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ORIGINAL ARTICLE

# Impact of different elicitors on grapevine leaf metabolism monitored by 1H NMR spectroscopy

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## Abstract

**Introduction** Grapevine protection is an important issue in viticulture. To reduce pesticide use, sustainable disease control strategies are proposed, including a promising alternative method based on the elicitor-triggered stimulation of the grapevine natural defense responses. However, detailed investigations are necessary to characterize the impact of such defense induction on the primary metabolism.

**Objectives** Our aim was to use a metabolomics approach to assess the impact on grapevine of different elicitors dependent on the salicylic acid (SA) and/or jasmonic acid (JA) pathway. For this purpose, leaves of grapevine foliar cuttings were treated with methyl jasmonate, acibenzolar-S-methyl or phosphonates.

**Methods** According to the elicitor, common and discriminating metabolites were elucidated using $^{1}$H NMR measurements and principal component analysis.

**Results** A wide range of compounds including carbohydrates, amino acids, organic acids, phenolics and amines were identified. The score plots obtained by combining PC1 versus PC2 and PC1 versus PC3 allowed a clear separation of samples, so metabolite fingerprinting showed an extensive reprogramming of primary metabolic pathways after elicitation.

**Conclusion** The methods applied were found to be accurate for the rapid determination and differential characterization of plant samples based on their metabolic composition. These investigations can be very useful because the application of plant defense stimulators is gaining greater importance as an alternative strategy to pesticides in the vineyard.

**Keywords** Metabolomic analysis · *Vitis vinifera* · MeJA · ASM · Phosphonates · Nuclear magnetic resonance spectroscopy

## 1 Introduction

Grapevine (*Vitis vinifera* L.) is one of the major fruit crops worldwide. Like all cultivated plants, the susceptibility of grapevine to diseases is high due to its uniform background. The greatest yield losses are due to damage caused by microbial agents such as *Plasmopara viticola* (downy mildew), *Erysiphe necator* (powdery mildew), *Botrytis cinerea* (gray mold), and phylloxera (Armijo et al. 2016). Therefore, pest control is a leading issue in viticulture and it is currently achieved by using synthetic fungicides. Despite their relatively high efficacy against numerous pathogens, these chemicals contribute significantly to environmental pollution and pose a threat to human health. Among the alternative strategies for plant protection, induced natural resistance has become one of the most promising strategies since it is based on the use of substances less toxic for the environment and human health, and providing relative long-lasting

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and wide-spectrum resistance. During the past decade, more and more elicitor-like products have been launched in viticulture (Delaunois et al. 2014). These naturally derived or synthetic molecules stimulate the plant immune system by mimicking a pathogen attack, thus triggering several chemical defense strategies such as the accumulation of secondary metabolites. Elicitors, which are also called plant defense stimulators (PDS), act mainly through pathways mediated by salicylic acid (SA) or jasmonic acid (JA) with ethylene (ET), which are regarded as the critical defense phytohormones. These molecules can lead to basal resistance against multiple pathogens (Robert-Seilaniantz et al. 2011; Pieterse et al. 2012). Furthermore, thanks to subtle interactions between these two pathways, the plant is also able to fine-tune its defense responses against specific pathogens (Beckers and Spoel 2006; Figueiredo et al. 2015). Both SA- and JA-mediated defense responses occur not only locally at the recognition site of a microbe or pest but also in distal plant parts, thus allowing the protection of undamaged tissues against subsequent pathogen invasion (Pieterse et al. 2012).

Hence, plants treated by SA or its functional analogs like β-aminobutyric acid (BABA), 2,6-dichloroisonicotinic acid (INA), acibenzolar-S-methyl (ASM) or benzo-(1,2,3)-thiadiazole-7-carbothioic acid S-methyl ester (benzothiadiazole, BTH), respond by the onset of systemic acquired resistance (SAR) with the up-regulation of typical SA defense reactions (Cao et al. 1994). The stimulation of these defense responses leads to a correlated broad-spectrum resistance. For example, BTH and ASM have been used to control gray mold and *Phytophthora* in strawberry (Terry and Joyce 2000; Eikemo et al. 2003), and to fight against powdery mildew in wheat and Arabidopsis (Görlach et al. 1996; Lawton et al. 1996). The performance of ASM in combination with a soft fungicide management program was shown by Marolleau et al. (2017) to be an effective system for controlling natural apple scab infection in the orchard. In grapevine, BTH treatment triggers the up-regulation of many PR-protein genes, especially those coding for glucanases and chitinases, as well as genes encoding VvSTS (stilbene synthase) and VvROMT (resveratrol *O*-methyl transferase), two enzymes involved in stilbene biosynthesis (Bellée et al. 2018). Overall, BTH induces resistance of grapevine to downy and powdery mildews (Dufour et al. 2013), and gray mold (Iriti et al. 2005; Bellée et al. 2018).

Exogenous application of methyl jasmonate (MeJA), the methyl ester of JA, induces a large number of defense responses. In grapevine this molecule up-regulates transcript levels and/or proteins such as PR-proteins (e.g. chitinase, β-1,3-glucanase), peroxidase and enzymes involved in the phytoalexin pathway such as phenylalanine ammonia lyase (PAL) and stilbene synthase (STS) (Repka et al. 2004; Belhadj et al. 2006). PAL and STS induction correlates with the accumulation of stilbenes, the grapevine phytoalexins, thus conferring an enhanced tolerance, for instance against powdery mildew (Belhadj et al. 2006).

On the other hand, phosphonates have a complex mode of action: (i) direct inhibition of pathogen development, mainly against oomycetes (ii) indirect action as inducers of the release of stress metabolites from the pathogen to elicit defense responses, and (iii) direct stimulation of plant defense responses (Lobato et al. 2010). They have been used to control *Phytophthora* diseases in several plants for more than 30 years (Hardy et al. 2001). Regarding phytohormones mediating phosphite resistance, their effectiveness is thought to be dependent or not on the SA and/or JA/ET pathways (Eshraghi et al. 2011; Massoud et al. 2012; Burra et al. 2014). Consequently, a wide range of host defenses are activated by phosphite treatment. Importantly, ASM and phosphonates act as priming compounds, i.e. allowing a more rapid, efficient, and/or intense activation of defense responses to secondary biotic or abiotic stresses (Conrath 2009).

Elicitor treatments trigger many plant defense responses and, as tight interconnections exist between primary and secondary metabolism, changes in primary metabolism can occur following elicitation. Indeed, stresses are known to alter plant growth and development due to the energy drained from growth toward the production of defensive metabolites (Heil 2002; Dietrich et al. 2005). However, little is known about the influence of common elicitors on grapevine primary metabolism, yet it is important to know whether they do heavily affect the plant vigor, and hence the quality of grapes and wine.

The aim of this initial study was to evaluate the early effect of different types of elicitors (SA- and/or JA-dependent) by measuring metabolomic responses in grapevine leaves at 24 h post-treatment (hpt). The leaves of *Vitis vinifera* L. cuttings were treated with MeJA, ASM or a mix of potassium phosphonates (PHOS). To assess and compare global changes in metabolite pool under each condition, we used proton nuclear magnetic resonance spectroscopy ($^1$H NMR) as it affords reproducibility, convenience for quantification, and straightforward metabolite identification (Kim et al. 2010). In grapevine, at least three studies using NMR have reported alterations in grapevine leaf metabolism: in Esca disease-affected leaves (Lima et al. 2010), in leaves inoculated with *Plasmopara viticola* (Ali et al. 2012), and after infection with Flavescence Dorée Phytoplasma (Prezelj et al. 2016). NMR analysis has also been performed to investigate metabolic changes in grapes during *B. cinerea* infection (Hong et al. 2012). However, to our knowledge no research has been carried out to examine the impact of elicitors in grapevine and experimental evidence is needed. In order to test the feasibility of using NMR to detect differences in metabolism of elicited leaves, we carried out a proof-of-concept analysis by studying a limited number

of plants per modality (four). Principal component analysis (PCA) and partial least-squares discriminant analysis (PLS-DA) were used on NMR quantified data as it reveals variables involved in differentiating the samples.

## 2 Materials and methods

### 2.1 Plant material

Grapevine plants (*Vitis vinifera* L. cv. Cabernet Sauvignon) were propagated from wood cuttings in a greenhouse (INRA, Villenave d'Ornon, France). Wood cuttings were provided by Château Couhins (Gironde, France) clone 191. After 3 weeks, rooted cuttings were potted in sandy soil and were grown with 16 h light per day. Foliar cuttings were grown under controlled conditions at ± 25/20 °C day/night air temperature, 75% relative humidity and a 16 h photoperiod (350 µmol/$m^2$/s). Two-month-old plants with 10–12 leaves were used for the experiments.

### 2.2 Elicitation

#### 2.2.1 Preparation of MeJA, ASM and PHOS solutions

Methyl jasmonate (MeJA, 95%, Sigma-Aldrich, St-Louis, USA) was dissolved in 100% EtOH to a final concentration of 5 mM and added to an aqueous solution containing Triton X-100 (Sigma-Aldrich) at 0.1% (v/v). Formulated acibenzolar-S-methyl (S-methyl benzo[1,2,3]thiadiazole-7-carbothioate, ASM, Bion 50% WG, Syngenta) was dissolved in water and added at 2 g/L final concentration. A mix of potassium phosphonates (PHOS, LBG-01F34, De Sangosse, 730 g/L) was dissolved in water and added at 1.5 g/L final concentration. Control plants received distilled water (Untreated) or Triton (0.1%, v/v).

#### 2.2.2 Treatments

For this proof-of-concept analysis, four plants per condition were entirely sprayed using a micro-diffuser with a pressure reserve (Ecospray®). At least 1 mL of solutions was sprayed on each leaf, on the adaxial and abaxial face. The third and fourth leaves below the apex were harvested at 24 hpt, thoroughly rinsed with tap water, dried, then lyophilized before extraction.

### 2.3 Extraction of plant material

Extraction buffer was composed of 750 µL methanol-$d_4$ (99.80% D, Euriso-Top, St-Aubin and Gif-sur-Yvette, France) plus 750 µL of pH 6.0 $KH_2PO_4$ buffer (0.1 M), in $D_2O$ (99.90% D, Euriso-Top) containing 0.3 mM 3-(Trimethylsilyl) propanoic-2,2,3,3-$d_4$ acid sodium salt (TMSP, 98% D, Euriso-Top), as NMR chemical shift reference, and 4 mM calcium formate (Sigma-Aldrich), as a reference (internal standard) for the quantitation of metabolites. The eight freeze-dried leaves for each modality (sampling time and treatment) were ground to a fine powder. Three batches were established. Fifty mg of leaf tissue were extracted by adding extraction buffer (1000 µL). Samples were vortexed at room temperature for 1 min, ultrasonicated for 20 min and centrifuged at 17 500 rpm for 10 min. Then 600 µL of the supernatant were transferred to a 5-mm NMR tube and analyzed by NMR the same day.

### 2.4 NMR spectroscopy

All $^1$H-NMR spectra were recorded on a 600 MHz AVANCE III spectrometer (Bruker, Wissembourg, France) operating at a proton frequency of 600.27 MHz using a 5-mm TXI probe with Z-gradient coils. Methanol-$d_4$ was used as the internal lock. The samples were analyzed using the zgesgp pulse sequence (Bruker, Biospin, Germany) at 293 K with 32 scans, a time domain (TD) of 65,536 real data points and a spectral width (SW) of 16.0183 ppm (9615.385 Hz). The acquisition time (AQ) and the relaxation delay (RD) were 3.4 s and 5 s, respectively. The free induction decays (FIDs) were multiplied by an exponential function corresponding to line broadening (LB) of 0.3 Hz. NMR spectra were calibrated to trimethylsilane propionic acid sodium salt (TMSP) at 0.0 ppm using Topspin software version 3.2 (Bruker Biospin, Germany). The resulting spectra were manually phased and baseline-corrected using the Whittaker Smoother method with the MestReNova NMR software version 11.0.3 (Mestrelab Research, Spain). The spectral peaks were assigned by comparing chemical shifts and multiplicity with the literature and by metered addition in leaf extracts of the various standards (the list of standards used is specified in Table S1). Classical 2D-NMR experiments including COSY, TOCSY, ROESY, HSQC and HMBC were used to identify phenolic compounds (quercetin-3-*O*-glucoside and *trans*-feruloyl acid derivative). Then, a semi-automatic quantification with Simple Mixture Analysis plugin (SMA, MestReNova) was performed. Peak deconvolutions were performed using the Global Spectral Deconvolution (GSD) method. An example of applying the GSD function is presented in Figure S1. Semi-quantification of metabolites was performed by utilizing the internal standard (calcium formate) added at a known concentration. Compounds were quantified by the relative ratio of the intensities of their peak integrals and those of the internal standard. Appropriate analyte signals were evaluated according to the formula developed by Godelmann et al. (2016) presented below, where $m_x$ and $m_{std}$ are the masses (g) of the analyte and the standard, $MW_x$ and $MW_{std}$, the molecular weights (g/mol)

of the analyte and the standard, $n_x$ and $n_{std}$, the numbers of protons of the analyte and the standard, $A_x$ and $A_{std}$, the integral values of the analyte and the standard, respectively.

$$m_x = \frac{MW_x}{MW_{std}} \times \frac{n_{std}}{n_x} \times \frac{A_x}{A_{std}} \times m_{std} \times CF$$

### 2.5 Multivariate and statistical analysis

The data presented consists of the results obtained from samples analyzed in triplicate. Error bars of graphs show the standard deviation (SD) of mean values. Principal component analyses (PCA) with unit variance scaling, hierarchical clustering on principal components (HCPC) with Manhattan distance measure and 3D partial least squares-discriminant analysis (3D PLS-DA) were performed with R software version 3.4.3 using the FactoMineR plugin or SIMCA 15.0 (Umetrics, Sweden). The quality of the models was estimated by $R^2$ and $Q^2$ values both for PCA and PLS-DA analysis. Hotelling's T2 regions shown as an ellipse in the score plots define the 95% confidence interval of the modeled variation. The PLS-DA models were validated using the default SIMCA 7-fold cross-validation method and with permutation testing (200 permutations). Finally, the samples were classified into their corresponding groups in the SIMCA classification table.

Statistical analysis was carried out in Prism® 7.04 (GraphPad software, Inc.). Radar charts were made in Excel 2007. To identify significant differences between elicitor-treated leaves and control leaves, one-way analysis of variance (ANOVA) was applied ($p < 0.05$). Tukey's multiple comparisons test was performed to compare simultaneously the means of every sample.

## 3 Results

### 3.1 Identification of metabolites in differently elicited leaves

Extracts of grapevine leaves treated with MeJA, ASM or PHOS along with those of control leaves (Untreated and Triton) were subjected to $^1$H NMR analysis. Using NMR (1D and 2D) experiments and by comparison with reference data from the literature, we identified 29 metabolites including amino acids, organic acids, carbohydrates, phenolics and amines (Fig. 1, Table S2). The amino acids valine, threonine, alanine, γ-aminobutyric acid (GABA), proline, glutamine and glutamic acid were recognized in the area between δ 0.8–4.0, which also corresponds to that of some organic compounds such as acetic, pyruvic, succinic, malic and ascorbic acids. However, tyrosine was identified at δ 6.85 and 7.16, and fumaric acid at δ 6.65. The aliphatic region also showed the signals of choline, *myo*-inositol, syringic and shikimic acids. Despite the high overlapping of the signals in the carbohydrate region (δ 4.0–5.5 ppm), we identified the signals of the anomeric protons of fructose, sucrose, β- and α-glucose, as well as the third signal of malic acid (δ 4.38) and that of tartaric acid (δ 4.41). The remaining region, i.e. δ 5.5–8.5, which is known as the phenolic region, showed the signals of (+)-catechin, quercetin-3-*O*-glucoside (Q3OG), *trans*-feruloyl acid derivative, gallic and shikimic acids. Two amines were also found in this region: adenine at δ 8.13 and trigonelline at δ 8.85 and 9.14.

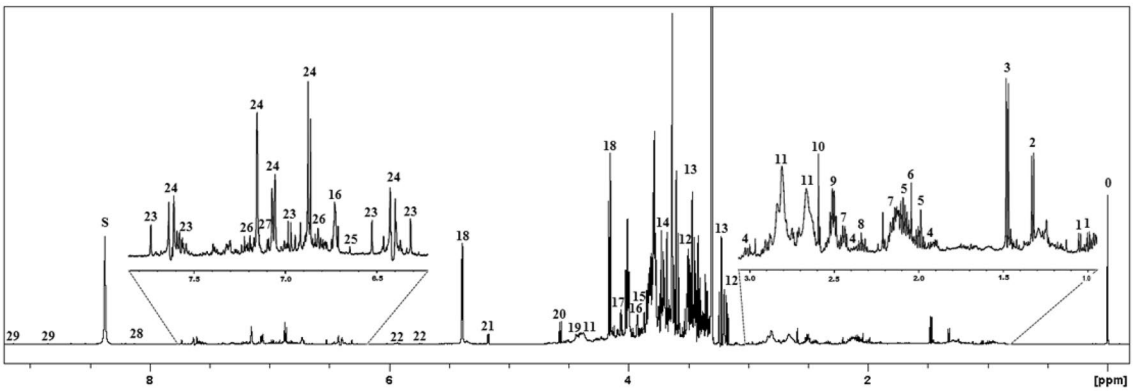


**Fig. 1** Representative $^1$H-nuclear magnetic resonance (NMR) spectrum of *Vitis vinifera* cv. Cabernet Sauvignon ('Untreated') leaf extract in $CD_3OD_3$ in $D_2O$ ($KH_2PO_4$ buffer, pH 6.0). 0, chemical shift reference standard (TMSP); S, standard for quantification (calcium formate); 1, valine; 2, threonine; 3, alanine; 4, γ-aminobutyric acid (GABA); 5, proline; 6, acetic acid; 7, glutamine; 8, pyruvic acid; 9, glutamic acid; 10, succinic acid; 11, malic acid; 12, choline; 13, *myo*-inositol; 14, ascorbic acid; 15, syringic acid; 16, shikimic acid; 17, fructose; 18, sucrose; 19, tartaric acid; 20, β-glucose; 21, α-glucose; 22, (+)-catechin; 23, quercetin-3-*O*-glucoside; 24, *trans*-feruloyl derivative; 25, fumaric acid; 26, tyrosine; 27, gallic acid; 28, adenine; 29, trigonelline

### 3.2 Global view of the impact of elicitor treatment on grapevine leaves

To interpret the changes in metabolism of leaves challenged by different elicitors, a series of chemometric methods was used. A principal component analysis (PCA) was performed to explore the whole data set obtained by relative quantification of all the metabolites identified in control leaves (Untreated and Triton) and elicitor-treated leaves (MeJA, ASM and PHOS). PCA score plot showed high statistical values of $R^2 = 0.963$, and $Q^2 = 0.592$, and clearly discriminated the samples by the first three principal components (PC1, PC2 and PC3) which cumulatively accounted for 81.93% of the variation in all variables (Fig. 2a). PC1 explained 46.24% of the variation and separated the elicitor samples from controls, while PC2 which accounted for 26.33% showed that the MeJA sample was different from the controls, ASM and PHOS treatments. Moreover, Untreated and Triton, the two controls, tended to be grouped together, as they occurred similarly in the three elicitor conditions (ASM, PHOS and MeJA). Although PC3 represented only 9.36% of the variation, it facilitated a slight separation of ASM and PHOS samples both between each other and from controls (Fig. 2a, Fig. S2a).

To gain more insight into the metabolic differences between control and elicitor-treated leaves, the correlation coefficient of the factor loadings and the square of the cosine of the variables were calculated for each PC (Figure S2 and Table S3). This allowed us to identify the compound that contributed the most to the separation of all the samples and to establish how significantly each metabolite was correlated to the axis of PC1, PC2 and PC3. Consequently, syringic acid (correlation coefficient of 0.972), threonine (0.968) and (+)-catechin (0.750)) showed the greatest influence on the scores for the three initial components, respectively (Table S3). Furthermore, the PCA factor loadings indicated that the control conditions (Untreated and Triton), and mainly the Untreated one, were concentrated by the majority of the identified metabolites, and particularly sugars (*myo*-inositol, fructose, α-glucose, β-glucose and sucrose), some organic acids (malic, pyruvic, tartaric, ascorbic and fumaric acids), and some phenolics (quercetin-3-*O*-glucoside, syringic, gallic and shikimic acids) (Fig. S2). The combination of PC1 and PC3 confirmed this grouping of ASM-, MeJA-, and PHOS-treated leaves (Fig. S2). The level of fumaric acid was the highest in Untreated leaves. Some specificity according to the type of applied elicitor was also noted. For instance, levels of glutamine, valine and acetic acid seemed to be higher in the MeJA sample. The ASM and PHOS samples displayed the highest amount of choline, despite their low contribution to the construction of the main components.

PCA was followed by partial least squares-discriminant analysis (PLS-DA) which improved the separation between treatments (Fig. 2b1). To identify the metabolites responsible for the discrimination in the PLS-DA score plot, a loading plot was generated (Fig. 2b2). The PLS-DA used the information contained in the descriptor X data matrix (metabolites) to predict the behavior of the response Y data matrix (treatments) (Fig. 2b2). The three-component PLS-DA model applied was evaluated by cross-validation. A high $Q^2$ (cumulative) value of 0.946 and high $R^2$ (cumulative) values ($R^2_X = 0.954$; $R^2_Y = 0.989$) indicated both strong predictability and the good fit of the model. Following prediction, all the samples were classified according to their group (100% of appropriate classification). The permutation test applied to the 6-component PLS-DA model produced intercepts of $R^2 < 0.3$ and $Q^2 < 0.05$, indicating the validity and the absence of over-fitting in the model.

Finally, the dendrogram of hierarchical clustering on principal components (HCPC) (Fig. 2c) confirmed the results obtained by the PCA and PLS-DA. The three elicitor conditions were clustered together with relative similarities. PHOS- and ASM-treated leaves had a similar metabolic profile.

### 3.3 Relative quantification of metabolites

To confirm the statistical significance of the results obtained by multivariate data analysis, we performed a one-way analysis of variance (ANOVA) followed by Tukey's multiple comparisons test. Relative quantification of identified compounds was based on the measurement of the average peak areas of the characteristic signals in comparison with the internal reference (calcium formate). The relative abundance of the compounds, as well as the levels of significant differences in all experimental conditions ($p < 0.05$), are reported in the supplementary materials (Fig. S3 and Table S4). To compare multiple variables and illustrate the comprehensive performance of the five different conditions, a radar chart was used by normalizing the data (Fig. 3, mean values in Table S4).

Some distinct metabolomic alterations were observed across the different classes of compounds. Changes were particularly apparent in the metabolism of sugars and organic acids. Two characteristic patterns of sugars emerged. The first included sucrose, β-glucose and α-glucose. Their relative quantity decreased after treatment of all the elicitors. Triton, the co-formulant of MeJA, affected these three sugars the least, whereas MeJA impacted them more drastically (around 2.5-fold). On the other hand, MeJA, PHOS and ASM affected fructose negatively to the same degree. *Myo*-inositol followed the same pattern, its amount decreasing significantly after all elicitor treatments. However, Triton also caused a decrease in the amount of *myo*-inositol.

All the organic identified acids were impacted (Fig. 3 and Fig. S3). Unlike sugars, no shared pattern emerged as

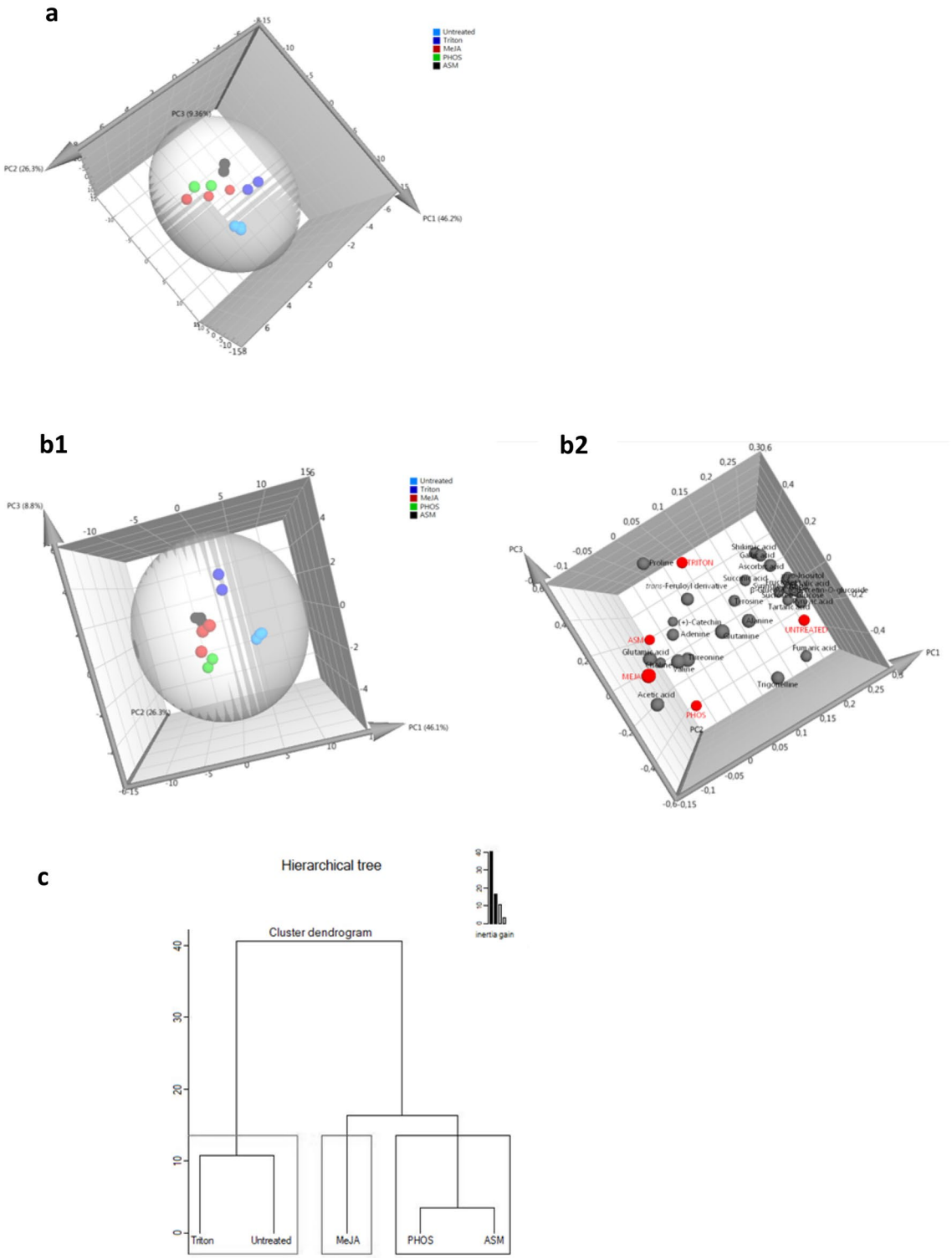


**Fig. 2** Score plot (**a**) of principal component analysis (PCA) on quantifications of all identified metabolites in grapevine leaves discriminating each condition at 24 h after elicitation: 'Untreated'—control leaves treated with distilled water; 'Triton'—control leaves treated with Triton X-100; 'MeJA'—leaves elicited with methyl jasmonate; 'PHOS'—leaves elicited with phosphonates (LBG-01F34); 'ASM'—leaves elicited with acibenzolar-S-methyl. The combination of PC1 and PC2, PC1 and PC3, and PC2 and PC3 is presented. Score (**b1**) and corresponding loading plot (**b2**) of partial least squares-discriminant (PLS-DA; model diagnostics were $R^2_X = 0.954$; $R^2_Y = 0.989$ and $Q^2 = 0.946$). The sphere indicates Hotelling T2 (0.95) for these models. Cluster dendrogram (**c**) performed by hierarchical clustering on principal components (HCPC)

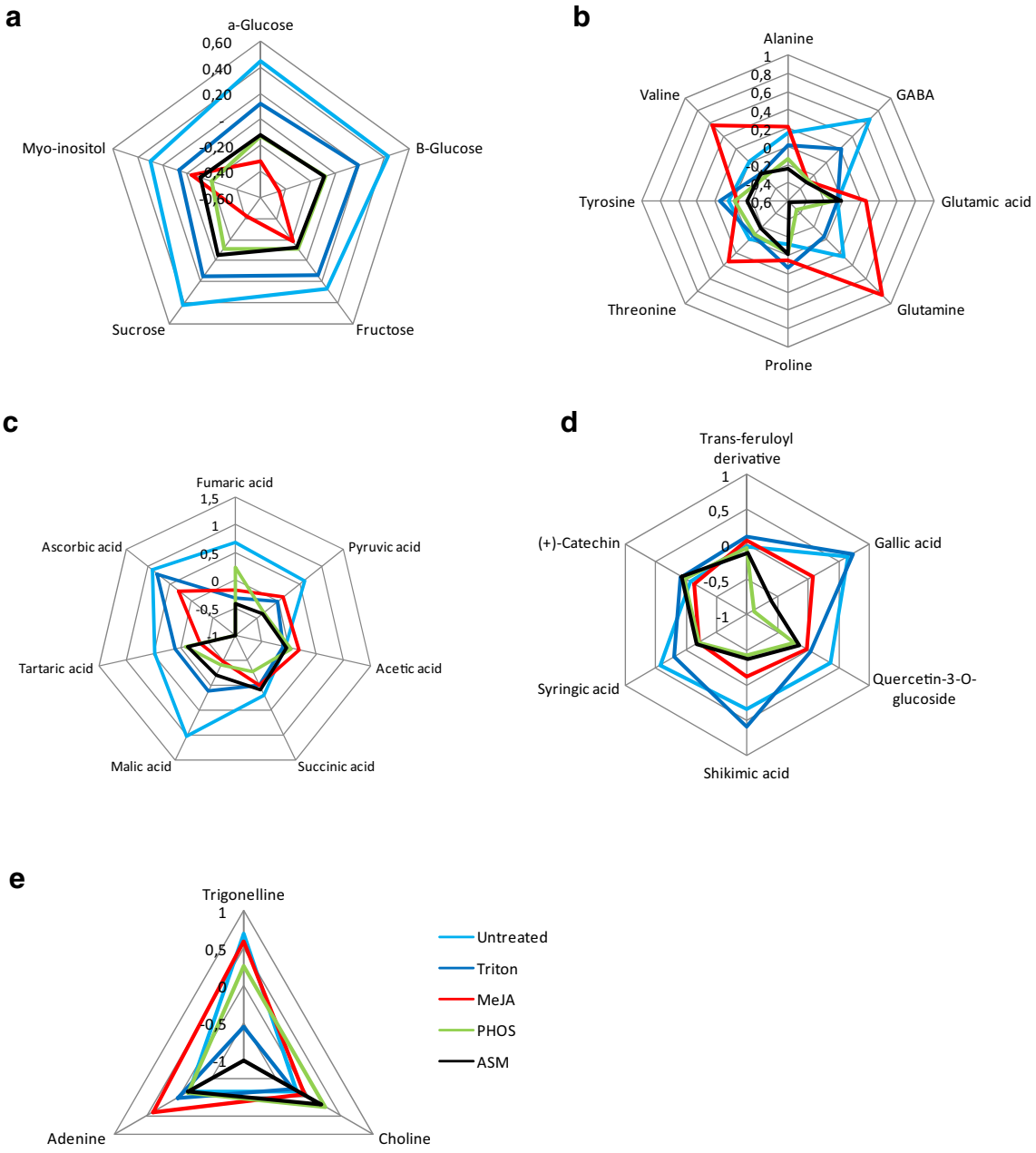


**Fig. 3** Radar charts comparing changes within classes of compounds identified in grapevine leaves differently elicited. Sugars (**a**), amino acids (**b**), organic acids (**c**), polyphenols (**d**), and amines (**e**)

both positive and negative changes occurred according to the treatment. Acetic acid remained at a basal level in all conditions, except in response to MeJA where its relative content increased. The relative quantity of succinic acid was lower in response to Triton, MeJA and PHOS, especially in comparison to the Untreated condition. A decline in fumaric and pyruvic acids was observed after all treatments, and even with Triton. This reduction by MeJA could be partially due to Triton, its co-formulant. All elicitors including Triton reduced the content of malic acid, whereas the levels of tartaric and ascorbic acids decreased significantly in leaves treated with MeJA, PHOS and ASM in comparison to both controls.

Among the amino acids identified, the relative content of glutamic acid, tyrosine and threonine did not change regardless of the treatment (Fig. S3b). However, all elicitors significantly reduced the quantity of GABA about 2.5-fold compared to the Untreated condition. Alanine occurred in nearly equal amounts in all conditions except ASM, where it slightly decreased. The relative content of proline increased

after Triton. MeJA was the only elicitor which increased the level of valine and glutamine. On the other hand, PHOS and ASM considerably inhibited glutamine while valine was not affected.

Unlike amino acids and organic acids, the profile of the relative quantity of gallic, shikimic and syringic acids, and Q3OG, was quite similar with a reduction when compared to the two controls. Nevertheless, in the case of Q3OG, Triton acted as the elicitors did. The relative quantity of gallic acid was affected neither by MeJA nor by its co-formulant, but it was highly impacted by ASM (5-fold) and even more by PHOS (10-fold). The *trans*-feruloyl derivative and (+)-catechin showed a similar level in all conditions.

Among the three identified amines, adenine was unaffected. The level of choline slightly increased after PHOS and ASM treatment, whereas the amount of trigonelline drastically decreased in the ASM sample but only slightly after Triton treatment.

## 4 Discussion

For the successful development of a natural defense stimulation strategy using elicitors as an alternative to pesticides in the vineyard, the metabolic profiling of plants treated with such compounds is required. The induction of plant defensive traits by PDS is known to involve costly metabolic changes. It forces plants to allocate their resources optimally to various competing demands and pathways, thereby creating trade-offs, i.e. promoting some functions and neglecting others as an inverse relationship (Caretto et al. 2015).

This present study is a proof-of-concept study carried out with a limited number of plants (four per modality) that aimed to give preliminary evidence of the effect of common elicitor compounds on the primary metabolism. For that, we investigated the impact of three elicitors, MeJA, PHOS and ASM on the foliar metabolism of *Vitis vinifera* cv. Cabernet Sauvignon cuttings during the first hours post-treatment (24 hpt). The changes in the metabolic profile of the elicited leaves were monitored by $^{1}$H NMR spectroscopy and compared to control samples, i.e. Untreated leaves or leaves sprayed with Triton, the MeJA co-formulant. Figure 4 shows the most likely metabolic interconnections of the compounds identified in this study.

The choice between growing and defending is a key point for plant survival, thus diverting carbon skeletons from the primary to the secondary metabolism. To establish a favorable energy balance for defense, the increase in defense-associated pathways is compensated by a reduction in other metabolic pathways. Photosynthesis-related genes and chlorophyll biosynthesis have been reported to be down-regulated following pathogen attack or elicitor treatment (Bolton 2009), leading to a reduction in plant fitness (growth and yield). In our study, this effect is reflected by a clear decrease in carbohydrates in all elicitor-treated leaves. A decrease in the level of sugars as a result of elicitation is known to occur with MeJA (Liang et al. 2006). As expected, *myo*-inositol was affected by all treatments since it is involved in the biogenesis of the cell wall, the phospholipid signaling pathway and the regulation of the cytoskeletal structure. Its reduction as a result of a stress response is not clear but was already reported in *Nicotiana tabacum* leaves infected with tobacco mosaic virus (Choi et al. 2006).

Similarly, most of the identified phenolics were significantly decreased after elicitor treatments. A reduction in the levels of phenolic metabolites as a result of ASM application was observed in *Arabidopsis thaliana* (Hien Dao et al. 2009). The decrease in shikimic acid, which is the crucial molecule in the phenylpropanoid pathway, may indicate an enhanced biosynthesis of the phenolic derivatives involved in defense responses. We hypothesize that this is also the case of the other phenolics, i.e. Q3OG, gallic and syringic acids, since they are the precursors of the phenylpropanoid metabolism. Unlike sugars and phenolics, amino acids were characterized by different patterns according to the condition of treatment. Some changes within this class of molecules were linked to nitrogen signaling, especially the GABA and glutamine metabolism. Although the mode of action of GABA in plant defenses is not clear, its production might be activated by biotic and abiotic stresses via the induction of glutamate decarboxylase, an enzyme catalyzing its synthesis (Lima et al. 2010). The accumulation of glutamine is considered as evidence of activation of the PAL pathway (Figueiredo et al. 2008). An increase in glutamine was noted in a wheat-resistant cultivar upon infection (Hamzehzarghani et al. 2005). Indeed, the glutamine content increased in MeJA-treated leaves, but decreased significantly in PHOS and ASM samples. Moreover, MeJA triggered an accumulation of valine. To our knowledge, the role of valine in plant stress has never been reported. The pool of amino acids was prominently changed in phosphite-treated Arabidopsis plants, in accordance with the literature (Berkowitz et al. 2013).

Large changes occurred in the metabolism of organic acids in treated leaves. There was a remarkable decrease in pyruvic, fumaric and malic acids, and a moderate one in succinic acid. The simultaneous decrease in succinic, fumaric and malic acids may result either from the stronger demand for the Krebs cycle intermediates required for the biosynthesis of other metabolites, including those involved in defense, or from the increased consumption of pyruvic acid. The latter mechanism might be due to the allocation of pyruvic acid to the polyphenol biosynthetic pathway for the purpose of elicitation/defense. A decrease in pyruvic acid and 2-oxoglutarate was also observed in *V. vinifera* cv. Gamay in vitro cultures elicited with oligogalacturonide

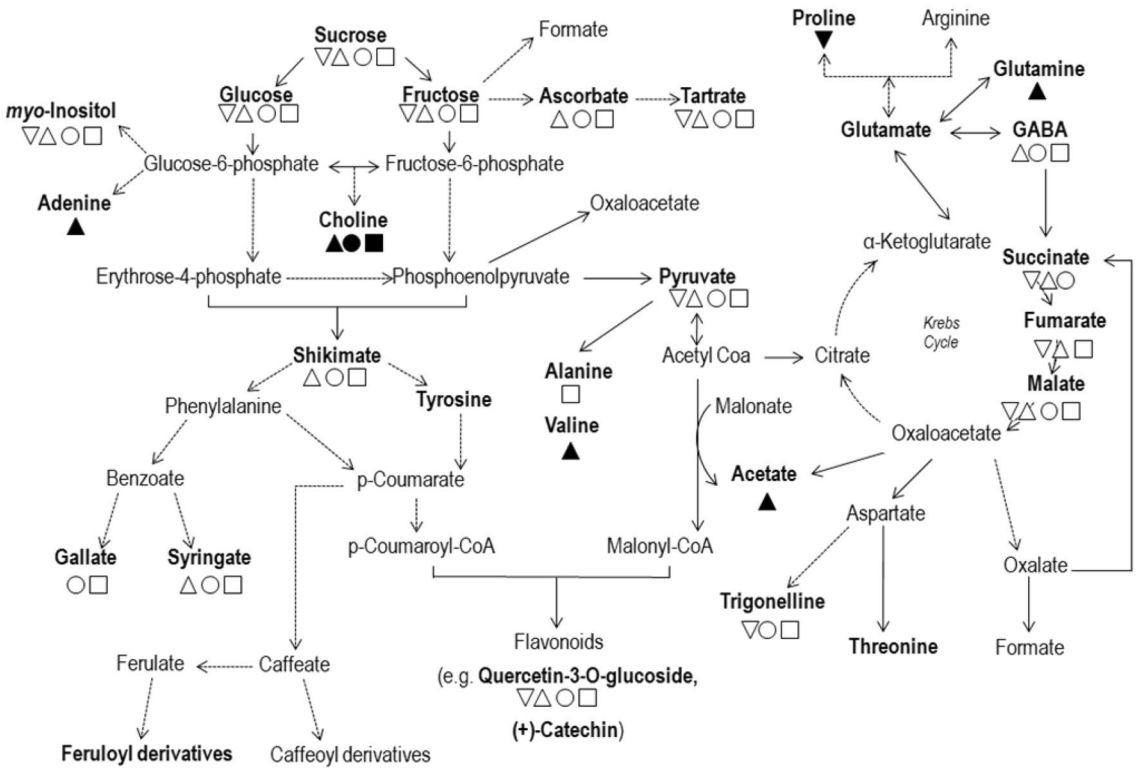


**Fig. 4** Simplified metabolic pathways influenced by different elicitors in *Vitis vinifera* cv. Cabernet Sauvignon leaves at 24 h after treatment. Compounds identified in the experiment by $^{1}$H NMR are shown in bold. Symbols associated with each metabolite show the significant changes with comparison to control leaves assessed at $p < 0.05$ through one-way ANOVA test. Metabolites that are set with empty symbols are significantly decreased after elicitation with respect to control leaves whilst those set with filled symbols are significantly increased. Lack of symbol indicates metabolites unaffected by elicitation. Reversed triangle—'Triton', triangle—'MeJA', circle—'PHOS', square—'ASM'. Kyoto Encyclopedia of Genes and Genomes—KEGG database was used to elucidate metabolic networks (https://www.genome.jp/kegg/)

(Krzyzaniak et al. 2018). Modifications of the concentrations of fumaric, malic, succinic and 2-oxoglutaric acids were monitored in opium poppy (*Papaver somniferum*) cell cultures treated with a fungal elicitor (Zulak et al. 2008). The levels of malate, succinate and fumarate were generally stable, reduced or even increased after treatment, depending on the moment of harvest (Zulak et al. 2008). Based on all these data, kinetic studies should be performed in order to monitor the evolution of organic acid levels according to the elicitor and the plant model investigated.

The biosynthesis of tartaric acid was inhibited by all treatments, mainly elicitors, and was accompanied by a reduced level of its precursor, ascorbic acid, which plays a role in plant stress and physiology (growth and development). A possible stress generated by elicitation could contribute to its degradation. Such a phenomenon was already observed in salad leaves and broccoli as an effect of harvest and treatments with hormones like MeJA (Dewhirst et al. 2017; Nishikawa et al. 2003). Acetic acid was the only organic acid whose level did not change or even increase after application of MeJA. Acetic acid is also known to inhibit some plant pathogenic fungi (Sehirli and Saydam 2016). Finally, the level of choline increased in PHOS and ASM conditions. This was probably a defense response since its role in plant stress has already been demonstrated (Mou et al. 2002; Zhang et al. 2010).

Taken together, our observations are in accordance with the literature data. However, the direction of the changes is sometimes different, perhaps due to the plant species investigated as well as the experimental model, the elicitors used and their concentration and/or time of incubation. Overall, this study gives first hints towards the effects of early (24 hpt) PDS treatments on grapevine plants: they are able to shift their major metabolites significantly, as occurs in response to pathogenic infection (Parvaiz et al. 2018).

## 5 Conclusion

To maintain plant fitness, advances in understanding global metabolic changes occurring under specific PDS conditions are fundamental. Our findings provide valuable preliminary

insights into the primary metabolic modifications of grapevine triggered by three elicitors (MeJA, ASM, and PHOS). Similar and/or specific metabolic modifications occur depending on the elicitor applied and the signaling pathway used for defense. We hypothesize that the induction of resistance to plant pathogens that correlates with primary metabolic modifications could negatively impact the overall fitness of plants. In the grapevine, this could influence the quality of grapes and wine. Therefore, elucidation of the metabolic responses that occur after elicitation would be helpful for developing strategies based on natural defense stimulation. Finally, the NMR technique applied in this initial study was found to be relevant as it covers a wide range of the metabolome. The long-term consequences of these three elicitors on the grapevine metabolome will now be studied.

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**Author's contribution** SC and MFCC conceived and designed the research. AlB, AnB, SC and MFCC conducted the experiments. AlB, GDC, LG, ILM and TR performed the metabolomics study and data analysis. AlB, JMM, AS and SC wrote and/or revised the manuscript. All authors read and accepted the final manuscript.

## Compliance with ethical standards

**Conflict of interest** The authors declare that they have no conflict of interest.

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