (stilbenes, *cis*-form), 306 (stilbenes, *trans*-form), and 360 nm (flavonols). The phenolic compounds present in the samples were characterized according to their UV and mass spectra. Quercetin 3-O-glucuronide was quantified by a calibration curve using quercetin at 360 nm, and values were expressed in mg per g quercetin equivalent of dry weight extract (mg/g DW). Means for each treatment were calculated from three technical replicates.

Gene Expression Analysis by RT-qPCR. Three grapevine leaves were sampled on each block (3 leaves × 5 blocks × 4 modalities) 2 days and 7 days after the second treatment in 2014 (June 4). Sampling was carried out on the fourth leaf away from the stem apex to collect leaves of a similar physiological age. Fifteen leaves were frozen in liquid nitrogen and stored at −80 °C. After being crushed in liquid nitrogen for each modality (sampling time and treatment), three batches were established and total RNA was extracted according to the protocol previously described by Dufour et al.[27] Leaf powder was added to an extraction buffer (20 g/mL) preheated to 56 °C (300 mM Tris HCl, pH 8.0; 25 mM EDTA; 2 mM NaCl; 2% CTAB; 2% poly vinyl poly pyrrolidone (PVPP); 0.05% spermidine trihydrochloride; and 2% β-mercaptoethanol added extemporaneously). The mixture was stirred vigorously and incubated in a water bath at 56 °C for 10 min under regular stirring. An equal volume of chloroform/isoamyl alcohol (24:2, v/v) was added and then centrifuged at 3500*g* for 15 min. The following steps were conducted using the Spectrum Plant Total RNA kit (Sigma) according to the manufacturer's instructions. Finally, samples were incubated for 15 min with the DNase I Digestion Set (Sigma). RNA concentrations were determined with a Denovix DS-11 spectrophotometer. Ten micrograms of total RNA was reverse-transcribed using 2 μM oligo-d$(T)_{15}$, ribonuclease inhibitor and M-MLV reverse transcriptase (Invitrogen), according to the manufacturer's instructions in a final volume of 900 μL. The cDNAs obtained were stored at −20 °C. High-throughput gene expression quantification was done by using microfluidic dynamic array (Fluidigm) technology, and specific primer sets included in the "NeoViGen96" chip, designed previously by Dufour et al..[27] were used. Details of genes are listed in Table S1. Five genes (*VvEF1γ*, *VvGAPDH*, *VvTIP41*, *VvTUA*, and *VvTHIORYLS8*) were used as internal standards to normalize the starting template of cDNA because they are not involved in the plant response to infections and they are very stable. cDNA was first preamplified before being analyzed by qPCR with Fluidigm technology: it was diluted to 5 ng/μL and preamplification was carried out by adding the reaction mixture containing all the pairs of primers (primers pool, 50 mM) and the TaqMan PreAmp Master Mix (1:2, Applied Biosystems) with 14 cycles of 95 °C for 15 s and 60 °C for 4 min. The preamplified cDNA was diluted with TE buffer (1:5) and used for qPCR analysis in a reaction mixture containing TaqMan Gene Expression Master Mix (Applied Biosystems), DNA Binding Dye Sample Loading Reagent (Fluidigm, Issy-les-Moulineaux, France), and EvaGreen (Interchim, Montluçon, France).

Real-time qPCR was performed using a BioMark HD system (Fluidigm Corporation). The 96.96 dynamic array was used for qPCR, according to the manufacturer's protocol (http://www.fluidigm.com/user-documents). Five microliters of mixture was prepared for each sample containing 1× TaqMan Universal Master Mix (without UNG), 1× GE sample loading reagent (Fluidigm PN 85000746), and each diluted preamplified cDNA. The loaded chip was placed in the BioMark system for PCR at 95 °C for 10 min, followed by 40 cycles at 95 °C for 15 s and 60 °C for 1 min. The data were analyzed by using real-time PCR BioMark 2.0 analysis software (Fluidigm Corporation, France) as the cycle of quantification (C_q) and by applying the same principle of classical real-time PCR with the Stratagene MX3005P system where the fluorescence signal of the amplified DNA intersected with the background noise.

C_q values >30 were regarded as invalid and treated as missing data. Expression levels were calculated based on a multiple gene normalization method and using the principles and formulas of Vandesompele et al.[28] The geometric mean of several carefully selected reference genes (Table 1) was used as an accurate normalization factor. The lowest gene stability value (M values) indicates genes with the highest gene expression stability. In all the experiments carried out with Fluidigm, all M values of the five reference genes were collected to obtain a data set sufficient to assess their stability. After completion of the run, a melting curve of the amplified products was determined to confirm the specificity of the reactions.

Table 1. Mycelial Growth (mm) of Fungal Isolate CCB16 on Malt-Agar Medium Supplemented with Concentrations (mg/L) of Pyrimethanil or BTH 4 Days after Mycelial Plug Deposit[a]

concentration (mg/L)	*B. cinerea* growth with Pyrimethanil (mm)	*B. cinerea* growth with BTH (mm)
0	80.0 (±0.3)a	79.7 (±0.3)a
0.1	76.6 (±0.3)b	79.8 (±0.3)a
1	73.9 (±0.0)c	79.6 (±0.2)a
10	64.6 (±0.0)d	79.6 (±0.3)a
100	26.1 (±0.0)e	71.6 (±0.2)b
1000	0.0 (±0.0)f	59.6 (±0.3)c
5000	0.0 (±0.0)f	27.5 (±0.4)d

[a]Different letters in same column indicate significant differences according to LSD test ($P \leq 0.05$).

The relative expressions obtained for all the genes and modalities were finally transformed into Log2. Differential gene expression was subjected to statistical analyses by nonparametric multiple comparisons with the "nparcomp" package in the R software, and significant differences compared to untreated control were determined by Dunnet's test at the 5% significance level. Principal component analysis was performed to analyze plant defense after treatments with the RCMD package and the plug-in FactoMiner of R statistical software.

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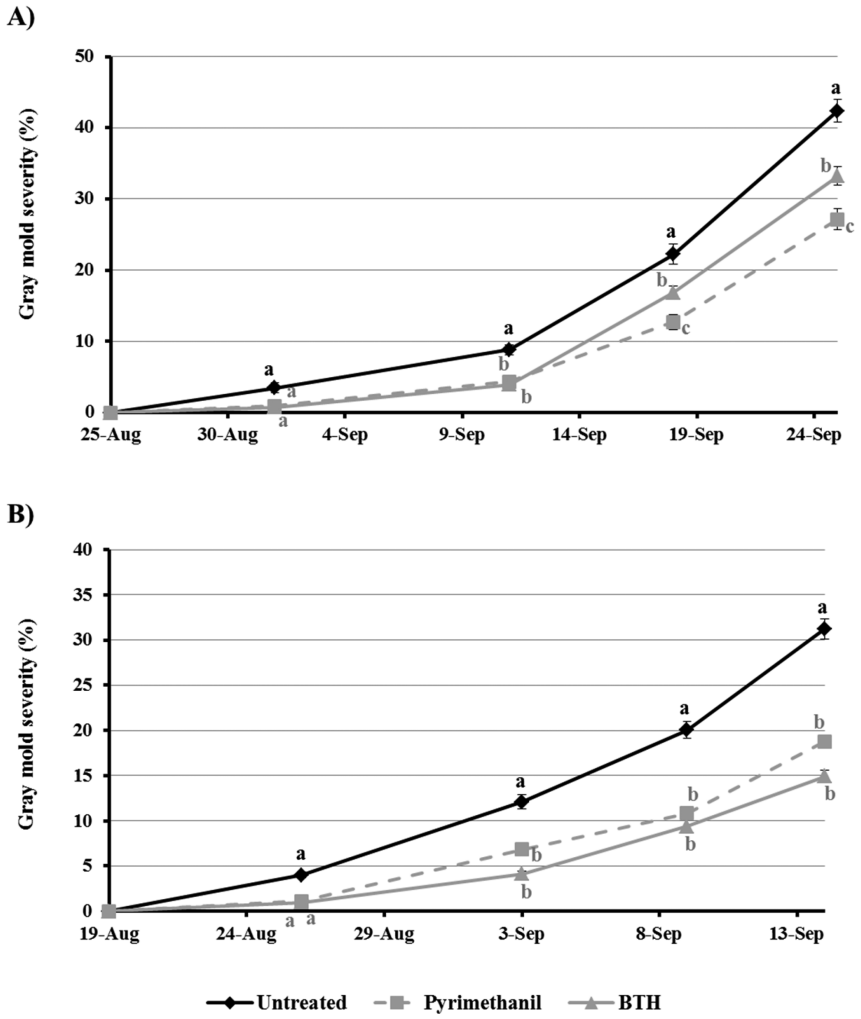

Figure 1. Protection of grapevine (cv. Sémillon) in response to Pyrimethanil and BTH treatments against *B. cinerea* in (A) 2014 and (B) 2015, expressed as disease severity. Control plants were untreated. Black line, untreated; gray line, BTH; gray dotted line, Pyrimethanil. For each disease point on epidemic curves, statistical differences ($P \leq 0.05$) between treatments are indicated using lowercase letters.

RESULTS

Antifungal Activity of Pyrimethanil and BTH on *B. cinerea in Vitro*. The direct effect of the compounds was evaluated *in vitro* after 4 days of growth. In control (without product), mycelial growth ranged from 79.7 to 80.0 mm (Table 1), with an average growth of 20 mm per day. With Pyrimethanil, the weakest concentration (0.1 mg/L) already led to a significant reduction of growth (4.25%). All concentrations of Pyrimethanil inhibited *B. cinerea* mycelium growth to reach 80% of inhibition at 100 mg/L. When the medium was supplemented with 1000 or 5000 mg/L of Pyrimethanil, the inhibition was total. With BTH, results were different. Indeed, at concentrations ranging from 0.1 to 10 mg/L, no significant effect was observed (79.6 ± 0.3 and 79.8 ± 0.3 mm). At 100 mg/L of BTH, a weak but significant reduction in growth (10.16%) was obtained. At higher concentrations (1000 and 5000 mg/L), the growth inhibition increased to reach only 65.49%. Compared to inhibition with Pyrimethanil, BTH had a poor direct fungicidal effect. Establishing the effective concentration inhibiting 50% of *B. cinerea* (EC_{50}) growth showed that Pyrimethanil (EC_{50} = 67.5 mg/L) was 51-times more efficient than BTH (EC_{50}= 3450 mg/L) (data not shown).

Effects of Pyrimethanil and BTH on Grapevine Protection in the Vineyard. Pyrimethanil and BTH were tested against *B. cinerea* in a Bordeaux vineyard in field conditions. Concentrations were 1000 g/ha for Pyrimethanil and 400 g/ha for BTH. Field trials were performed in 2014 and 2015 on the same plot. The severity (disease intensity) and incidence (disease frequency) of *B. cinerea* were quantified (Figures 1A,B and 2A–D). In 2014, at the beginning of disease scoring, a low natural infection was detected on August 25. After two rainy periods, the disease severity from 1 September ranged from 0.91 ± 0.25 to 3.46 ± 0.69% without a significant difference between control, BTH, and Pyrimethanil treatments (Figure 1A). After September 4, the severity increased gradually and strongly on the untreated vines and reached 42.38 ± 1.58% at harvest. Conversely, with the two treated modalities, the severity had significantly decreased at harvest on September 25, with a much lower severity on bunches treated with the fungicide (27.15 ± 1.46%) compared to those treated with BTH (33.22 ± 1.27%). The severity decrease was 35% for Pyrimethanil and 20% for BTH. The incidence of disease was already 39.18 ± 3.51% on September 1 and increased to reach 99.53 ± 0.47% at harvest on the untreated bunches. No significant reduction was found with the treatments at harvest, with an average incidence of 98.40 ± 0.92% (data not shown).

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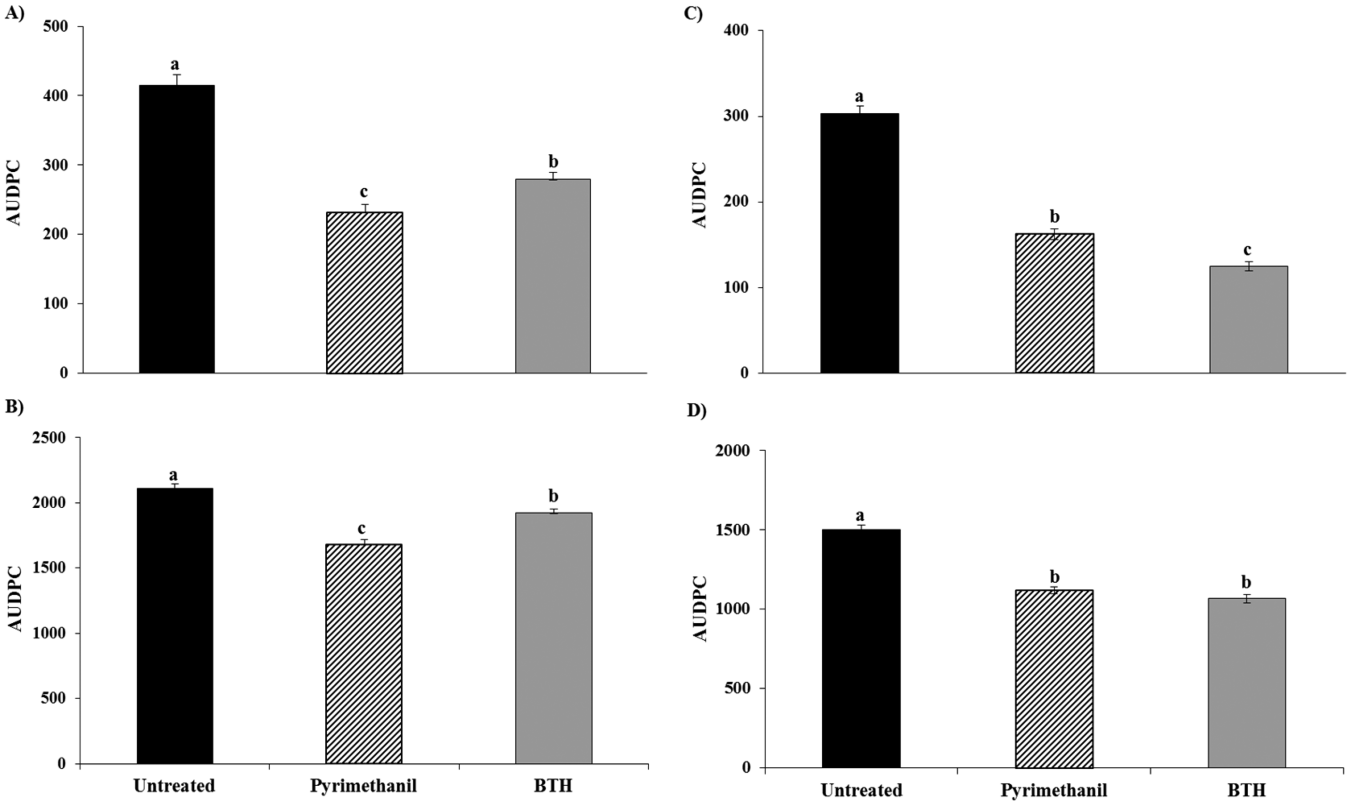

Figure 2. Effect of treatments (BTH and Pyrimethanil) on (A, C) severity and (B, D) incidence of gray mold during (A, B) 2014 and (B, C) 2015. Results are expressed as area under the disease progress curve (AUDPC). Statistical differences between treatments are indicated using lowercase letters ($P \leq 0.05$).

Table 2. Physicochemical Determination of Grapes at Harvest in 2014 and 2015[a]

	number of bunches per vine	average weight of bunch (g)	sugar concentration (g/L)	potential alcoholic strength (% vol)	pH	total acidity (g/L)
2014						
untreated	3.8a	143.4a	168.5a	10.3a	3.7a	4.7a
Pyrimethanil	4.5a	140.7a	170.0a	10.4a	3.8a	4.4a
BTH	4.5a	116.9b	174.4b	10.7b	4.0b	3.7b
2015						
untreated	11.2a	168.0a	179.6a	10.9a	3.5a	4.1a
Pyrimethanil	11.2a	194.5a	177.3a	11.0a	3.5a	3.8a
BTH	9.4a	146.7a	173.6a	10.7a	3.5a	4.2a

[a]Numbers of bunches per vine, average weight of bunch, sugar concentration of must, potential alcoholic strength, pH, and global acidity were calculated for each modality. Different letters in same column indicate significant differences according to LSD test ($P \leq 0.05$).

In 2015, gray mold symptoms began to appear on 26 August after a rainy period (Figure 1B). The progression of severity was similar to that observed in 2014, with a severity of 3.99 ± 0.32% on the untreated bunches at the beginning of the epidemic, and reaching 31.24 ± 1.12% at harvest. On plots treated with Pyrimethanil and BTH, severity was significantly lower on bunches 1.10 ± 0.17 and 0.99 ± 0.13%, respectively, and it was 14.90 ± 0.71% for BTH and 18.78 ± 0.69% for Pyrimethanil at harvest. No significant difference was found between the two treatments. The reduction in severity exceeded 40% compared to the untreated plots. In 2015, the severity of untreated bunches at harvest (31.24%) was lower than in 2014 (42.38%, Figure 1A). The incidence at harvest was 89.83 ± 1.39% on untreated bunches and was lower on treated bunches (from 74.31 to 76.92%). Compared to 2014, the effects of treatment on the incidence in 2015 were more efficient until harvest (data not shown).

AUDPC of severities and incidences were calculated for the two seasons (Figure 2A–D). Overall, the AUDPC were higher in 2014 than in 2015 according to the severity curves (Figure 1). In 2014 (Figure 2A) and 2015 (Figure 2C), all treatments led to a significant reduction in severity compared to the untreated control. Pyrimethanil was the most efficient in 2014 (severity decrease of 44%), while BTH exhibited better efficacy in 2015 (severity decrease of 59%). Concerning incidence, all treatments led to a significant reduction compared to the control (Figure 2B,D). In 2015, the two treatments led to a similar reduction of incidence (25 to 29%) that was greater than in 2014 (Figure 2D). These results showed the efficacy of the elicitor and the fungicide treatments.

Effect of Treatments on Physicochemical Composition of Grapes at Harvest. Grapes were harvested on September 25, 2014 and on September 14, 2015. Physicochemical determinations were performed on the control and

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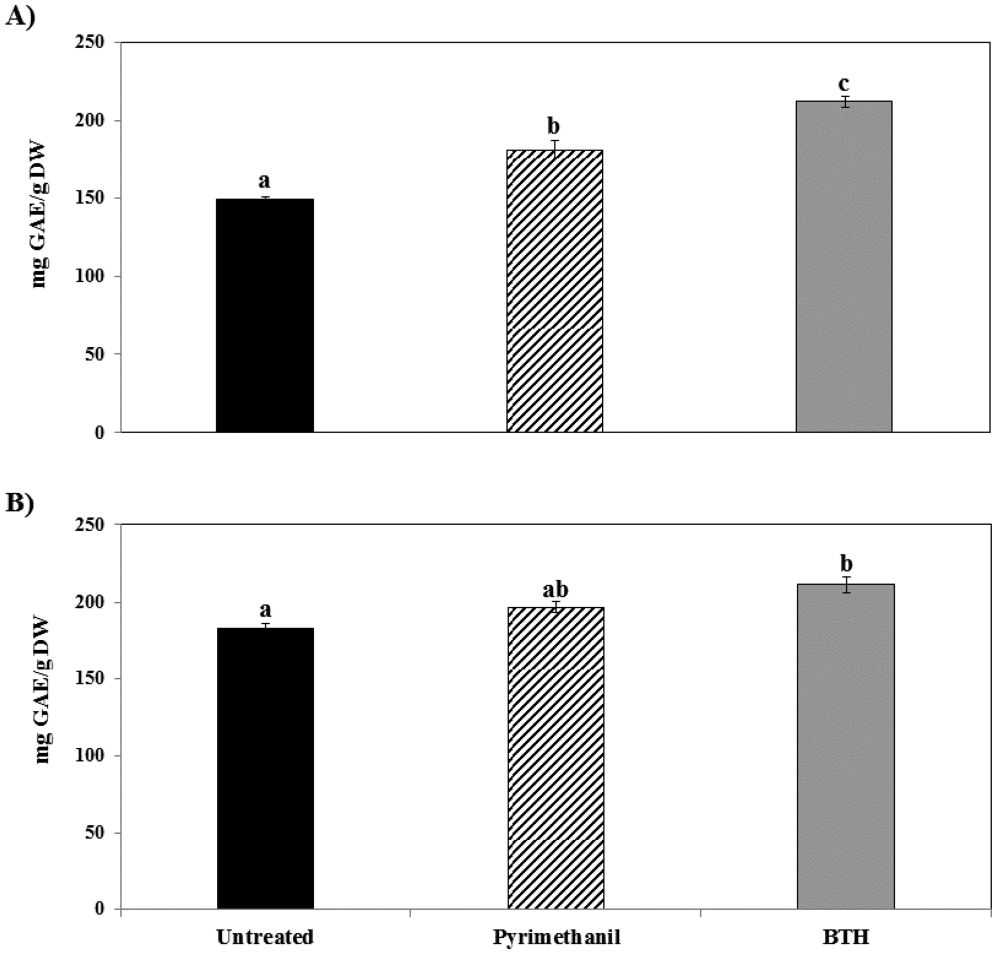

Figure 3. Total polyphenol (TP) content of untreated, Pyrimethanil- and BTH-treated leaves at (A) 2 and (B) 7 dpt in 2014. TP content is expressed in mg of gallic acid equivalent (GAE, g^{-1} DW). Statistical differences between treatment modalities are indicated using lowercase letters ($P \leq 0.05$).

treated grapes at harvest (sugar concentration, potential alcoholic strength, pH, total acidity) (Table 2). Regarding the effect of the treatments, no effect was observed on the number of clusters per vine (3.8 to 4.5 in 2014 and 9.4 to 11.2 in 2015) and only the vintage had an effect on the number of clusters. The average cluster weight, sugar concentration, percent proof, pH, and total acidity of the must were significantly modified by BTH treatments in 2014 compared to the other conditions. Indeed, after BTH applications, the cluster weight was reduced (−18.5%), but this was counterbalanced by an increase in sugar concentration (+3.5%), alcoholic strength (+3.9%), and total acidity (+21.3%). In 2015, no significant differences were observed even though the cluster weight was the lowest. Concerning treatments with Pyrimethanil in the two vintages, no changes were noted in the five parameters measured.

Total Polyphenol Quantification. The effects of Pyrimethanil and BTH on total polyphenol (TP) content in leaves, 2 and 7 days after the second treatment in 2014, were assessed by spectrophotometric analysis (Figure 3). TP content was expressed as mg of gallic acid equivalent per g of dry weight (GAE/g DW). The two treatments increased TP content significantly in leaves at 2 dpt compared to untreated leaves. Thus, BTH induced an increase of 42% (212.28 mg GAE/g DW) compared to untreated leaves (149.34 mg GAE/g DW), while Pyrimethanil induced an increase of 22% (181.60 mg GAE/g DW) (Figure 3A). The increase triggered by BTH was significantly higher than that induced by Pyrimethanil. At 7 dpt (Figure 3B), foliar TP contents were almost identical in untreated leaves (183.28 mg GAE/g DW) and in Pyrimethanil-treated leaves (196.74 mg GAE/g DW). In leaves treated with BTH, TP content was significantly different from that obtained with untreated leaves (211.25 mg GAE/g DW), with an increase of 15%.

Quercetin-3-O-glucuronide Quantification. Analyses by a reverse phase HPLC system coupled with an MS allowed us to monitor the main phenolic compounds present in the treated leaves and to identify them. According to HPLC chromatograms, a major peak was noted. On the basis of its UV−vis absorption maxima, mass spectra obtained in negative mode, fragmentation patterns, and comparison with literature, we identified this molecule as quercetin 3-O-glucuronide (Q3OG). Indeed, this peak, with a retention time of 2.6 min, exhibited UV maxima at 372 nm characteristic absorbance for flavonols and a precursor ion at m/z 477 $[M + H]^-$ and an MS/MS spectrum with product ion at m/z 301. The loss of 176 Da corresponds to the elimination of a glucuronic acid. These fragmentation data were in accordance to those previously reported in the literature for this compound.[29,30] The HPLC−MS analysis of the foliar polyphenol content allowed us to detect slight stilbene content (only *cis*-piceid at trace level) and no major difference was detected 2 and 7 days after treatments. However, one compound quercetin-3-O-glucuronide (Q3OG)

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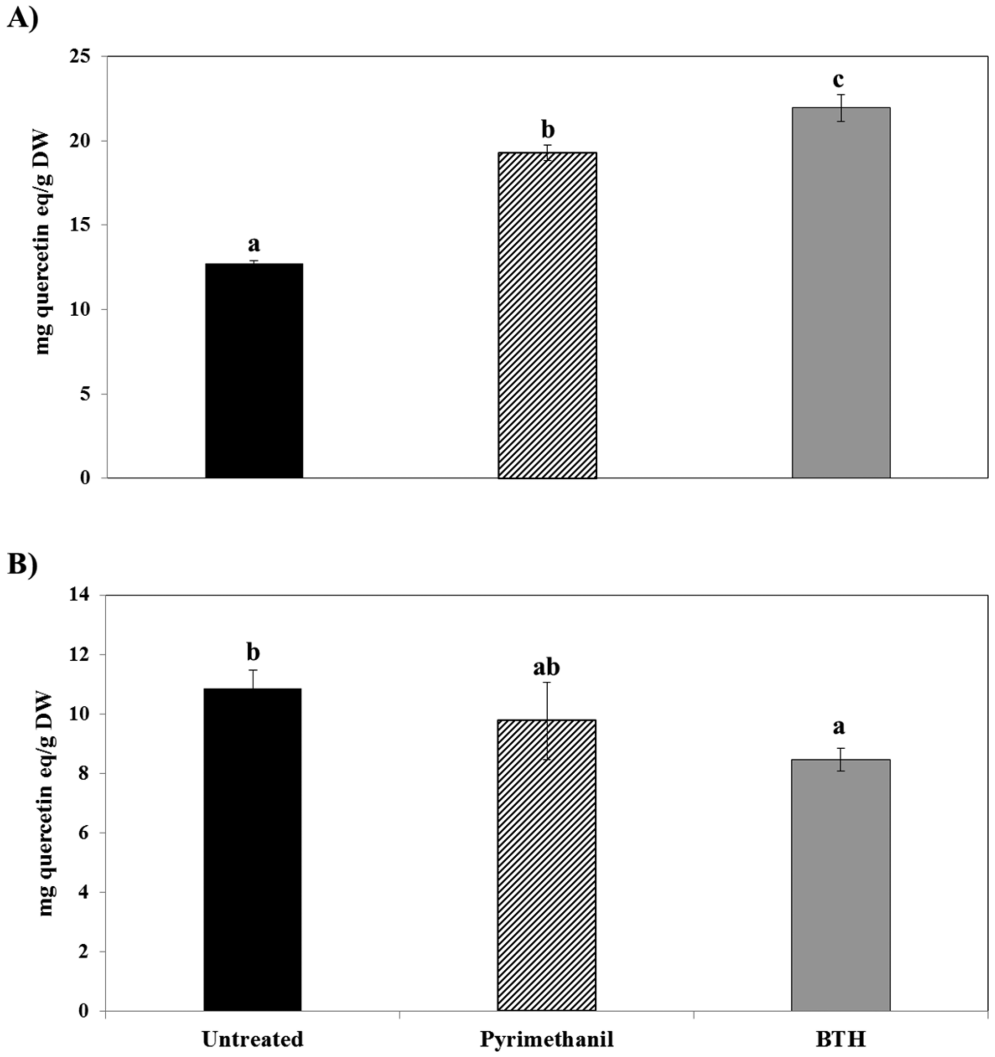

Figure 4. Total quercetin 3-O-glucuronide (Q3OG) content of untreated, Pyrimethanil-, and BTH-treated leaves at (A) 2 and (B) 7 dpt in 2014 based on HPLC−MS analysis. Q3OG content is expressed as mg of quercetin equivalent per g of dry weight (QE/g DW). Statistical differences between treatment modalities are indicated using lowercase letters ($P \leq 0.05$).

flavonoid (Figure 4A,B) was very present, and its content varied according to the treatment. Q3OG content was expressed as mg of quercetin equivalent per g of dry weight (QE/g DW). At 2 dpt, the two treatments increased the Q3OG content significantly in leaves compared to untreated leaves. Thus, BTH induced an increase of 72% (21.95 mg QE/g DW) compared to untreated leaves (12.71 mg QE/g DW), while Pyrimethanil induced an increase of 51% (19.27 mg QE/g DW) (Figure 4A). The increase triggered by BTH was significantly higher than that induced by Pyrimethanil. At 7 dpt (Figure 4B), the contents of Q3OG were similar in untreated leaves (34.32 mg QE/g DW) and in Pyrimethanil-treated leaves (28.71 mg QE/g DW). In leaves treated with BTH, Q3OG content was significantly different from that obtained with untreated leaves (27.77 mg QE/g DW), with a decrease of 20%.

Effect of Treatments on Foliar Gene Expression in the Vineyard. In the same samples as those used to quantify TP contents in 2014, transcripts (Table 1) were measured by high-throughput qPCR 2 and 7 days after the second treatment and compared to transcripts of untreated leaves. The expressions of 48 genes involved in plant defense were modulated (up- or down-regulated) from 27.66% to 56.25%, depending on the treatment compared to untreated leaves (Figure 5). At 2 dpt, a few genes were commonly up-regulated or down-regulated in Pyrimethanil- and BTH-treated leaves: *VvPR3* (Chitinase, PR-protein), *VvGST1* (redox status), *VvCALS* (callose synthase), and *VvICS* (SA pathway) were commonly up-regulated. Conversely, *VvPR10* (Ribonuclease), *VvPAL* and *VvSTS* (phenylpropanoid pathway), *VvAPOX* (cell wall reinforcement), and *VvEIN3* (ethylene pathway) were commonly down-regulated. In BTH-treated leaves, some PR protein genes coding for chitinases (*VvPR4*, *VvPR8*, and *VvPR11*) or glucanase (*VvPR2*) were specifically up-regulated. As mentioned above, stilbenoid genes were down-regulated with the two treatments. In contrast, with regard to flavonoid and isoprenoid biosynthesis, some genes (*VvLDOX*, *VvF3H*, and *VvHMGR*) were up-regulated only with Pyrimethanil. Some glutathione-S-transferase genes (*VvGST4* and *VVGST5*) were down- or up-regulated in BTH- and Pyrimethanil-treated leaves. BTH had no effect on oxylipin genes, while Pyrimethanil induced down-regulation of two of them (*VvLOX9* and *VvLOX3*). Concerning phytohormone pathways, some up- or down-regulation was noticed in BTH- and Pyrimethanil-treated leaves, especially a strong up-regulation of the SA (*VvSAMT*,

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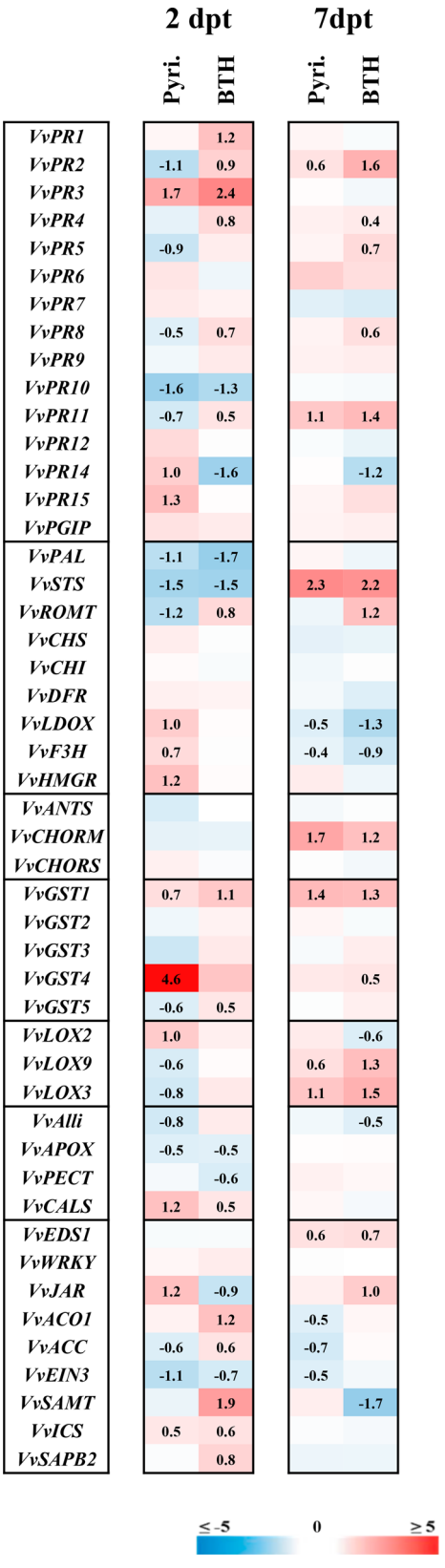

Figure 5. Pattern of relative expression of defense genes in grapevine leaves 2 and 7 days after second treatment (dpt) with BTH and Pyrimethanil in vineyard in 2014. Expression data are given after log2 transformation. Gene expression of untreated leaves was used as reference to calculate the relative expression. Each column represents the time point after treatment (BTH or Pyrimethanil), 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 expression level higher than 5 in bright red, while those down-regulated appear in shades of blue, with intensity lower than −5 in dark blue. Numbers in boxes represent the significant changes in gene expression ($P \leq 0.05$) compared to untreated control.

VvICS, and *VvSAPB2*) and the ethylene (*VvACO1* and *VvACC*) pathway following BTH treatment and a down-regulation of the ethylene pathway (*VvACC* and *VvEIN3*) and an up-regulation of the JA pathway (*VvJAR*) following Pyrimethanil treatment.

Seven days after treatment, the expression pattern of BTH- and Pyrimethanil-treated leaves was more consistent with many comparable response patterns. However, as at 2 dpt, some PR protein genes coding for chitinases (*VvPR4* and *VvPR8*) were up-regulated only with BTH. The response was the opposite in relation to 2 dpt, with an up-regulation of a stilbenoid biosynthesis gene (*VvSTS*) and a down-regulation of flavonoid biosynthesis genes (*VvLDOX* and *VvF3H*), even with BTH. Concerning oxylipin genes, an up-regulation of *VvLOX9* and *VvLOX3* was observed after Pyrimethanil and BTH treatments. Concerning phytohormone pathways, as at 2 dpt, a global down-regulation of ethylene (*VvACO1*, *VvACC*, and *VvEIN3*) was observed following Pyrimethanil treatment. In contrast, and unlike at 2 dpt, no up-regulation of the SA pathway was observed with BTH but the JA pathway was up-regulated.

Combined Analysis of Plant Responses and Field Protection. Principal component analysis (PCA) was used to summarize the gene responses after elicitation and fungicide treatments (Figure 6A,B). The two principal components explained 76.12% of the total data variability at 2 dpt and 61.77% at 7 dpt. At 2 dpt (Figure 6A), explanatory quantitative variables (TP content and severity AUDPC) were projected on the second axis (28.16% of the variability). Confidence ellipses around the treatments revealed three significant clusters corresponding to each modality, with the two treatments separated on axis 1 (47.96% of the variability). The explanatory variable severity (AUDPC) as expected was projected in the direction of the untreated leaf responses and two gene expressions were correlated (*VvPR10* and *VvEIN3*). Although TP content projected to the BTH ellipse and was linked with *VvPR3*, *VvGST1*, *VvWRKY*, *VvICS*, and *VvACO1* gene expression, TP content was not correlated with *VvPAL* or *VvPECT* gene expression.

At 7 dpt (Figure 6B), the AUDPC variable was correlated with one flavonoid gene (*VvLDOX*) and the *VvPAL* gene, but also with *VvSAMT* (SA methyltransferase) and *VvPR14* (a lipid transfer protein). At 2dpt, TP content was correlated with BTH treatment and with numerous PR-protein genes (*VvPR2*, *VvPR4*, *VvPR5*, *VvPR8*, and *VvPR15*), stilbene synthase (*VvSTS*), and the resveratrol-*O*-methyl transferase gene (*VvROMT*).

Clearly the effects of Pyrimethanil were different at 2 and 7 dpt, especially with a modulation of genes involved in flavonoid and isoprenoid biosynthesis (*VvHMGR*, *VvF3H*, and *VvLDOX*) at the first sampling, and less specific modulations of genes at 7dpt, with only a transcription factor (*VvWRKY*) and a gene involved in the ethylene pathway (*VvACC*) modulated.

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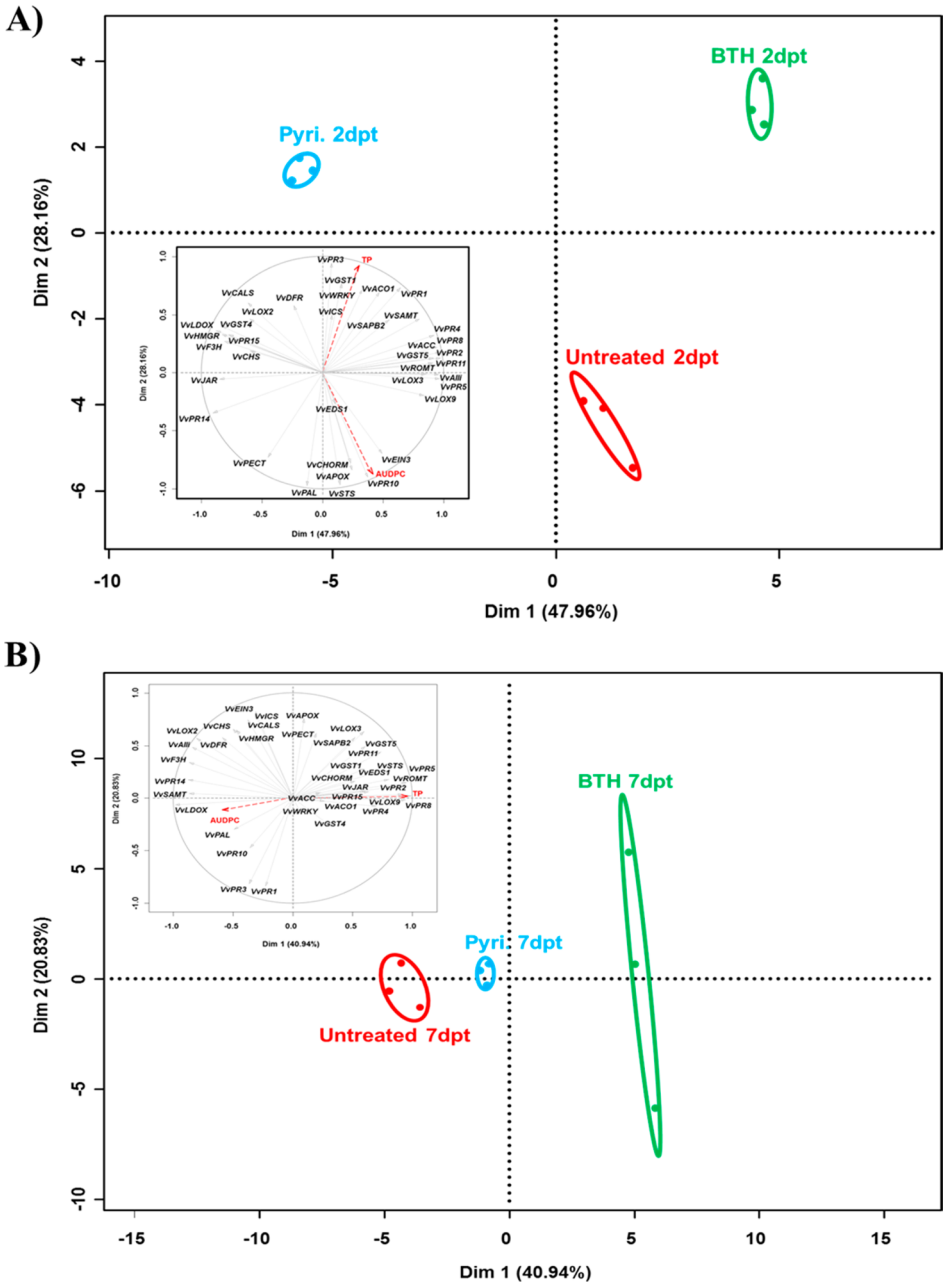

Figure 6. Principal component analysis and cluster of genes differentially expressed, disease severity (AUDPC) and total polyphenol (TP) obtained (A) 2 days and (B) 7 days after treatment or not with BTH and Pyrimethanil. Projections on standard unit circle of quantitative variables (gene) and quantitative illustrative variables (AUDPC and TP) are represented. Distribution of plant responses and severity data on principal planes defined by two axes of gene expression profiles obtained by PCA using all treatment modality data. Ellipses represent the 95% confidence intervals calculated for each modality. The different groups are indicated by different colors (red, untreated; green, BTH; blue, Pyrimethanil).

DISCUSSION

As alternatives to synthetic chemical pesticides in the vineyard, elicitors have been investigated by many authors,[9] but pest management assessment in the field has often proved disappointing with these molecules.[8] In the vineyard, many studies have assessed elicitors against powdery or downy mildew,[9] but few have been tested against *Botrytis*, the causal agent of gray mold.[24] In our study, we investigated the ability of BTH to provide a satisfactory level of protection against *B. cinerea* in a context of strong epidemic pressure. We also analyzed plant responses with the expression of defense genes and TP content to identify markers for elicitor or even Pyrimethanil treatment.

Various studies have demonstrated the ability of BTH to enhance plant defenses[23,31] including gray mold management on crops like grapevine and tomato.[10,24,32] In addition, a direct fungicide effect was described against *Botrytis in vitro*, but the EC_{50} was high.[33] A similar control experiment performed here with an isolate sampled and tested in the vineyard led to an EC_{50} in agreement with the concentration of 3.45 g/L described in the above-mentioned study. In comparison, Pyrimethanil with an EC_{50} of 67.50 mg/L was 50-times more effective than BTH. This EC_{50} found for Pyrimethanil is close to that obtained by Kim et al. of 50 mg/L for a *B. cinerea* isolate sampled on strawberry.[34] This suggests that the isolate present in our plot had no particular resistance against this fungicide.

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This first experiment clearly demonstrated that the direct fungicidal effect of BTH was minimal compared to the proven fungicidal effect of Pyrimethanil. This conclusion is also reinforced by the minimal fungicidal effects of BTH found by others on grapevine downy and powdery mildew.[35]

BTH has been found to act as a plant defense stimulator on many plants against several pathogen agents.[10,23,24,31,36] A finding corroborated by our experiments in the vineyard. Indeed, only three BTH applications were required to trigger significant protection against *B. cinerea*, with efficiency close to that of Pyrimethanil. On average over the two years of experiments, the reduction in severity of *B. cinerea* obtained with BTH and Pyrimethanil was similar, that is, 45% compared to untreated plots. Although fungicide efficacy was very stable during the two years (44% in 2014 and 46% in 2015), its efficacy varied with BTH from 32% in 2014 to 58% in 2015. This could be due to the complexity of plant−pathogen−environment interactions.[8] The reduction in severity that we obtained with BTH treatment was similar to the 36% reduction reported by Iriti et al.[24] Nevertheless, their BTH concentration was weaker, and the number of treatments was higher.

Enhanced plant defenses may induce protection against fungal pathogens but may also affect plant and berry development.[37] Previous studies with BTH showed that it can delay fruit-ripening[31,38] and even lead to changes in aromatic profiles of wine,[39] depending on the concentration and number of treatments. Here, three treatments were performed to manage *B. cinerea*, but no effect was observed in fruit ripening after BTH treatment. However, the physiochemical composition of grapes was altered with a decrease in cluster weight and an increase in sugar concentration depending on the year. This finding is consistent with previous studies and suggests that according to the nature of the inducer, dose applied, species and variety of plant, pathosystem and crop conditions, BTH at high doses could have a slight effect on fitness cost. However, since BTH acts as an analogue of salicylic acid, hormonal disturbances on the auxin and ethylene pathways may also be involved in the observed effects, in relation to the phenological stages of the plant (flowering) during treatments. Concerning the increase in sugar content, BTH treatment is known to increase fructose levels.[25] These changes in grape composition were rather vintage-dependent and were not correlated with the efficiency of the elicitor.

Elicitors are molecules or microorganisms capable of inducing innate plant immunity by activating plant defenses by gene modulation and antimicrobial compound production. A known inducible resistance mechanism against *B. cinerea* in grapevine is the synthesis of PR-proteins or antimicrobial phytoalexins like resveratrol.[13] Investigations *in vitro* on plant defense gene expression after induction by elicitors with protection against *B. cinerea* showed up-regulation of *VvPR2* (glucanase), *VvPR3* (Chitinase), *VvPAL*, and *VvSTS* (resveratrol biosynthesis) genes until 72 h after elicitor application.[40−43] These gene overexpressions were corroborated by Chitinase and glucanase activities in the fruit.[44,45] Specific use of BTH on grapevine showed that the increase in resveratrol and anthocyanin levels plays a role in resistance against *B. cinerea*.[24]

As expected, BTH treatment at 2 and 7 dpt up-regulated many PR-protein genes, in particular those coding for glucanase and chitinases (*VvPR2*, *VvPR3*, *VvPR4*, *VvPR8*, and *VvPR11*), as reported in previous studies on various plants.[10,23,25,45,46] Up-regulation of *VvROMT*, which is involved in the methylation of resveratrol in pterostilbene at 2 and 7 dpt, and the up-regulation of *VvSTS* at 7 dpt are in accordance with the increase in pterostilbene and resveratrol found in leaves treated with BTH[23] and could explain the increase in TP observed at 2 and 7 dpt after BTH treatment. However, at 2 days, the *VvPAL* and *VvSTS* genes were significantly repressed, and those of the flavonoid biosynthesis pathway were rather overexpressed (*VvCHS* and *VvF3H*), especially in leaves treated with Pyrimethanil. Increases in TP and Q3OG contents were noted in leaves treated with the two products. This point is consistent with current knowledge of the polyphenol biosynthesis pathway, with the stilbene synthase and the chalcone synthase sharing the same substrates. It is also in agreement with the fact that, under certain conditions, the transcriptional response of *VvSTS* and *VvCHS* genes appears to be diametrically opposed, suggesting a tight regulation at the transcriptional level.[47] Therefore, we suggest that the flavonoid pathway may be activated 2 days after the induction of plants leading to an increase in total polyphenol content in leaves treated with Pyrimethanil and BTH, and an increase in Q3OG and the absence of stilbene accumulation. The fact that the *VvROMT* gene was overexpressed in the leaves treated with BTH, but without any pterostilbene accumulation, could be explained by a biphasic and alternating stimulation of *VvSTS* and *VvCHS* genes, as reported by Borie et al. and Faurie et al.[48,49] The results of another experiment that we performed in 2015 corroborate and complement this observation. In that experiment, we noted a rapid activation of *VvSTS* only 24 h after treatment and the suppression of the flavonoid pathway, before reversal of the process (data not shown). On the other hand, at 7 dpt, the stilbene pathway was again be stimulated with overexpression of *VvSTS* and *VvROMT* genes and repression of the flavonoid pathway genes. This resulted in a significant decrease in Q3OG levels in leaves treated at 7 dpt. However, given the high content of Q3OG in the leaves that we studied, we believe that the content varies according to the environmental conditions in which leaves grow. Indeed, several authors such as Latouche and collaborators have noted that leaves from grapevine grown in the vineyard, like our leaves, display high flavonol content.[50] Flavonols are naturally synthesized compounds that accumulate in leaves exposed to full sunlight, as in the vineyard. They play a primary role of protection against UV radiations, and their accumulation is dependent on UV light regime.[51] Besides, flavonol induction has also been reported following downy mildew infection.[52,53]

Monitoring of other genes of the chip is innovative and is known to date about their involvement in plant defenses after elicitor application. The up-regulation of the oxylipin pathway at 7 dpt (*VvLOX*) and glutathione-S-transferase genes (*VvGST*) that we obtained was greater than that found by Dufour et al. and Harel et al., and stronger than that obtained in the study by Sapple et al. in which the *GST* gene was induced by SA.[10,23,54] In addition, induction of the signaling gene was particularly strong at 2 dpt with up-regulation of SA and ethylene biosynthesis and regulation (*VvACO1*, *VvACC*, *VvSAMT*, *VvICS*, and *VvSAPB2*). This attests to the interconnection between SA and ethylene signaling and is in accordance with the global up-regulation of phytohormone signaling gene expression demonstrated after BTH treatment.[23,55]

To our knowledge, no study to date has investigated Pyrimethanil elicitation on grapevine. Its fungicide mode of action is known to inhibit methionine biosynthesis and laccase activity of pathogens.[3,4] Its ability to stimulate plant defenses

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has now been demonstrated. Surprisingly, in addition to its direct fungicidal action, Pyrimethanil acted as an elicitor, leading to an increase in TP and Q3OG contents in leaves 2 days after treatment and to the modulation of many defense genes. This finding requires further investigation. Indeed, it is difficult to link these results to a direct effect of Pyrimethanil on the plant or to an indirect effect due to the release of PAMPs as a result of its fungicidal action.[56]

Global PCA analyses showed that TP content and AUDPC were negatively correlated, suggesting that this increase in secondary metabolite content could be a strong marker of protection against *B. cinerea* related to elicitor applications.[57] The projections of BTH and Pyrimethanil were different between 2 and 7 dpt, suggesting that the gene modulation they induced were different in time and depending on the treatment. At 2 dpt, some genes were specifically projected in the direction of BTH or Pyrimethanil and were linked to the specific mode of action of each product. However, similar genes were commonly regulated at 2 dpt such as PR-Protein genes (*VvPR3* and *VvPR10*), stilbenoid genes (*VvPAL*, *VvSTS*, *VvAPOX*, *VvGST1*), and a gene involved in callose synthase (*VvCALS*). At 7 dpt, the common modulation was on different PR-Protein genes (*VvPR2* and *VvPR11*), on the genes involved in flavonoid biosynthesis (*VvLDOX*, *VvF3H*) and on oxylipin genes (*VvLOX9* and *VvLOX3*). The common regulation suggested that the two compounds had a partially similar effect on plant defense. Some of the genes in the opposite direction of the severity variable could be markers of interest for protection against *B. cinerea*, especially some involved in SA and ethylene metabolism and some PR protein genes.

Taken together, the present findings suggest that BTH could be a valuable elicitor to manage *B. cinerea* in the vineyard. Two consecutive years of field assays showed stable efficacy equivalent to that of Pyrimethanil, an approved fungicide. A detailed study of the outcome of the clusters after harvesting would be interesting, and especially microwinemaking, which would provide information on the effect of BTH on the winemaking process and wine quality. Plant response analysis after BTH treatment provided many insights into its action as an elicitor with increases in TP and Q3OG contents and the regulation of many genes involved in plant defenses. Unexpectedly, our results seem to demonstrate that Pyrimethanil could induce plant defenses in addition to its fungicide action. This hypothesis deserves further investigation to better understand its mode of action. Usually, plant responses are studied in controlled conditions after elicitor treatment. Here, data from vineyard trials showed that the Neovigen chip is useful in the vineyard to assess the defense status of plants and to supply specific markers of protection. Understanding the mechanism of action and having markers of grapevine resistance status will be a prerequisite for using elicitors in integrated pest management. Future optimizations of BTH use in association or alternation with fungicides are still necessary, but the tools reported here would allow their application for safer agricultural practices including the use of fungicides with double mode of action as Pyrimethanil.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jafc.7b05725.

Selected genes and corresponding primer sets used (PDF)

■ AUTHOR INFORMATION

Corresponding Authors

*E-mail: marie-france.corio-costet@inra.fr. Phone: +33(0) 557122625.

*E-mail: anthony.bellee@inra.fr. Phone: +33(0)557122624.

ORCID

Jean-Michel Mérillon: 0000-0002-9626-2149

Marie-France Corio-Costet: 0000-0003-2206-9482

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Notes

The authors declare no competing financial interest.

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