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Grapevine Stimulation: A Multidisciplinary Approach to Investigate the Effects of Biostimulants and a Plant Defense Stimulator

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ABSTRACT: The increasing use of plant defense stimulators (PDS) and biostimulants (BS) to make agriculture more sustainable has led to questions about their action on plants. A new PhysBioGen approach is proposed with complementary tools: PHYSiological (root weight); BIOchemical and BIOlogical (secondary metabolite quantification and *Plasmopara viticola* development) and expressions of 161 GENes involved in metabolic plant functions. The proposed approach investigated the effects of three phytostimulants on *Vitis vinifera*: one PDS (ASM) and one BS chelated (CH) and another enriched with seaweed (SW). Distinct responses were obtained between the PDS and the two BS. In particular, we observed the persistence of anti-mildew efficacy over time, correlated with differentiated expressions of defense genes (*VvROMT*, *VvSAMT*, *VvPR8*). As expected, the two BS displayed more similarities to each other than to the PDS (flavonols, anthocyanins, free salicylic acid). However, the two BS revealed differences in the modulation of genes involved in defense and primary metabolism and some genes were identified as potential markers of their action (*VvWRKY1*, *VvLOX9*, *VvPOD*, *VvPDV1*, *VvXIP1*, *VVDnaJ*). Our results highlight the common and the specific effects of the two BS and the PDS. These new tools could help in understanding the mode of action of phytostimulants in order to achieve better quality and production yield and/or as a way to limit chemical inputs in the vineyard.

KEYWORDS: *acibenzolar-S-methyl, biostimulant, benzothiadiazole, gene expression, Plasmopara viticola, polyphenols, salicylic acid, Vitis vinifera*

INTRODUCTION

Agricultural inputs in viticulture lead to problems in ecotoxicology and human health. To reduce chemical inputs and ensure sustainability, complementary or alternative methods are in constant development, including plant defense stimulators (PDSs). The same applies to conventional fertilizers whose inputs are being reduced in favor of the development of biostimulants (BSs). These two types of products (PDSs and BSs) that stimulate biological and biochemical plant processes could be defined as "phytostimulants". Various subcategories have been described in the literature including defense stimulator products, elicitors,[1] biostimulants, and fertilizers.[2] However, PDSs and BSs include all products or extracts capable of inducing a plant's metabolic response, leading to better protection against biotic and abiotic stresses or a better growth. Their properties are clearly defined: PDSs stimulate the secondary metabolism and induce defense or protection mechanisms against biotic stresses, while BSs stimulate primary functions and plant growth and induce tolerance against abiotic stresses.[1−3]

PDS compounds are defined by their mode of action rather than by their biological or synthetic composition. They are living and nonpathogenic substances or microorganisms able to induce higher resistance in plants under biotic stress. They elicit specific responses from the innate immunity of plants, and their effects are well-described in the literature.[4] Certain PDSs mimic the attack of a bioaggressor and are perceived by extracellular receptors of plants called pattern recognition receptors (PRRs), through the signature of pathogen-associated molecular patterns (PAMPs). Many PDS compounds have been described and used in viticulture over the years to fight a wide spectrum of bioaggressors:[1] chitosan against *Botrytis cinerea*,[5] the *Trichoderma harzianum* T39 or sulfated laminarin extract against *Plasmopara viticola*,[6,7] and the association of chito-oligosaccharides with oligogalacturonides (COS-OGA) against *Erysiphe necator*.[8] PDSs can also be phytohormones that play a central action in defense pathways such as salicylic acid (SA), jasmonic acid, or ethylene, which trigger defense responses against downy and/or powdery mildew of *Vitis vinifera*.[4,6,9,12] The recognition of elicitors in grapevine induces signaling pathways that lead to the establishment of systemic-acquired resistance (SAR) and defense responses.[12,13]

Another way to stimulate the metabolic pathways of plants is using BSs. In the literature, they are defined as products or extracts that improve the quality of yield or growth, the bioavailability of nutrients, and the tolerance to abiotic stresses, independent of nutrient supply.[2,3] Like PDSs, BS compounds are defined by their mode of action rather than their composition. Many terms are used to define products with BS properties: "fertilizers", "nutritional agent", "agronomic

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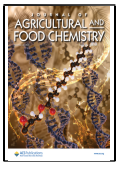

additive", or even "phytostimulant". The term "biostimulant" has also recently been used to describe PDS.[14]

The use of phytostimulants is thought to offer a global solution for plants subjected to abiotic and biotic stresses. This is particularly the case for grapevine, which, as a perennial plant, is in constant interaction with its environment. Since it is treated rather heavily with pesticides, the physiology of grapevine may be affected (*e.g.*, negative impact on photosynthesis).[15] Global warming brings increasing temperature variations with late frosts or very dry summers, making the grapevine an ideal candidate for more sustainable protection and production thanks to phytostimulants. However, certain issues have to be addressed, such as the potential physiological cost of the impact of PDSs[16] and the indirect consequences of BSs on the innate immunity of plants.[17] The trade-off between growth and protection has been described in several studies[18] and suggests various indirect consequences of BS and PDS treatments.

Our main objective was to investigate and compare the effects of various phytostimulant treatments on *V. vinifera* cuttings, with one plant defense stimulator (acibenzolar-*S*-methyl (ASM) as the elicitor) and two commercial BSs, one containing seaweed (SW) extract and the other containing chelated (CH) iron. To achieve this, a multidisciplinary approach, "PhysBioGen", was developed to better understand their modes of action and characterize them through physiological (root biomass), biological (*P. viticola* inhibition), and biochemical (chlorophyll, flavonols, anthocyanins, salicylic acid quantifications) measurements, complemented by the monitoring of gene expression (161 genes) involved in responses to biotic or abiotic stresses.

The results provide new insights into the characteristics of these two categories of phytostimulants (BSs and PDSs) to better discriminate them and into the identification of potential markers of the effects of BSs.

MATERIALS AND METHODS

Plant Material. Grapevine cultivars (*V. vinifera* cv. Cabernet Sauvignon) were propagated in a greenhouse from wood cuttings. After three weeks, rooted cuttings were planted in sandy soil and were grown under controlled conditions (15/9 h light/dark photoperiod). Two-month-old plants with 8−10 leaves were used for the experiment in individual pots (11 × 11 × 11 cm). During the entire experiment, the cuttings did not receive any fertilization.

Fungal Material. *P. viticola* isolate (ORG) was collected on *V. vinifera* in a commercial vineyard (Les Lèves, France) in 2014. As previously described,[19] the isolate was multiplied by depositing droplets (15 μL) of a spore suspension (10 000 sporangia/mL) onto the abaxial face of leaves and incubated for 7 days, at 22 °C with a 16/8 h light/dark photoperiod and relative humidity (RH) > 90% in a growth chamber. The isolate was subcultured weekly on fresh grapevine leaves (*V. vinifera* cv. Cabernet Sauvignon).

Chemicals. Formulated acibenzolar-*S*-methyl ((*S*)-methyl benzo-[1,2,3]thiadiazole-7-carbothioate, ASM, (Bion 50WG, Syngenta)) or benzothiadiazole (BTH) was used as the elicitor. Two commercial biostimulant products were used. The first BS, named CH, contained various oligoelements [N (100 g/L), MgO (30 g/L), B (10 g/L), Fe−ethylenediaminetetraacetic acid (EDTA) (35 g/L)]. The second, named SW, contained more varied oligoelements [N (90 g/L), P_2O_5 (55 g/L), K_2O (55 g/L), MgO−EDTA (3 g/L), B (0.5 g/L), Fe−EDTA (0.3 g/L), Cu−EDTA (0.14 g/L), Mn−EDTA (0.5 g/L), Mo (0.05 g/L), Zn−EDTA (0.4 g/L)] enriched with 0.5% of brown algae *Ascophyllum nodosum* extract.

Bioassays and Treatment. Four independent grapevine cutting blocks of 24 plants were established. The first untreated block was the control block, a second block received an ASM treatment at 1 g/L of a.i., and the third and fourth blocks were treated with the CH (10 g/L) or SW biostimulant (50 g/L). Two measures were conducted on eight plants for each block, 2, 7, and 14 days post treatment (dpt). With a Dualex clip, pigment measurements were performed on the entire plant and leaves from the upper part of the shoots (third leaf below the apex) were collected for downy mildew bioassays, salicylic acid quantification, and gene expression. Finally, the cuttings were sacrificed to assess the root biomass.

Nondestructive Measurements of Chlorophyll, Flavonols, and Anthocyanins. Chlorophyll, flavonols, and anthocyanins were indirectly quantified with Dualex 4 Scientific Leaf-Clip (FORCE-A, Orsay, France). The Dualex system instantly assessed the pigments in situ using this nondestructive portable leaf clip on eight plants, for each modality and for each sampling time. Near-infrared chlorophyll fluorescence was measured thanks to a first excitation reference not absorbed by polyphenols emitting at 650 nm (red). It was compared to a second specific probe excitation of a type of polyphenols (*e.g.*, green for anthocyanins (520 nm) or UV-A for flavonols (375 nm)). Then, the fluorescent infrared light emitted in response by the leaf pigments was recorded and the epidermal flavonol and anthocyanin contents were estimated by the following equations

$$\text{flavonols Dualex index} = \frac{\text{fluorescence}_{\text{excited by red}}}{\text{fluorescence}_{\text{excited by UV}-\text{A}}}$$

$$\text{anthocyanin Dualex index} = \frac{\text{fluorescence}_{\text{excited by red}}}{\text{fluorescence}_{\text{excited by green}}}$$

Leaf chlorophyll content could be assessed by light transmission. First, a red wavelength (720 nm) quantified the chlorophyll and, second, the near-infrared (860 nm) measured the effects of the leaf structure. These two wavelengths were used to estimate the chlorophyll content as follows

$$\text{chlorophyll Dualex index} = \frac{\text{near} - \text{infrared}_{\text{transmission}} - \text{red}_{\text{transmision}}}{\text{red}_{\text{transmision}}}$$

Chlorophyll, flavonols, and anthocyanins were measured at every foliar stage, from the youngest (second leaf below the apex) to the oldest leaves (Data S1). An increase in chlorophyll content was observed in the youngest leaves until the eighth foliar stage, followed by a plateau in the older leaves (Data S1A). For flavonols, it was in the youngest leaves that quantities varied the most. Unlike chlorophyll content, flavonol and anthocyanin contents decreased with the age of leaves and then stabilized from the sixth leaf from the apex (Data S1B,C).

***P. viticola* Bioassay.** Sporangia obtained as described above were harvested and suspended in sterile water at 4 °C before inoculation on the abaxial face of leaves. After a washing process under water and drying leaves with filter paper, foliar discs were made as described previously[11] and deposited in Petri dishes containing Whatman paper moistened with 3 mL of sterile water and three droplets (15 μL) per disc of a sporangia suspension at 10 000 mL^{-1}. The next day, the residual water was removed and discs were incubated for 7 days under controlled conditions (22 °C, RH > 90%) with a 16 h light day. Grapevine downy mildew development was measured according to the density of mycelium and sporulation.[19] By comparison with the control discs, a conversion to a percentage of inhibition was performed and was expressed as the mean ± standard deviation of eight replicates.

Salicylic Acid Quantification. Free salicylic acid was extracted and quantified as described by Vergnes et al.[20] Salicylic acid (SA) was extracted twice from 200 mg of eight leaves using 90% MeOH (400 μL), and 50 ng of an internal standard (*o*-anisic acid, *o*ANI) was added. Following centrifugations at 10 000 g (20 min), supernatants were recovered, pooled, and evaporated to dryness in a vacuum rotary evaporator at 35 °C and suspended in water (660 μL) and trichloroacetic acid (TCA) 5% (40 μL). After a partitioning phase (ether), the organic phase was evaporated and diluted with 100 μL of

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acetonitrile/water/orthophosphoric acid (95/5/0.1%, v/v/v). SA analyses were performed on a 1100 series high-performance liquid chromatograph (HPLC) (Agilent Technologies). Chromatographic separation was performed on a Zorbax C18 SBAq column (150 mm $L \times 4.5$ mm $L \times 5$ μm), and the temperature was maintained at 45 °C. The flow rate was set at 2 mL/min. Acidified water (0.1% orthophosphoric acid; v/v (solvent A)) and acetonitrile (solvent B) were used as mobile phases. The following binary gradient was programmed: 0 min 95% A, 5% B; from 2 to 5 min 75% A, 25% B; from 8 to 9 min 95% A and 5% B. Fluorimetric detection was performed at an excitation wavelength of 305 nm and emission wavelength of 410 nm. The SA content was estimated from the calibration curve with SA (≥99% Sigma). Corrections for losses were made for each individual sample according to recoveries of the internal standard. Statistical analysis was performed using R statistical software.

RNA Extraction and Reverse Transcription. RNA extraction was performed as described previously[11] on the other half of the leaves used for free-SA quantification. Three biological replicates per treatment per day (untreated, ASM, SW, and CH) were stored at −80 °C. After grinding in liquid nitrogen, leaf powder 166 mg/mL was added to an extraction buffer preheated to 56 °C (300 mM Tris−HCl, pH 8.0, 25 mM EDTA, 2 M NaCl, 20 g/L cetrimonium bromide (CTAB), 20 g/L poly(vinylpolypyrrolidone) (PVPP), 500 μL/L spermidine trihydrochloride (0.05%) (≥98% Sigma), and 10 g/L β-mercaptoethanol added extemporaneously). The mixture was stirred vigorously and incubated in a bath at 56 °C for 10 min. An equal volume of chloroform/isoamyl alcohol (24:1, v/v) was added and centrifuged at 3500*g* for 15 min at 4 °C. The following RNA extraction steps were conducted using the MagMax-96 total RNA isolation kit, according to the manufacturer's instructions. Total RNA was reverse-transcribed using 2 μM Oligo$(dT)_{12-18}$, ribonuclease inhibitor, and M-MLV reverse transcriptase (Invitrogen), according to the manufacturer's instructions. Three housekeeping genes (*VvGAPDH*, *VvTIP41*, *VvTHIORLYS8*) were used as internal standards to normalize the starting template. The cDNAs were stored at −20 °C. Each data point is based on three independent biological replicates and nontechnical replicates.

Gene Expression. Expression of genes was monitored by quantitative polymerase chain reaction (qPCR) as described previously with the NeoViGen microarray[11] and a newly designed chip called BioStim96. The NeoViGen Chip included PR proteins (N = 21), some genes involved in secondary metabolites (phenylpropanoids, N = 14) and the indole pathway (N = 5), and others involved in the oxidoreduction system (N = 5), in the SA, ethylene, or jasmonic acid pathways (N = 17), and in cell wall reinforcement (N = 11). The BioStim chip gene set included genes involved in primary pathways (N = 10) and phytohormones pathways (auxin, cytokinin, gibberellin, ABA) (N = 28), some involved in cell or plast division (N = 4), in ion or metal transport (N = 14), or aquaporin (N = 10), and in ionic homeostasis (N = 19). Details of genes are listed in Data S2, Supporting Information.

The specificity (appropriate specific target) of each primer set was established by checking the size of the amplified product on agarose gel (not shown) with a single peak in the melting curve after each qPCR run. The PCR efficiencies for each primer set ranged between 0.8 and 1.2, thereby allowing us to simplify Pfaffl's model formula for calculating the relative expression with $2^{-\Delta\Delta Cq}$ (data not shown). Hierarchical clustering with the Pearson correlation as metric and the average linkage cluster method was performed with TIGR MeV software, and a Venn diagram was plotted using online software jvenn (http://jvenn.toulouse.inra.fr/app/index.html).

Destructive Measures of Root Biomass. Root biomass measurements were carried out on eight plants at 7 and 14 dpt for each modality. Fresh root weights were measured after gently washing the root system. Then, roots were stored at −80 °C and root dry weights were determined after lyophilization (12 h).

Statistical Analyses. Statistical analysis was performed using R Studio software (3.6.2 version). Each treatment consisted of 24 plants, with each plant representing a replicate. Data were subjected to one-way analysis of variance (ANOVA), and means were separated by Tukey's test ($p < 0.05$) (glht function {multcomp}). Relative gene expression was observed as differentially expressed for a p-value <0.05 in rank-based nonparametric multiple comparisons (Dunnet_test, nparcom function {nparcomp}). To determine individual variability of the four different treatment modalities, multiple-factor analyses (MFA) were performed (MFA function {FactoMineR}).

RESULTS

Chlorophyll, Flavonol, and Anthocyanin Measurements. Chlorophyll, flavonol, and anthocyanin contents were analyzed on the third leaves of cutting, which were the same leaves used for pathogen bioassay and biochemical and molecular analyses (Figure 1). At 2 dpt, a significant decrease in chlorophyll content compared to the control was observed (Figure 1A). Indeed, there was a decrease to 25% of chlorophyll in both BS-treated leaves compared to untreated ones and a tendency to decrease compared to ASM-treated leaves. Interestingly, an opposite variation was observed at 7 and 14 dpt on SW-treated leaves, respectively, with 25 and

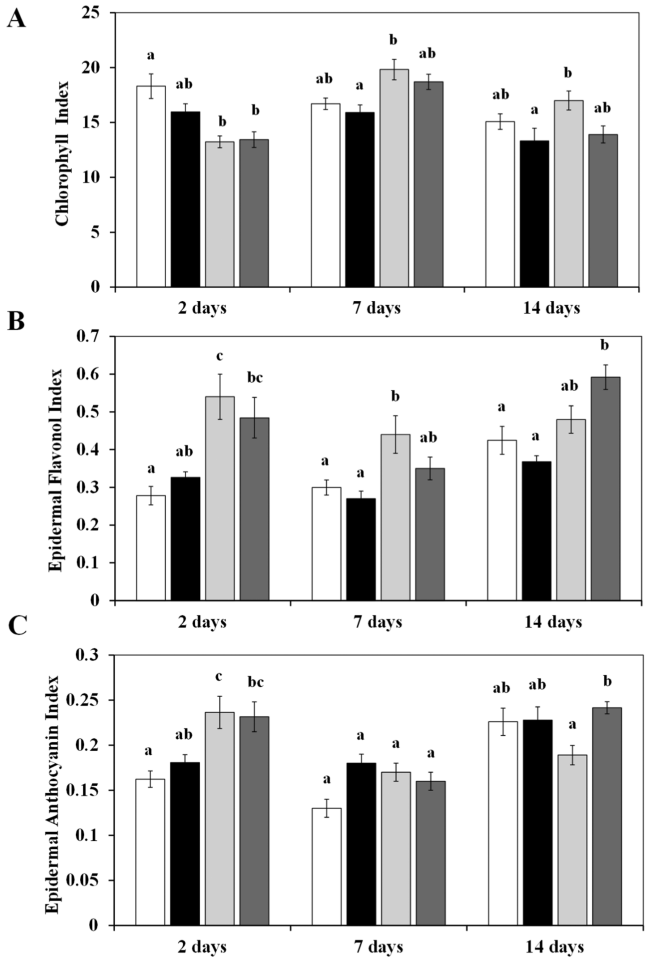

Figure 1. (A) Chlorophyll, (B) epidermal flavonol, and (C) epidermal anthocyanin contents in third leaves from the apex of untreated and ASM-, SW-, and CH-treated cutting, at 2, 7, and 14 days post treatment. Results are expressed as the Dualex index and are mean of eight replicates ± standard error of the mean (SEM). Significant difference between the four modalities on each day is indicated using letters (Tukey's HSD test, $p < 0.05$). White bar, untreated leaves; black bar, ASM-treated leaves; light-gray bar, SW-treated leaves; and dark-gray bar, CH-treated leaves.

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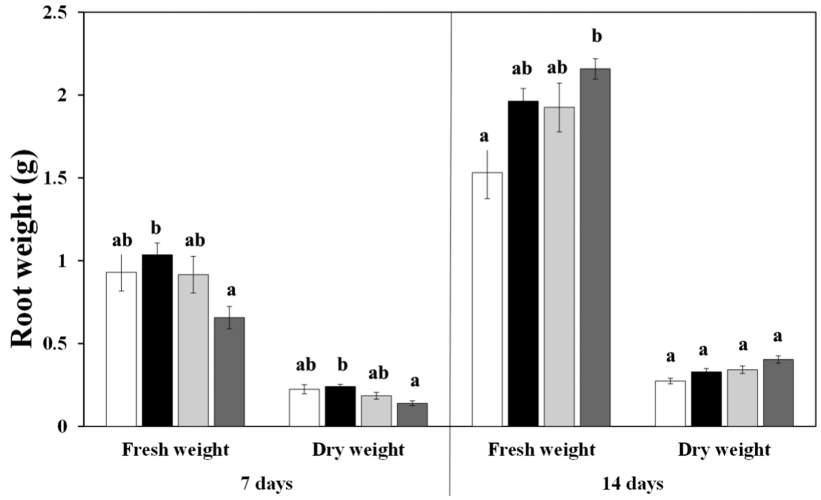

Figure 2. Root biomass at 7 and 14 dpt expressed in fresh and dry weight (g). Results are the means of eight replicates ± SEM. Letters indicate significant differences according to Tukey's test for each date independently ($p < 0.05$). White bar, untreated leaves; black bar, ASM-treated leaves; light-gray bar, SW-treated leaves; and dark-gray bar, CH-treated leaves.

15% of increase compared to ASM-treated leaves. Like BS, the elicitor ASM did not increase in chlorophyll compared to untreated leaves between 2 and 14 dpt. The most notable difference was between the lowest chlorophyll levels in ASM-treated leaves and those in SW-treated leaves at 7 and 14 dpt. This suggested that PDS (ASM) did not act as an activator of chlorophyll content, contrary to SW treatment, which increased the chlorophyll content compared to ASM-treated leaves.

Epidermal flavonol content increased strongly (1.77−2 times) in SW- and CH-treated leaves compared to untreated and ASM-treated leaves at 2 dpt (Figure 1B). Seven days post treatment, only the flavonol content of SW-treated leaves was higher than the control and ASM-treated leaves (+51.7 and +63%, respectively). On the other hand, at 14 dpt, only CH treatment induced a significant increase in flavonols compared to untreated (+40%) and ASM-treated leaves (+60%). Therefore, BS treatments increased the flavonol content, while ASM did not affect it. Finally, the epidermal anthocyanin content evolved differently from flavonol content over time in the same leaves. However, at 2 dpt, the distribution of anthocyanin content was similar to that of flavonol content, with a significant increase after biostimulant treatments (CH, SW; +29 to +68.8%) compared to control and ASM leaves (Figure 1C). Seven and 14 days after treatment, no significant difference was observed between untreated leaves and treated leaves. Nevertheless, the CH-treated leaves exhibited significantly more epidermal anthocyanins (+21%) than SW-treated leaves at 14 dpt. Again, the two BS behaved differently from the plant defense stimulator.

Root Biomass. Fresh and dry root weights of the treated plants were similar to those of control roots at 7 days after treatment (Figure 2). Nevertheless, at 14 dpt, the root weight of CH-treated plants had increased significantly by 40.96% (2.159 g) compared to controls. The dry root weights of treated plants were similar to those of controls, regardless of time. However, at 7 dpt, the fresh and dry root weights of ASM-treated plants were significantly higher than those of CH-treated plants. Indeed, CH treatment led to a slight decrease in fresh and dry root weights at 7 dpt, which was compensated by an increase in the fresh root weight at 14 dpt. This suggested significant variations in water content and, to a lesser extent, biomass over time after CH treatment.

Free Salicylic Acid Content. Free salicylic acid (SA) content in leaves was analyzed by HPLC, at 2, 7, and 14 dpt (Figure 3). In biostimulant-treated leaves (SW, CH) at 2 and 7 dpt, the free-SA quantities were significantly lower (−74 to −87%) than in control leaves (475.59 and 390.24 ng/g of fresh weight at 2 and 7 dpt, respectively). This difference in BS-treated leaves was even more marked at 2 dpt compared to ASM-treated leaves, the latter containing even more SA than control leaves (+23%, 586.28 ng SA/g FW). Indeed, ASM treatment rapidly increased the amount of free SA, clearly visible at 2 dpt, but not at other sampling times. Finally, at 14 dpt, leaves treated by phytostimulants (BS and PDS) exhibited SA contents similar to those of untreated leaves (63 ng SA/g FW). In conclusion, treatments with both BSs led to a decrease

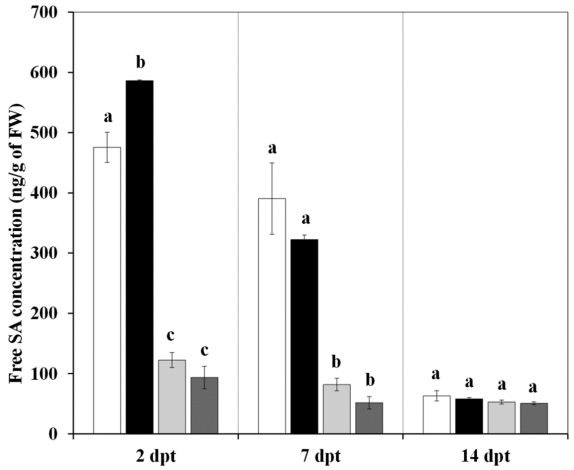

Figure 3. Free salicylic acid content in third leaves from the apex from untreated and ASM-, SW-, and CH-treated cuttings at 2, 7, and 14 days post treatment. Salicylic acid concentration is expressed in ng/g of fresh leaf weight. Results are the means of eight replicates ± SEM. Significant differences between the four treatment modalities on each day are indicated with letters (Tukey's HSD test, $p < 0.05$). White bar, untreated leaves; black bar, ASM-treated leaves; light-gray bar, SW-treated leaves; and dark-gray bar, CH-treated leaves.

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in the free-SA content in leaves at 2 and 7 dpt, while ASM increased the SA content only at 2 dpt.

Enhancement of Immunity against *P. viticola*. The effect of phytostimulants on the reinforcement of innate grapevine immunity was assessed by a leaf protection bioassay with *P. viticola* (Figure 4). All treatments at 2 dpt led to a

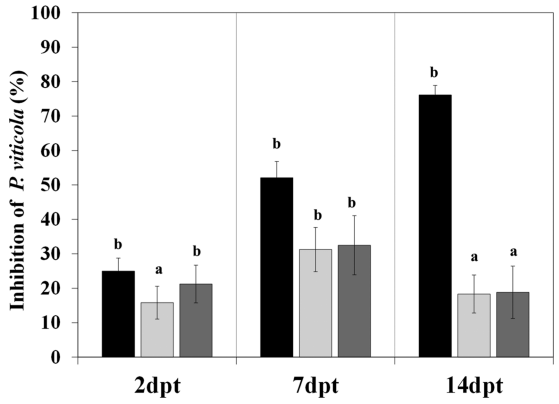

Figure 4. Inhibition of *P. viticola* after ASM, SW, or CH treatments compared to untreated cutting. *P. viticola* was inoculated on leaf discs, and development was assessed 7 days after inoculation. The data are the means of eight replicates with SEMs. Statistical differences between the three modalities are indicated with letters (Tukey's HSD test, $p < 0.05$). Black, ASM-treated leaves; light gray, SW-treated leaves; and dark gray, CH-treated leaves.

slightly significant reduction (15.8−25%) of downy mildew development compared to untreated control leaves. At 7 dpt, while all of the treatments were still effective against mildew, the effect of the two BSs was lower than that observed in the ASM-treated leaves (52 *vs* 31%). Finally, at 14 dpt, only ASM treatment had induced a significant inhibition (76%) of *P. viticola*. Therefore, treatments with BSs may transiently strengthen the plant's immunity, while PDS showed an increase in efficiency over time.

Gene Expression. The 161 genes used 2, 7, and 14 days after phytostimulant treatments were arranged in seven categories (reference genes not included) (Figure 5) as (i) phytohormone and signaling genes (32%), some of which are mainly known as growth regulators (cytokinin, gibberellin, abscisic acid, and auxin) and others more often as promoters of

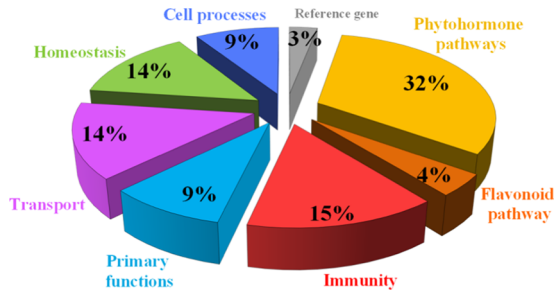

Figure 5. Function of genes used in "NeoViGen96" and in new "BioStim96" chips. Genes (161) coded for reference genes (gray; $N = 5$), phytohormone pathways (yellow; $N = 53$), flavonoid pathway (orange, $N = 7$), immunity genes (red; $N = 24$), some primary functions (light blue; $N = 15$), oligoelements or mineral transport genes (purple; $N = 24$), homeostasis and cell detoxification (green; $N = 23$), and cell integrity and cell processes (dark blue; $N = 15$).

defense and protection (ethylene, jasmonic acid, salicylic acid); (ii) genes involved in the flavonoid pathway (4%); (iii) immunity genes (15%) including PR-protein genes with different functions such as glucanase, chitinase, and peroxidase, or stilbene biosynthesis genes; (iv) primary functions (9%) with genes regulating photorespiration and the Krebs cycle; (v) transport of oligoelements or minerals (14%); (vi) homeostasis and detoxification (14%) including gene coding for chaperones and antioxidant enzymes; and (vii) cell integrity and cell processes (9%), including genes involved in cell cycle, plastid division, or cell wall reinforcement. Detailed information for each gene is given in Data S2, Supporting Information.

Overall gene expressions were significantly modulated (up- and downregulated) from 49 to 83% after treatment compared to untreated leaves (Figure 6). Modulation of expression was

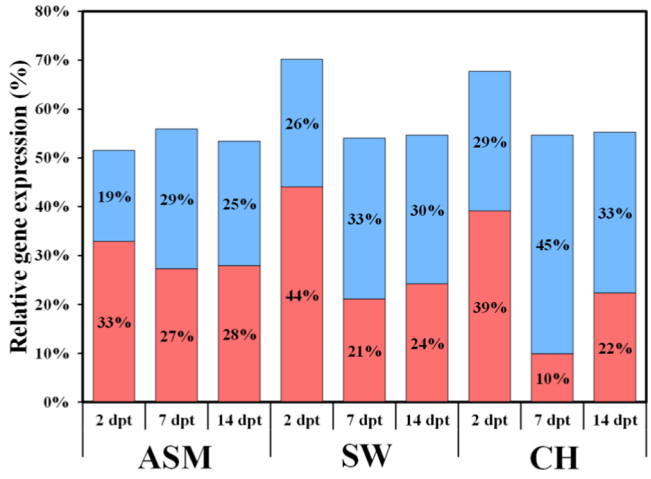

Figure 6. Significant gene modulation in ASM-, SW- or CH-treated leaves compared to untreated leaves at 2, 7, and 14 days after treatment. Genes upregulated are colored in red and those repressed in blue.

greater in BS-treated leaves than in ASM-treated leaves at 2 dpt (+18 and +16% for SW and CH, respectively, compared to ASM), suggesting a rapid impact of BS treatments on the overexpression and repression of selected genes. However, at 7 and 14 dpt, while the three phytostimulants had induced a similar percentage of significant gene-modulated expression (55 ± 1% at 7 dpt and 54 ± 1% at 14 dpt), the ASM-treated leaves exhibited higher levels of upregulated genes. At 7 dpt, the BS CH induced the strongest downregulation (45%) and the lowest upregulation (10%) compared to leaves treated with ASM or SW (29 and 33% *vs* 27 and 21%, respectively). Treatment with ASM induced rather stable modulations over time, unlike the two BS, which were strongly expressed until 2 dpt and then showed very variable modulations depending on the time and on the BS.

Hierarchical clustering analyses (HCAs) (Figures 7 and S3) revealed two clusters that separated gene expression at 2 and 7 dpt from 14 dpt. Expression profiles in CH- and SW-treated leaves were closer than in ASM-treated leaves at 2 dpt. At 7 dpt, the profile of ASM-treated leaves was closer to that of CH-treated leaves, while it was closer to that of SW-treated leaves at 14 dpt,

A Venn diagram was then plotted to highlight differences in gene expression (Figure 8). Several genes were expressed similarly in the leaves after the three treatments, and this set of

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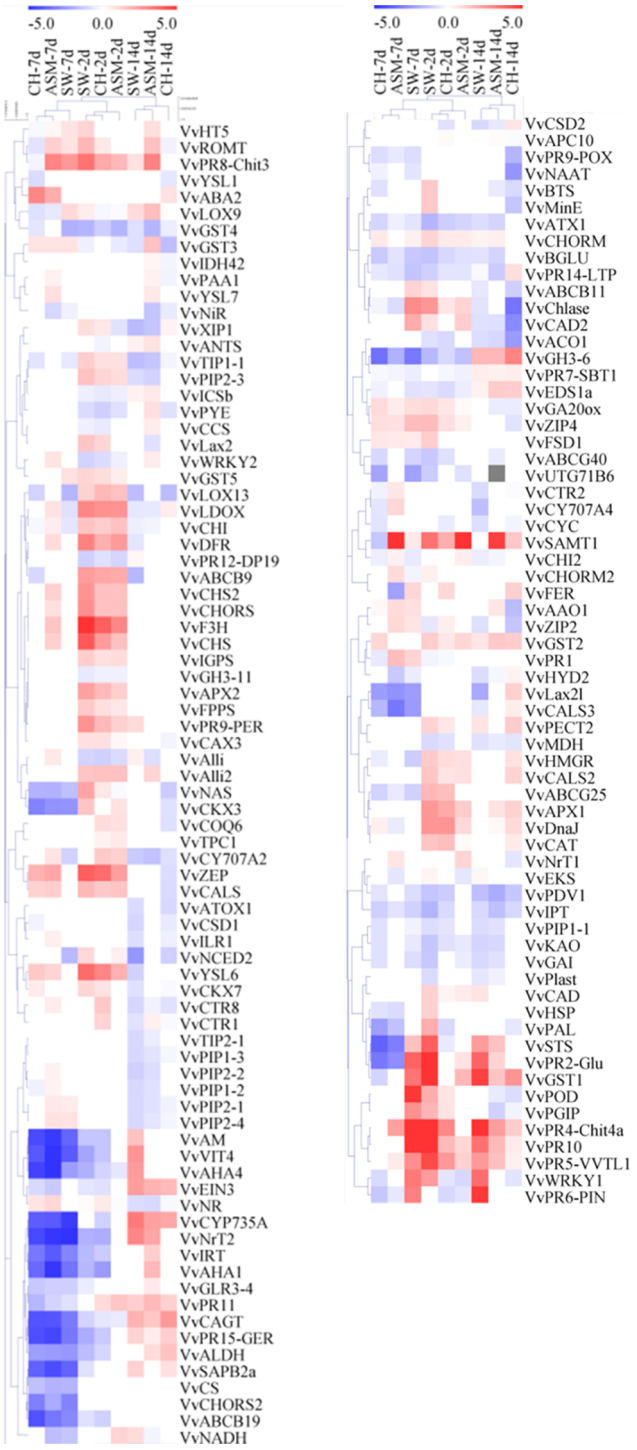

Figure 7. Hierarchical clustering of relative expression of genes (log 2) in phytostimulant-treated leaves (ASM, SW, CH) compared to untreated leaves at 2, 7, and 14 dpt. Each column represents treatments at different times, and each line corresponds to one gene (single row of boxes). Color scale bars indicate ratios corresponding to the mean of three independent experiments. Genes upregulated are in shades of red, with the expression level higher than 5 expressed in bright red. Genes downregulated are in shades of blue, with intensity lower than −5 expressed in dark blue.

common responsive genes decreased over time with 36.9% (N = 58) of the total chip genes at 2 dpt, 24.2% (N = 38) at 7 dpt, and 17.8% (N = 28) at 14 dpt. Moreover, this global decrease was mainly due to a large decrease in commonly overexpressed

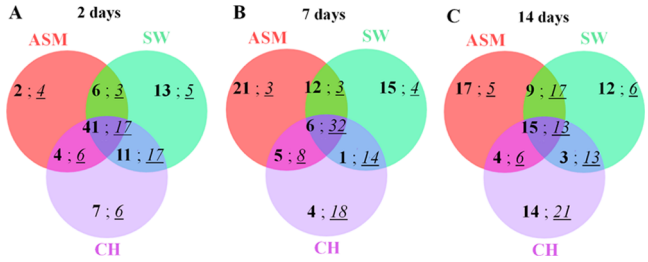

Figure 8. Venn diagrams of relative expression of genes significantly induced (bold) or repressed (underline italic), in phytostimulant-treated leaves compared to untreated leaves at 2 dpt (A), 7 dpt (B), and 14 dpt (C).

genes from 26.1 to 0.5% at 7 dpt and to 3.8% at 14 dpt. Detailed specific and common genes are listed in Data S2 and S4, Supporting Information. Among the common genes expressed, flavonoid pathway genes (*VvCHS*, *VvCHI*, *VvF3H*, *VvDFR*, *VvLDOX*) were upregulated at 2 dpt and rather downregulated at 7 and 14 dpt, especially for the two BSs (SW, CH) (Figures 7, S3, and S4). Other genes belonging to the PR-protein family (*e.g.*, *VvPR5*, *VvChit3*, *VvPR10*) were overexpressed for most treatments and sampling times. Genes involved in the phytohormone pathways (ethylene, *VvACO1*), abscisic acid (*VvABCB9*, *VvZEP*), and gibberellin (*VvGA20ox*) were commonly modulated at 2 dpt, as well as others from the isoprenoid pathway (*VvHMGR*, *VvFPPS*) and parietal reinforcements (*VvCAD*, *VvAPOX*, *VvCAL*). Moreover, genes involved in water transport (*e.g.*, tonoplastic and plasma membrane aquaporins (*VvTIP1-1*, *VvPIP-2-3*)) and zinc transport (*VvCax3*, *VvZIP4*) were also commonly overexpressed at 2 dpt. On the contrary, at 14 dpt, all aquaporin genes were commonly repressed in ASM- and SW-treated leaves. Genes overexpressed in response to treatment regardless of post-treatment time included glutathione-*S*-transferases, with *VvGST2* and *VvGST5* overexpressed at 2 dpt, *VvGST3* at 7 dpt, and *VvGST1* and *VvGST2* at 14 dpt.

In addition, some gene expressions were specific to one or to two phytostimulants. SW and CH treatments induced common expressions for many genes (28−16) that decreased over time. Conversely, genes expressed in common in the ASM- and SW-treated leaves increased over time (9, 15, and 26) (Figure 8). In addition, the common gene modulations between ASM and CH treatments remained steady (between 11 and 10).

Some genes showed modulations of expressions specific to a treatment. For example, in ASM-treated leaves, many genes involved in defenses were overexpressed, in common with at least one BS. Expressions of three PR-protein genes are noteworthy, *VvPR4*, *VvPR8* (chitinase genes) (Figure 8), and *VvPR5* (thaumatin-like gene).

However, time modulations were different, especially for the gene coding for a methyl resveratrol transferase (*VvROMT*), which leads to pterostilbene biosynthesis. This gene expression increased progressively over time in ASM-treated leaves, in contrast to SW-treated leaves (decrease in expression) and CH-treated leaves (repression) (Figure 9A). The pattern was similar for the gene coding for salicylic acid methylation, *VvSAMT*, and for the *VvPR8* gene (chitinase 3) (Figure 9B,C), whose overexpression increased progressively over time in ASM-treated leaves and decreased in other treatments.

In SW-treated leaves, in addition to the commonly expressed genes mentioned above, some more specific ones such as PR-

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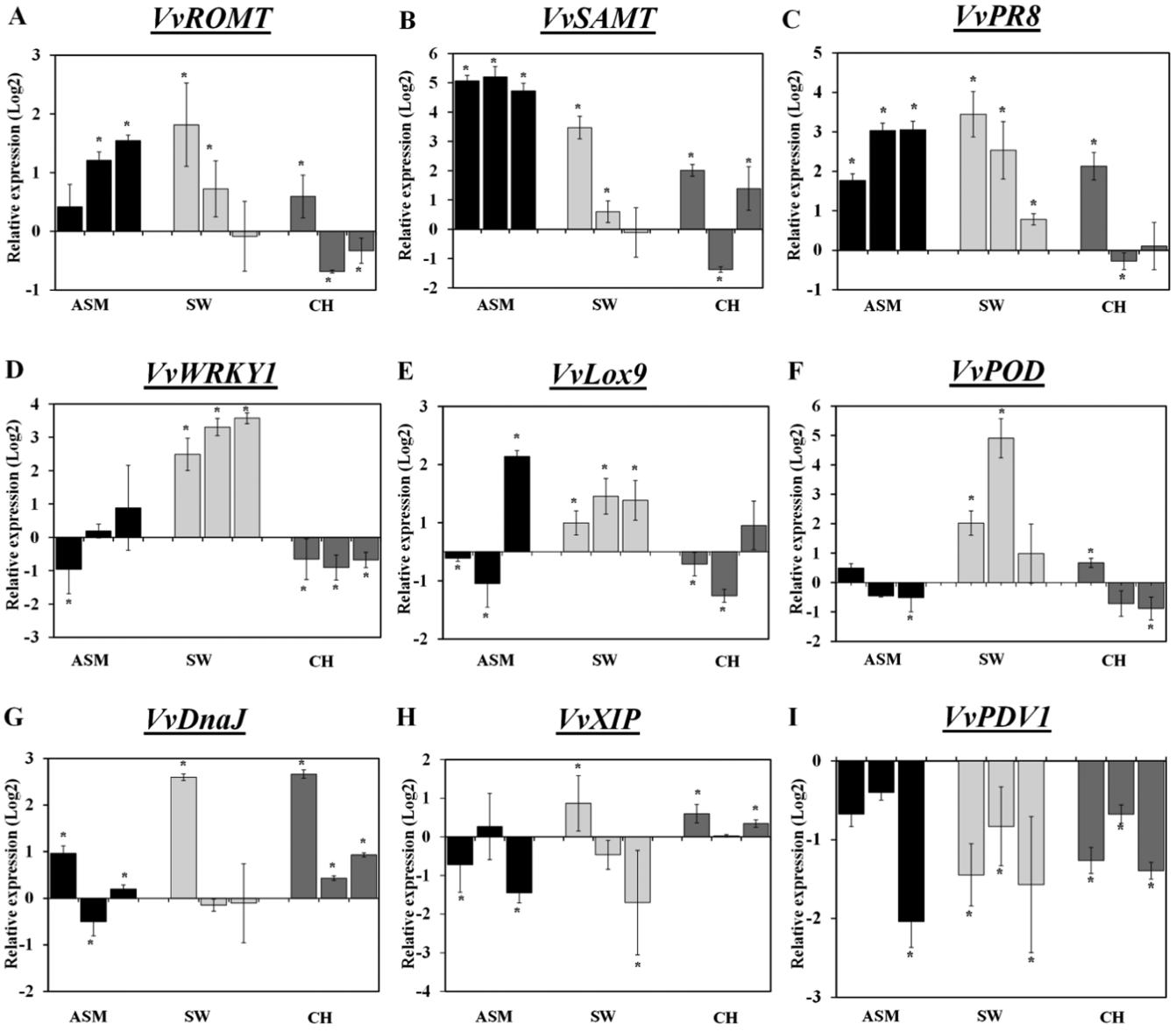

Figure 9. Relative gene expressions in ASM-, SW-, and CH-treated plants compared to untreated plants at 2, 7, and 14 dpt, with gene modulation specific to ASM treatment (A−C), to SW treatment (D−F), to CH treatment (G, H) and to BS treatment (I).

protein genes (*VvGlu*, (glucanase); *VvPR10*) were strongly overexpressed at all sampling times, accompanied by a major gene coding for stilbene synthase (*VvSTS*) and a glutathione-*S*-transferase (*VvGST1*) (Figures 8, S3, and S4). Several genes of the stilbene pathway were overexpressed in SW-treated leaves (*e.g.*, *VvPAL*, *VvSTS*, *VvROMT*) and were repressed after CH treatment. The transcription factor (*VvWRKY1*), the oxylipin biosynthesis gene (*VvLOX9*), glutathione-*S*-transferase 1 (*VvGST1*), and the overexpressed peroxidase (*VvPOD*) genes, appeared to be a marker of SW-treated leaves (Figure 9D−F).

Concerning specific genes of CH treatment, only *VvGST2* (glutathione-*S*-transferase) and *VvDnaJ* (chaperone protein) genes were still overexpressed over time, and even *VvXIP1* and *VvCYC* (cell mitotic cycle control) were constantly repressed (Figures 8, 9G,H, and S3). Among the PR-protein genes, only *VvPR14* coding for a lipid-transfer protein was more overexpressed over time than in ASM- and SW-treated leaves. Alongside the genes described above, the *VvPDV1* gene coding for a protein involved in plastid division was particularly repressed in SW- and CH-treated leaves and could be a marker of the action of both BSs (Figure 9I). In CH-treated leaves, genes involved in the storage and/or transport of metals (*VvFER*, *VvCTR*), the *XIP1* gene (tonoplastic aquaporin) (Figure 9H), and the nitrate reductase (*VvNR*) were more overexpressed. CH treatment globally repressed the genes involved in the abscisic acid (ABA) pathway, with the exception of *VvABA2* gene, which plays a role in its biosynthesis.

Focusing more specifically on the effect of time, the aquaporin gene modulations were of interest at 7 dpt, as either they were commonly repressed in the leaves treated with both BSs (*TIP1-1*; *PIP1-1*) or others were commonly overexpressed in the leaves treated with SW or ASM (*PIP2-1* and *PIP2-4*). Conversely, at 14 dpt, all of these genes were repressed in all modalities, especially with ASM and SW.

Multiple-factor analysis (Figure 10) summarizes results. Clearly, it significantly differentiated all treatment effects on

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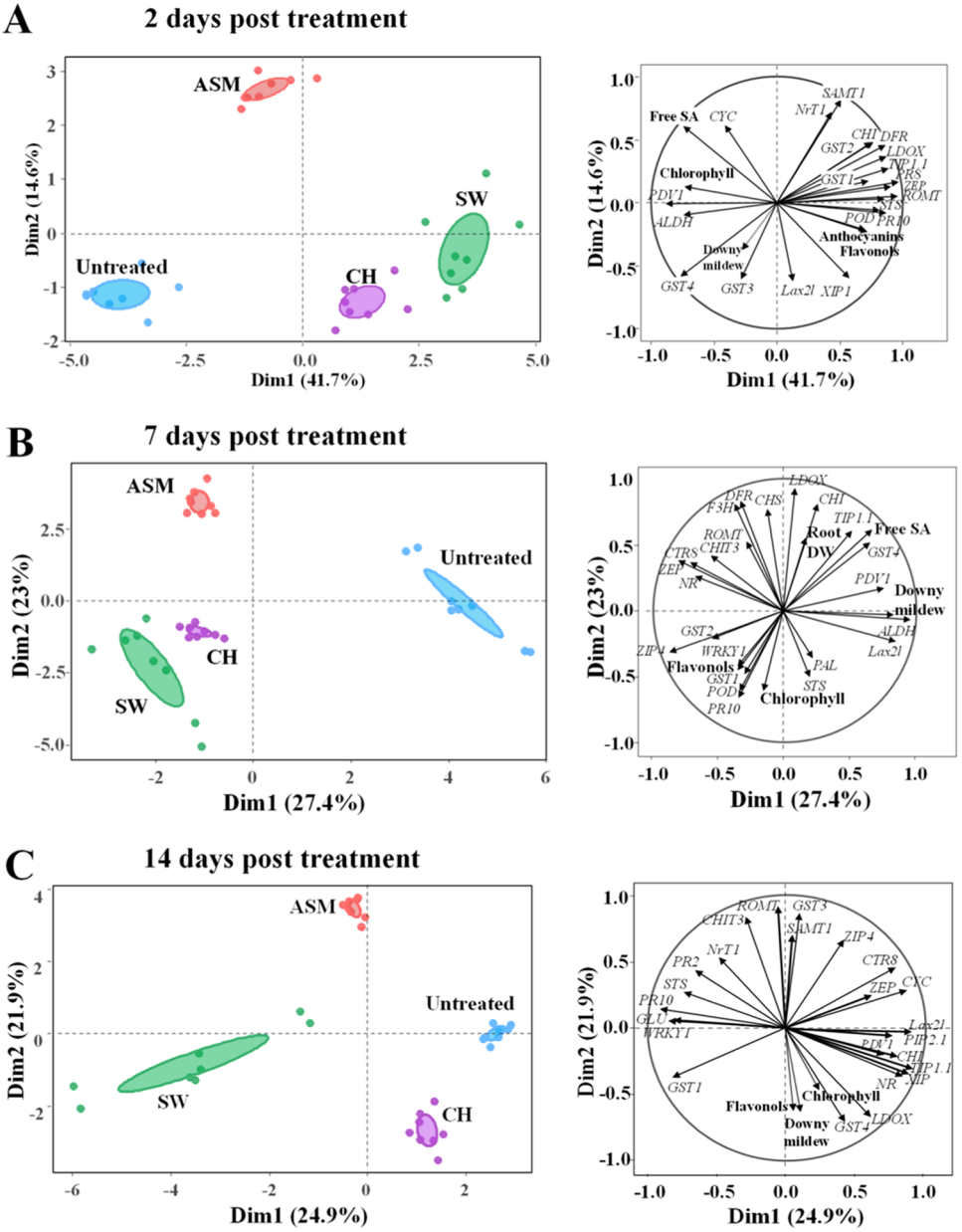

Figure 10. Multiple-factor analysis (MFA) of physiological, biochemical, and biological parameters and gene expression, at 2 dpt (A), 7 dpt (B), and 14 dpt (C). Distribution of individual plant responses on principal planes defined by the first two axes (dimensions 1 and 2) obtained with MFA. The two major principal components explained 56.3, 50.4, and 46.8% of the variability at 2, 7, and 14 dpt, respectively. Points represent values for each cutting. Ellipses represent 95% confidence intervals calculated for each treatment condition (ASM in red, SW in green, CH in purple, and untreated plants in blue). For each AFM, the projection on a standard unit circle of quantitative variables is presented. Physiological, biological, or biochemical parameters are represented by black arrows.

plants, including control for all time points. Dimensions 1 and 2 explained 56.3−46.8% of the variability between 2 and 14 dpt. Both BS treatments produced similar effects at 2 and 7 dpt, unlike the PDS treatment and the control (Figure 10A,B). However, at 14 dpt, while all of the treatments were quite distinct, CH was further away from SW and closer to the control (Figure 10C). Dimension 3 provided from 13.6 to 18.5% of the additional variability between 2 and 14 dpt (S5). Detailed analysis of treatments indicated that the variation in free SA was negatively correlated with flavonol content, like the negative correlation between root dry weight and chlorophyll content. Some genes related to inhibition of *P. viticola* emerged on correlation circles such as *VvROMT*, *VvSAMT1*, or *VvPR8*. Some genes, like *VvPOD*, *VvPR10*, and *VvGST1*, were related to the flavonol content at 2 and 7 dpt. Finally, genes associated with nitrogen metabolism (*VvNR*, *VvNrT1*), cell division (*VvCYC*) or plast division (*VvPDV1*), aquaporins (*VvXIP*, *VvTIP*, *VvPIP*), and genes involved in oligoelement transport (*e.g.*, *VvCTR8*, *VvZIP4*) had the greatest impact on the variability between treatment modalities.

DISCUSSION

Our starting point for this study was to take the term phytostimulant in its pure etymological sense, *i.e.*, a plant stimulator that includes PDSs and BSs. While the definition of a plant defense stimulator is now well-defined,[1] scientists are

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only beginning to reach a consensus about the definition of biostimulants.[2] The same is true for government agencies (*e.g.*, EU regulation, no. 2019/1009) and industrialists (European Biostimulants Industry Council (EBIC) in Europe, Biostimulant Coalition in the U.S.). Providing tools and approaches to assess and understand the mode of action of PDSs and BSs is a real challenge for their deployment in the field to reduce chemical inputs. While various methods are available to explore natural resources for biostimulants, few may be used to evaluate their effect on the vine, to probe their mode of action, and to characterize them. Here, the deciphering of *V. vinifera* responses to a known defense stimulator, ASM, and to two biostimulants with different compositions (SW, CH) made it possible to discriminate and to highlight their effects on the physiology and reinforcement of the plant's immunity.

PDSs enhance the plant's capacity to make active its immune network responses without being directly toxic to pathogens.[21] On the other hand, BSs have been widely used in vineyards to improve tolerance to abiotic stress, growth, and nutrient uptake and they trigger the metabolic response of plants.[2,3] Clearly, the effects of PDSs differed from those of BSs in the intensity and persistence of their antimildew efficacy over time. As expected, common responses between the products were noted between the two BSs, which displayed not only greater similarities between each other than with PDSs (content of flavonols and anthocyanins and free SA) but also specific responses to each BS.

Focusing on biotic stress and immune responses, ASM, an SA analog, was effective against *P. viticola* as expected, in accordance with its antipathogenic effects on grapevine,[6,10,22,23] and it was able to induce an SAR response, conferring induced resistance to many plants.[24,25] Here, ASM treatment was increasingly efficient against *P. viticola* over time, unlike the two BSs. This control of downy mildew was well-correlated with an increase in the expression of the *VvROMT* gene, which coded for a resveratrol-*O*-methyl transferase involved in the synthesis of pterostilbene (methylated resveratrol), which is known to be efficient against *P. viticola*. This is consistent with the overproduction of pterostilbene in the vineyard after ASM treatment and better protection against mildew.[12] Conversely, the low efficacy of the two BSs against *P. viticola* was correlated to a decrease in the overexpression or even repression of *VvROMT*. ASM treatment also led to a high concentration of free SA accompanied by a strong overexpression of the *VvSAMT* gene, coding for an SA-methyl transferase (4−8 times greater than other treatments), suggesting the storage of SA in the methylated form probably to limit the toxicity of the SA and/or to achieve better bioavailability of SA for defense responses.[26] In addition, the low free-SA concentrations quantified in BS-treated leaves are in accordance with vegetative and root growth and improved photosynthesis.[27] As expected, ASM overexpressed PR-protein genes (*VvPR4*, *VvPR8*-endochitinases, *VvPR2*-β-1,3-glucanase, *VvPR5*-thaumatin-like protein) and others,[6,10,11,25] including some that are common with biostimulant treatments, and in particular with SW (*VvPR2*, *VvPR4*, *VvPR5*, *VvPR8*). While treatment with CH only slightly stimulated the PR-protein overexpression, treatment with SW led to strong and more specific overexpressions of *VvPR10* and *VvPR6* genes. The *VvPR10* gene codes for a ribonuclease that is related to the formation of reactive oxygen species, along with nitric oxide, playing a role in biotic or abiotic (nutrient stress, water deficiency, salt) stresses. It is also likely that this protein plays a role in the growth and development of the plant.[28] Similarly, while the *VvPR6* gene is known to code for a protease in response to biotic stressors, it might also be involved in responses to a wide variety of environmental stressors.[29]

Flavonoids dichotomized the actions of PDSs in response to biotic stress and BSs in response to abiotic stress. Many genes involved in the flavonol and anthocyanin pathway were overexpressed at 2 and 7 dpt (*VvCHI*, *VvCHS*, *VvDFR*, *VvLDOX*, *VvF3H*) in ASM-treated leaves, but their expressions persisted at 7 dpt in ASM-treated leaves, unlike leaves treated with both BS. However, anthocyanin and flavonol contents increased significantly in BS-treated leaves compared to ASM-treated leaves. These results suggested that ASM did not promote the synthesis of flavonols such as quercetin and derivatives, or that other molecules (flavanols) such as catechin and derivatives were less well-detected at the wavelength used with the Dualex clip (480 nm) (Dr. Cluzet S. personal communication),[30] or even that as yet unknown post-transcriptional phenomena might limit the biosynthesis to promote another pathway. A recent study comparing the effects of various elicitors on lettuce confirmed the role of SA in favor of flavanol biosynthesis.[30] Concerning the biosynthesis of phenylpropanoids, it was surprising to observe no overexpression of *VvPAL* and *VvSTS* in ASM-treated leaves, suggesting that *VvSTS* was probably already overexpressed in the latter. However, *VvPAL* was overexpressed at 2 dpt in SW-treated leaves. This would be consistent with the overexpression of the *PAL* gene observed in tobacco leaves treated with oligosaccharide from seaweed.[31]

Regarding the effects of the two BSs and their features, SW contained various oligoelements and 0.5% of the algae *A. nodosum*. It is known for its biostimulant properties in the vineyard and increased yield, vegetative growth, and berry quality.[32,33] *A. nodosum* and its oligosaccharides have also been studied for their tolerance to biotic and abiotic stresses (salinity) on *Arabidopsis thaliana* and grapevine while improving growth and quality of grapes.[34] SW modulated defense genes (*VvPAL*, *VvLOX9*, and *VvGST1*), which could be markers of its effects. This result is potentially consistent with the effects of sulfated galactan (from seaweed) on treated tobacco leaves and on growth stimulation.[31] In addition, lipoxygenase (*VvLOX9*) catalyzes oxylipin and plays a significant role during development and in responses to various biotic and abiotic stresses and senescence (heat, drought, salt). Concerning the gene coding for a glutathione-*S*-transferase (*VvGST1*), this family of proteins responds to biotic and abiotic stresses and participates in the transport of phytohormone (auxin) and many secondary metabolites such as anthocyanins, flavonoids, or stilbenes.[35] Another gene coding for the transcription factor WRKY1 (*VvWRKY1*) appeared to correlate with *VvPR10*, as suggested by Eulgem et al.,[36] and might act as a marker of the specific effect of SW. Upregulation of the *WRKY1* gene, which responds to biotic and abiotic stresses,[37,38] had also been reported to induce an oxidative burst, as in H_2O_2 cellular accumulation. SW-treated plants overexpressed the *VvPOD* gene, which was involved in the detoxification of H_2O_2, like other genes of peroxidation with *VvAPX1* (ascorbate peroxidase), *VvCAT* (catalase), and *VvGST1* (glutathione-*S*-transferase). Together, all of these overexpressions suggest that the strong antioxidant activity of SW treatment triggers responses to abiotic stresses and that it might also respond to cold and drought stresses. SW might enable the plant to respond to different abiotic stresses and to a

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lesser extent to biotic stress. This might explain why several genes (antioxidant system) were common in responses to different stresses.

The second biostimulant, CH, contained 10-, 20-, and 100-fold more magnesium oxide (MgO), boron (B), and chelated iron (EDTA−Fe), respectively, than SW. Some studies showed its positive effects on plant growth and tolerance to abiotic stress by the application of micronutrients.[39−41] In contrast to the effect of SW treatment, CH treatment repressed the transcription factor *WRKY1*. However, the transcription factor WRKY1 might act as a negative regulator of tolerance to hydric stress.[42] We hypothesize that CH plays a role in water regulation, as supported by the significant increase in root water content at 14 dpt. This point was interesting to discriminate the effect of CH and BS, which was related to aquaporins and the ABA metabolism. Indeed, while SW decreased the ABA catabolism (*VvHYD2*, *VvCY707A2*, and *VvCY707A4*), CH increased the ABA biosynthesis more at 2 and 7 dpt (*VvAAO*, *VvZEP*, *VvABA2*, *VvNCED2*, *VvCOQ6*), but also decreased it at 14 dpt. In addition, the 10 aquaporin genes of our chip (*VvPIPs*, *VvTIPs*, *VvXIP*) coding for proteins involved in water transport play a role in specific plant developmental stages and in the regulation of osmotic stress by decreasing the water permeability.[43] *VvPIP2.1*, *VvTIP1.1*, and *VvTIP2.2* have been reported to play a role in water transport, unlike other *VvPIP*.[44] While SW and ASM had repressed all aquaporin genes 14 dpt (10), CH repressed only a few genes (*VvTIP1.1*, *VvPIP2.1*, *VvPIP1.3*, and *VvPIP2.4*). Another aquaporin, *VvXIP1*, involved in boron transport,[45] was specifically upregulated in CH-treated leaves at 2 and 14 dpt. In addition, expression of the auxin transporter gene *VvLax2l* increased in CH-treated plants and decreased in SW-treated plants. This gene, which is phylogenetically near *OsLax4*, is involved in auxin influx and intake in vascular tissues and promotes the growth of root apices.[46]

The modulation of different aquaporin genes, the potential role of boron, and the modulation of auxin metabolism genes might explain the significant increase in fresh root weight in CH-treated plants. On the other hand, this was not the case for dry weight, suggesting that the roots retained water and developed at the expense of differentiation. All of these results show that both BSs stimulated the developmental stages, particularly CH. Future experiments will study more physiological characteristics over longer periods of time to evaluate this biostimulant effect, particularly on growth. Another gene, *VvDnaj*, coding for a chaperone protein, appeared to be modulated specifically in CH-treated leaves and could play a role in the growth and in the response of the plant to heat stress.

Another difference between the two BSs concerned the accumulation of flavonols and anthocyanins over. Both BSs contained magnesium oxide (MgO), which participated in the increase in the anthocyanin content observed at 2 dpt in leaves, in accordance with anthocyanin accumulation described in the *Vitis* cell.[47] The GST genes, which act as flavonoid or anthocyanin transporters and play a role in oxidative response to various biotic and abiotic stresses,[34,48] also promoted the differences between the two BSs. Each treatment preferentially overexpressed different GST genes: in SW-treated leaves, *VvGST1* and to a lesser extent *VvGST5* and *VvGST2*; *VvGST2* in CH-treated leaves; and *VvGST3*, *VvGST1*, and *VvGST2* in ASM-treated leaves. SW treatment upregulated *VvGST1* 27-fold more than in control and 10-fold more than in the other treated leaves. Concerning the specific role of different GSTs, while *VvGST4* is thought to transport anthocyanins, *VvGST1* might preferentially transport other flavonoids and be more expressed during the early development of *V. vinifera* and particularly in berry skins.[48]

Some common genes induced by BS treatments might act as developmental-stage markers. First, the *VvPDV1* gene, which plays a role in chloroplast division, was highly repressed after BS treatment and might be characterized by a lower chloroplast division.[49] The repression that we evidenced may explain the transient decrease in the chlorophyll content observed in SW- and CH-treated leaves. Likewise, the *VvCYC* gene, which is involved in the regulation of the G2 phase of mitosis, was specifically repressed in both BS-treated leaves, suggesting cell differentiation or elongation in leaves.

Concerning the genes of primary metabolism, few appeared to be selectively modulated in ASM-treated leaves, except for genes involved in nitrogen metabolism. *VvNRT1*, a nitrate/nitrite transporter, was overexpressed from 2 to 7 dpt in ASM-treated leaves, while *VvNiR*, a nitrite reductase, was repressed from 7 to 14 dpt. This suggests the potential activation of the uptake and transport of nitrate/nitrite in cytosol from 2 to 7 days after treatment, combined with the repression of nitrite reductase in plasts. In addition, genes involved in copper and zinc transport were more expressed in ASM-treated leaves than in BS-treated leaves. *VvCTRs* have been reported to be upregulated in the event of an excess or deficit in copper concentration.[50] The downregulation induced by BS treatment suggests that copper homeostasis is better regulated by BS than by ASM.

Globally, the present results validate and optimize the development of the "PhysBioGen" method, so that complex commercial products may be tested in the future instead of single molecules or components. While the two BSs behaved similarly, gene expression analysis provided a more in-depth view of their action on the plant's metabolism. Despite some similar effects, it was possible to differentiate the mode of action of each product. It is thus now possible to provide guidance to professionals, allowing them (i) to improve the composition of their products and (ii) to explore their mode of action in more detail. Research is now underway in our laboratory to establish even more efficient measures of plant life traits and to test specific hypotheses. In the world of viticulture nowadays affected by various environmental factors, the use of phytostimulants involved in abiotic stress and biotic stress is opening new pathways of exploration. The "PhysBioGen" method constitutes an innovative set of tools to better understand the mechanisms underlying the effects of phytostimulants on resistance to abiotic stress and its impact on the transient resistance to biotic stress. An intriguing future issue for the research community is to investigate the possibility to associate phytostimulants with other products to reduce the harmful effects of chemical inputs.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.jafc.0c05849.

Chlorophyll, flavonol, and anthocyanin contents in all leaves (Data S1); list of genes analyzed (Data S2); heatmap or relative expression of 161 genes (defense and metabolism) in grapevine leaves (Data S3); list of

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common and specific genes expressed after treatments (Data S4); multiple-factor analysis (MFA in three dimensions) of all measures (Data S5) (PDF)

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Author Contributions

E.B. and A.B. carried out the experiments and analysis/interpretation of data. E.B. designed the primer and wrote the paper. M.-C.D. performed gene expression experiments (primer efficacy). M.-F.C.-C. carried out the experimental design and coordination of the work, interpretation of data, drafting, and cowriting of the manuscript. O.A. critically revised the manuscript. All authors have given approval to the final version of the manuscript.

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Notes

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ABBREVIATIONS

ABA, abscisic acid; ANOVA, analysis of variance; ASM, acibenzolar-*S* methyl; BS, biostimulant; B, boron; EDTA, ethylenediaminetetraacetic acid; HPLC, high-performance liquid chromatography; MFA, multiple-factor analysis; MgO, magnesium oxide; PDS, plant defense stimulator; SA, salicylic acid

REFERENCES

(1) Delaunois, B.; Farace, G.; Jeandet, P.; Clément, C.; Baillieul, F.; Dorey, S.; Cordelier, S. Elicitors as Alternative Strategy to Pesticides in Grapevine? Current Knowledge on Their Mode of Action from Controlled Conditions to Vineyard. *Environ. Sci. Pollut. Res.* **2014**, *21*, 4837−4846.

(2) du Jardin, P. Plant Biostimulants: Definition, Concept, Main Categories and Regulation. *Sci. Hortic.* **2015**, 3−14.

(3) Yakhin, O. I.; Lubyanov, A. A.; Yakhin, I. A.; Brown, P. Biostimulants in Plant Science: A Global Perspective. *Front. Plant Sci.* **2017**, *7*, No. 2049.

(4) Lyon, D. L. Agents That Can Elicit Induced Resistance. In *Induced Resistance for Plant Defense*, 2nd ed.; Walters, D. R.; Newton, A. C.; Lyon, G. D., Eds.; Wiley Blakwell: Chichester, U.K., 2014; pp 11−31.

(5) Trotel-Aziz, P.; Couderchet, M.; Vernet, G.; Aziz, A. Chitosan Stimulates Defense Reactions in Grapevine Leaves and Inhibits Development of *Botrytis cinerea*. *Eur. J. Plant Pathol.* **2006**, *114*, 405−413.

(6) Banani, H.; Roatti, B.; Ezzahi, B.; Giovannini, O.; Gessler, G.; Pertot, I.; Perazzolli, M. Characterization of Resistance Mechanisms Activated by *Trichoderma harzianum* T39 and Benzothiadiazole to Downy Mildew in Different Grapevine Cultivars. *Plant Pathol.* **2014**, *63*, 334−343.

(7) Trouvelot, S.; Varnier, A.-L.; Allègre, M.; Mercier, L.; Baillieul, F.; Arnould, C.; Gianinazzi-Pearson, V.; Klarzynski, O.; Joubert, J.-M.; Pugin, A.; et al. A β-1,3 Glucan Sulfate Induces Resistance in Grapevine against *Plasmopara viticola* Through Priming of Defense Responses, Including HR-like Cell Death. *Mol. Plant−Microbe Interact.* **2008**, *21*, 232−243.

(8) van Aubel, G.; Buonatesta, R.; Van Cutsem, P. COS-OGA: A Novel Oligosaccharidic Elicitor That Protects Grapes and Cucumbers against Powdery Mildew. *Crop Prot.* **2014**, *65*, 129−137.

(9) Belhadj, A.; Saigne, C.; Telef, N.; Stéphanie, C.; Jérôme, B.; Corio-Costet, M.-F.; Mérillon, J. M. Methyl Jasmonate Induces Defense Responses in Grapevine and Triggers Protection against *Erysiphe necator*. *J. Agric. Food Chem.* **2006**, *54*, 9119−9125.

(10) Dufour, M. C.; Lambert, C.; Bouscaut, J.; Mérillon, J. M.; Corio-Costet, M. F. Benzothiadiazole-Primed Defence Responses and Enhanced Differential Expression of Defence Genes in *Vitis vinifera* Infected with Biotrophic Pathogens *Erysiphe necator* and *Plasmopara viticola*. *Plant Pathol.* **2013**, *62*, 370−382.

(11) Dufour, M.-C.; Magnin, N.; Dumas, B.; Vergnes, S.; Corio-Costet, M.-F. High-Throughput Gene-Expression Quantification of Grapevine Defense Responses in the Field Using Microfluidic Dynamic Arrays. *BMC Genomics* **2016**, *17*, No. 957.

(12) Aziz, A.; Poinssot, B.; Daire, X.; Adrian, M.; Bézier, A.; Lambert, B.; Joubert, J.-M.; Pugin, A. Laminarin Elicits Defense Responses in Grapevine and Induces Protection Against *Botrytis cinerea* and *Plasmopara viticola*. *Mol. Plant−Microbe Interact.* **2003**, *16*, 1118−1128.

(13) Pieterse, C. M. J.; Zamioudis, C.; Van der Does, D.; Van Wees, S. In Signalling Networks Involved in Induced Resistance. *Induced Resistance for Plant Defense*, 2nd ed.; Walters, D. R.; Newton, A. C.; Lyon, G. D., Eds.; Wiley Blakwell: Chichester, U.K., 2014; pp 58−72.

(14) Gutiérrez-Gamboa, G.; Romanazzi, G.; Garde-Cerdán, T.; Pérez-Álvarez, E. P. A Review of the Use of Biostimulants in the Vineyard for Improved Grape and Wine Quality: Effects on Prevention of Grapevine Diseases. *J. Sci. Food Agric.* **2019**, *99*, 1001−1009.

(15) Petit, A. N.; Fontaine, F.; Clément, C.; Vaillant-Gaveau, N. Photosynthesis Limitations of Grapevine after Treatment with the Fungicide Fludioxonil. *J. Agric. Food Chem.* **2008**, *56*, 6761−6767.

(16) Walters, D. R.; Havis, N. D.; Sablou, C.; Walsh, D. J. Possible Trade-off Associated with the Use of a Combination of Resistance Elicitors. *Physiol. Mol. Plant Pathol.* **2011**, *75*, 188−192.

(17) Frioni, T.; Tombesi, S.; Quaglia, M.; Calderini, O.; Moretti, C.; Poni, S.; Gatti, M.; Moncalvo, A.; Sabbatini, P.; Berrìos, J. G.; et al. Metabolic and Transcriptional Changes Associated with the Use of

Ascophyllum nodosum Extracts as Tools to Improve the Quality of Wine Grapes (*Vitis vinifera* cv. Sangiovese) and Their Tolerance to Biotic Stress. *J. Sci. Food Agric.* **2019**, *99*, 6350−6363.
(18) Ning, Y.; Liu, W.; Wang, G. L. Balancing Immunity and Yield in Crop Plants. *Trends Plant Sci.* **2017**, *22*, 1069−1079.
(19) Corio-Costet, M. F.; Dufour, M. C.; Cigna, J.; Abadie, P.; Chen, W. J. Diversity and Fitness of *Plasmopara viticola* Isolates Resistant to QoI Fungicides. *Eur. J. Plant Pathol.* **2011**, *129*, 315−329.
(20) Vergnes, S.; Ladouce, N.; Fournier, S.; Ferhout, H.; Attia, F.; Dumas, B. Foliar Treatments with *Gaultheria procumbens* Essential Oil Induce Defense Responses and Resistance against a Fungal Pathogen in Arabidopsis. *Front. Plant Sci.* **2014**, *5*, No. 477.
(21) Walters, D. R.; Newton, A. C.; Lyon, G. D. *Induced Resistance for Plant Defense: A Sustainable Approach to Crop Protection*; Wiley Blackwell: Chichester, U.K., 2014; p 332.
(22) Bellée, A.; Cluzet, S.; Dufour, M. C.; Mérillon, J. M.; Corio-Costet, M. F. 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). *J. Agric. Food Chem.* **2018**, *66*, 3338−3350.
(23) Iriti, M.; Rossoni, M.; Borgo, M.; Ferrara, L.; Faoro, F. Induction of Resistance to Gray Mold with Benzothiadiazole Modifies Amino Acid Profile and Increases Proanthocyanidins in Grape: Primary versus Secondary Metabolism. *J. Agric. Food Chem.* **2005**, *53*, 9133−9139.
(24) Tripathi, D.; Jiang, Y. L.; Kumar, D. SABP2, a Methyl Salicylate Esterase Is Required for the Systemic Acquired Resistance Induced by Acibenzolar-S-Methyl in Plants. *FEBS Lett.* **2010**, *584*, 3458−3463.
(25) Marolleau, B.; Gaucher, M.; Heintz, C.; Degrave, A.; Warneys, R.; Orain, G.; Lemarquand, A.; Brisset, M. N. When a Plant Resistance Inducer Leaves the Lab for the Field: Integrating ASM into Routine Apple Protection Practices. *Front. Plant Sci.* **2017**, *8*, No. 1938.
(26) Park, S.-W.; Kaimoyo, E.; Kumar, D.; Mosher, S.; Klessig, D. F. Methyl Salicylate Is a Critical Mobile Signal for Plant Systemic Acquired Resistance. *Science* **2007**, *318*, 113−116.
(27) Rivas-San Vicente, M.; Plasencia, J. Salicylic Acid beyond Defence: Its Role in Plant Growth and Development. *J. Exp. Bot.* **2011**, *62*, 3321−3338.
(28) Sinha, R. K.; Verma, S. S.; Rastogi, A. Role of Pathogen-Related Protein 10 (PR 10) under Abiotic and Biotic Stresses in Plants. *Phyton* **2020**, *89*, 167−182.
(29) Myagmarjav, D.; Sukweenadhi, J.; Kim, Y. J.; Jang, M. G.; Rahimi, S.; Silva, J.; Choi, J. Y.; Mohanan, P.; Kwon, W. S.; Kim, C. G.; et al. Molecular Characterization and Expression Analysis of Pathogenesis Related Protein 6 from Panax ginseng. *Russ. J. Genet.* **2017**, *53*, 1211−1220.
(30) Moreno-Escamilla, J. O.; Jimeńez-Hernández, F. E.; Alvarez-Parrilla, E.; De La Rosa, L. A.; Martĺnez-Ruiz, N. D. R.; González-Fernández, R.; Orozco-Lucero, E.; González-Aguilar, G. A.; García-Fajardo, J. A.; Rodrigo-García, J. Effect of Elicitation on Polyphenol and Carotenoid Metabolism in Butterhead Lettuce (*Lactuca sativa* Var. Capitata). *ACS Omega* **2020**, *5*, 11535−11546.
(31) Laporte, D.; Vera, J.; Chandía, N. P.; Zúñiga, E. A.; Matsuhiro, B.; Moenne, A. Structurally Unrelated Algal Oligosaccharides Differentially Stimulate Growth and Defense against Tobacco Mosaic Virus in Tobacco Plants. *J. Appl. Phycol.* **2007**, *19*, 79−88.
(32) Sabir, A.; Yazar, K.; Sabir, F.; Kara, Z.; Yazici, M. A.; Goksu, N. Vine Growth, Yield, Berry Quality Attributes and Leaf Nutrient Content of Grapevines as Influenced by Seaweed Extract (*Ascophyllum nodosum*) and Nanosize Fertilizer Pulverizations. *Sci. Hortic.* **2014**, *175*, 1−8.
(33) Salvi, L.; Brunetti, C.; Cataldo, E.; Niccolai, A.; Centritto, M.; Ferrini, F.; Mattii, G. B. Effects of *Ascophyllum nodosum* Extract on *Vitis vinifera*: Consequences on Plant Physiology, Grape Quality and Secondary Metabolism. *Plant Physiol. Biochem.* **2019**, *139*, 21−32.
(34) Frioni, T.; Sabbatini, P.; Tombesi, S.; Norrie, J.; Poni, S.; Gatti, M.; Palliotti, A. Effects of a Biostimulant Derived from the Brown Seaweed *Ascophyllum nodosum* on Ripening Dynamics and Fruit Quality of Grapevines. *Sci. Hortic.* **2018**, *232*, 97−106.
(35) Marrs, K. A. The Functions and Regulation of Glutathione S-Transferases in Plants. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **1996**, *47*, 127−158.
(36) Eulgem, T.; Rushton, P. J.; Schmelzer, E.; Hahlbrock, K.; Somssich, I. E. Early Nuclear Events in Plant Defence Signalling: Rapid Gene Activation by WRKY Transcription Factors. *EMBO J.* **1999**, *18*, 4689−4699.
(37) Marchive, C.; Mzid, R.; Deluc, L.; Barrieu, F.; Pirrello, J.; Gauthier, A.; Corio-Costet, M. F.; Regad, F.; Cailleteau, B.; Hamdi, S.; et al. Isolation and Characterization of a *Vitis vinifera* Transcription Factor, VvWRKY1, and Its Effect on Responses to Fungal Pathogens in Transgenic Tobacco Plants. *J. Exp. Bot.* **2007**, *58*, 1999−2010.
(38) Guo, C.; Guo, R.; Xu, X.; Gao, M.; Li, X.; Song, J.; Zheng, Y.; Wang, X. Evolution and Expression Analysis of the Grape (*Vitis vinifera* L.) WRKY Gene Family. *J. Exp. Bot.* **2014**, *65*, 1513−1528.
(39) Cole, J. C.; Smith, M. W.; Penn, C. J.; Cheary, B. S.; Conaghan, K. J. Nitrogen, Phosphorus, Calcium, and Magnesium Applied Individually or as a Slow Release or Controlled Release Fertilizer Increase Growth and Yield and Affect Macronutrient and Micronutrient Concentration and Content of Field-Grown Tomato Plants. *Sci. Hortic.* **2016**, *211*, 420−430.
(40) Singh, S.; Chauhan, D. K.; Sharma, S.; Yadav, V.; Gaur, S.; Tripathi, D. K.; Singh, V. P.; Dubey, N. K.; Singh, S.; Liu, S.; et al. Acquisition and Homeostasis of Iron in Higher Plants and Their Probable Role in Abiotic Stress Tolerance. *Front. Environ. Sci.* **2018**, *5*, No. 86.
(41) Tripathi, D. K.; Singh, S.; Singh, S.; Mishra, S.; Chauhan, D. K.; Dubey, N. K. Micronutrients and Their Diverse Role in Agricultural Crops: Advances and Future Prospective. *Acta Physiol. Plant.* **2015**, *37*, No. 139.
(42) Qiao, Z.; Li, C. L.; Zhang, W. WRKY1 Regulates Stomatal Movement in Drought-Stressed *Arabidopsis thaliana*. *Plant Mol. Biol.* **2016**, *91*, 53−65.
(43) Maurel, C.; Verdoucq, L.; Luu, D.-T.; Santoni, V. Plant Aquaporins: Membrane Channels with Multiple Integrated Functions. *Annu. Rev. Plant Biol.* **2008**, *59*, 595−624.
(44) Sabir, F.; Leandro, M. J.; Martins, A. P.; Loureiro-Dias, M. C.; Moura, T. F.; Soveral, G.; Prista, C. Exploring Three PIPs and Three TIPs of Grapevine for Transport of Water and Atypical Substrates through Heterologous Expression in Aqy-Null Yeast. *PLoS One* **2014**, *9*, No. e102087.
(45) Noronha, H.; Araujo, D.; Conde, C.; Martins, A. P.; Soveral, G.; Chaumont, F.; Delrot, S.; Geros, H. The Grapevine Uncharacterized Intrinsic Protein 1 (VvXIP1) Is Regulated by Drought Stress and Transports Glycerol, Hydrogen Peroxide, Heavy Metals but Not Water. *PLoS One* **2016**, *11*, No. e0160976.
(46) Chai, C.; Subudhi, P. K. Comprehensive Analysis and Expression Profiling of the OsLAX and OsABCB Auxin Transporter Gene Families in Rice (*Oryza sativa*) under Phytohormone Stimuli and Abiotic Stresses. *Front. Plant Sci.* **2016**, *7*, No. 593.
(47) Sinilal, B.; Ovadia, R.; Nissim-Levi, A.; Perl, A.; Carmeli-Weissberg, M.; Oren-Shamir, M. Increased Accumulation and Decreased Catabolism of Anthocyanins in Red Grape Cell Suspension Culture Following Magnesium Treatment. *Planta* **2011**, *234*, 61−71.
(48) Pérez-Díaz, R.; Madrid-Espinoza, J.; Salinas-Cornejo, J.; González-Villanueva, E.; Ruiz-Lara, S. Differential Roles for VviGST1, VviGST3, and VviGST4 in Proanthocyanidin and Anthocyanin Transport in Vitis vinífera. *Front. Plant Sci.* **2016**, *7*, No. 1166.
(49) Osteryoung, K. W.; Pyke, Ka. Division and Dynamic Morphology of Plastids. *Annu. Rev. Plant Biol.* **2014**, *65*, 443−472.
(50) Martins, V.; Bassil, E.; Hanana, M.; Blumwald, E.; Gerós, H. Copper Homeostasis in Grapevine: Functional Characterization of the *Vitis vinifera* Copper Transporter 1. *Planta* **2014**, *240*, 91−101.

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