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Short Notes

Metabolite fingerprints of Chardonnay grapevine leaves affected by esca is both clone- and year-dependent

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Summary. Esca is one of the most widespread grapevine trunk diseases affecting vineyards. This complex disease leads to leaf alterations, wood necrosis and eventually to plant death. Esca symptoms are caused by several fungi inhabiting the xylem of host plants and degrading the wood structure. The main pathogens causing the disease are *Phaeomoniella chlamydospora*, *Phaeoacremonium minimum*, *Fomitiporia mediterranea* and other wood-rotting basidiomycetes. Grapevine susceptibility to esca can be predisposed by several factors, especially climate, vine age, and cultivar. An experiment was carried out (in 2015) to assess if esca expression on leaves could also be clone-dependent. Chardonnay clones 76 and 95 grown in the same plot were compared according to their developmental and physiological traits, metabolome, and foliar symptom expression. Leaves were sampled during summer on visually healthy vines as controls (C), and from asymptomatic (D-) and symptomatic (D+) shoots of esca-affected vines. Analysis of their metabolomes highlighted a clone-dependent metabolite fingerprint associated to esca expression. Opposite variations of specific metabolites were found between C and D+ leaves of both clones. The experiment was repeated (in 2018). Leaf samples could be discriminated, especially the C and D+ samples for each clone, but the differences were less marked than in the first experiment. Discriminant compounds were all different between the two experiments, and showed no opposite variations between C and D+ samples of both clones, which indicated variable metabolite responses from year to year for both clones. These results confirm that the leaf metabolite fingerprint associated to esca expression is clone-dependent, and is year-dependent in intensity and nature.

Keywords. *Vitis vinifera*, grapevine trunk diseases, metabolomics, clone.

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INTRODUCTION

Grapevine trunk diseases (GTDs) are widespread and progressively cause vine death in all wine producing countries (De la Fuente *et al.,* 2016). They affect and weaken vines, eventually leading to their death, and adversely affect vineyard yields and wine quality causing economic losses for wine industries. Gramaje and Armengol (2011) observed that increase of GTD incidence may be partly due to the worldwide grapevine planting "boom" during the 1990s, as it contributed to the propagation of potentially contaminated planting material and increased the area of vineyards reaching the age of esca symptom expression. Other factors also contributed to GTD spread, such as changes of production methods that favoured fungal infection, and the banning in 2003 in some countries of sodium arsenite and other chemicals that were widely used to control GTDs (Gramaje and Di Marco, 2015; Gramaje *et al.,* 2018; Mondello *et al.,* 2018). Since these bans, no efficient products have been available for effective GTD control, leading to important economic losses in all major vine-growing countries, including Spain, France, Italy, and the United States of America (De la Fuente *et al.,* 2016; Guerin-Dubrana *et al.,* 2019). In 2012, it was estimated that if only one percent of vines within vineyards were replaced each year because of GTDs, the worldwide annual financial cost would exceed one and a half billion $US (Hofstetter *et al.,* 2012).

Esca is one of the main GTDs, and is caused by several fungus genera and species, including the Ascomycetes *Phaeomoniella chlamydospora*, *Phaeoacremonium minimum* and Basidiomycetes such as *Fomitiporia mediterranea* (Surico *et al.,* 2006). These pathogens induce wood symptoms, including black spots, vascular streaking, discolouration and necrosis. This complex disease is also associated with foliar and berry symptoms (Mugnai *et al.,* 1999; Surico, 2009; Mondello *et al.,* 2018). Foliar symptoms, known as Grapevine Leaf Stripe Disease (GLSD), consist of tiger-stripe patterns leaves, and this is considered as a chronic form. Apoplectic symptoms can also occur, and these include leaf wilting rapidly followed by partial or total death of plants (Mugnai *et al.,* 1999). The mechanisms leading to the appearance of esca foliar symptoms are unclear, since on individual vines they can appear in one growing season, but not the next. It is also difficult to reproduce external symptoms in artificial conditions (Mondello *et al.,* 2018; Reis *et al.,* 2019).

Several factors can affect the symptom appearance and the level of expression, including region, soil, vine training system, cultivar, rootstock, vine age, and climatic conditions, which vary from one vintage to another (Mugnai *et al.,* 1999; Van Niekerk *et al.,* 2011; Lecomte *et al.,* 2012; Andreini *et al.,* 2014; Murolo and Romanazzi, 2014). In addition, the grapevine clone is important, as symptom expression is modulated by rootstock and clone combination (Murolo and Romanazzi, 2014). We showed (in an experiment in 2015) that different metabolite fingerprints are associated with expression of esca symptoms between leaves of vines of Chardonnay clones 76 and 95, with opposite variation of specific secondary metabolites between control and diseased leaves of both clones (Moret *et al.,* 2019). Due to the erratic occurrence of the esca foliar symptoms we repeated the experiment to validate the previous observations in another vintage. The present study thus assessed the metabolite fingerprint of leaves from symptomatic and asymptomatic vines of Chardonnay clones 76 and 95 in a second growing season (in 2018), and results were compared to those of the 2015 study (Moret *et al.,* 2019).

MATERIALS AND METHODS

Experimental plot

The experimental plot was located in Chablis vineyard, Burgundy, France (126 m above sea level, GPS coordinates 47_47046.9600N, 3_47026.1700E), and the vines were planted in 2002, half with *Vitis vinifera* cv. Chardonnay clone 76 and the other half with clone 95, both grafted on Fercal rootstocks (Moret *et al.,* 2019). The plot was on a clay soil, and the vines were trained in a double Guyot system. The plot was of total area of 0.7 ha, and contained a total of 4361 vines growing at spacing of 1 × 1.5 m.

Climate data

Climatic parameters were recorded by a meteorological station located at Saint-Bris le Vineux (Burgundy, France) <8 km from the experiment plot. The station is representative of plot climate conditions, and is used as a reference by the Chambre d'Agriculture. Rainfall and daily temperatures were recorded. Available data were from year 2005 to year 2018.

Incidence of Esca symptoms

The occurrence of diseased vines within the plot was established by visual observations of esca symptom expression. Diseased (D) and healthy (as controls, C) vines were counted at the end of August every growing season since 2005. On diseased vines, asymptomatic (D-)

and symptomatic (D+) shoots were distinguished. Control vines were considered healthy when they did not display foliar symptoms during the year of sampling or during any other year.

Agronomic and physiological parameters

Two series each of ten successive visually healthy (asymptomatic) vines were selected in the plot to be representative of the whole plot, and were used to follow agronomic parameters and for $\Delta^{13}C$ analyses.

Agronomic parameters

Bud burst, fertility and vine vigour were determined as described in Moret *et al.,* (2019), only on visually healthy vines (C). Bud burst and fertility were calculated as follows:

Bud burst percentage = (number of shoots / number of buds remaining after Chablis pruning) × 100

Fertility = number of clusters/number of shoots

Vine vigour was determined by measurements of cane diameter using calipers. Measurements were performed between the first and second node of the third shoot starting from the old part of the trunk (Chablis pruning).

Water status

The water stress of control and diseased vines, was assessed using $\Delta^{13}C$ measurements, which were made at the end of summer on must obtained from 150 fruit bunches collected from the four grapevine modalities (Farquhard *et al.,* 1989). The GISMO Platform (University of Burgundy, France) performed these analyses.

METABOLIC ANALYSIS

Sampling

Asymptomatic green leaves were sampled from randomly selected vines, from five healthy (C) and five esca-affected (D) vines showing partial apoplexy symptoms, for each clone, on July 24, 2018, during symptom expression at the early veraison vine growth stage. For each clone, five control leaves were collected, and for diseased samples, five leaves were sampled on asymptomatic canes (D-), plus three leaves on symptomatic canes (D+). Leaves were taken from the same foliar rank (approximately the same age), and were conserved in liquid nitrogen during sampling then ground and stored at -80°C.

GC-MS analyses

GC-MS analyses were carried out as described by Krzyzaniak *et al*., (2018) and Moret *et al.,* (2019). Briefly, 50 mg of ground leaves were resuspended in 1 mL of frozen water:acetonitrile:isopropanol (2:3:3) containing ribitol (4 µg mL^{-1}), and were then extracted for 10 min at 4°C with shaking (1400 rpm). Insoluble material was removed by centrifugation (20,000×g for 5 min). Three blank tubes underwent the same steps as the samples and a quality control was made by pooling an equal volume of each sample type. Thirty µL were collected and dried overnight at 35°C in a Speed-Vac vacuum centrifuge. For derivatization, 10 µL of 20 mg mL^{-1} methoxyamine in pyridine were added to each sample, and the reaction was performed for 90 min at 28°C under continuous shaking. Ninety µL of N-methyl-N-trimethylsilyl-trifluoroacetamide (MSTFA) (Aldrich 394866–10 × 1 mL) were then added and the reaction continued for 30 min at 38°C. After cooling, 45 µL were transferred to an Agilent vial for injection. Four h after the end of derivatization, the whole sample series was first injected (1 µL) in splitless mode, and then in split mode (1/30), in an Agilent 7890A gas chromatograph coupled to an Agilent 5977B mass spectrometer, as previously detailed (Krzyzaniak *et al*., 2018).

Data processing and statistical analyses

Data processing and statistical analyses were carried out using R-3.6.1 and RStudio software. For climate data assessments, mean temperature and the sum of rainfall were calculated for the 2008-2017 period, and for the year 2018. The differences of foliar disease expression count data were analyzed with Chi-square tests of proportions (at $P < 0.05$).

For processing of GC-MS data, the data files in NetCDF format were analyzed with AMDIS software. A home retention indices/mass spectra library built from the NIST, Golm2, and Fiehn databases and standard compounds were used for the identification of metabolites. Chromatogram peak areas were also determined with the Targetlynx software (Waters) after conversion of the NetCDF file in Masslynx format. AMDIS, Target Lynx in splitless and split 30 mode data were compiled into a single Excel file for comparison. After blank mean subtraction, peak areas were normalized to ribitol and fresh weight (µg mg^{-1} fresh weight). These data were fil-

tered by removing entries with 50% or more missing values across samples, except for those that were significant at $P < 0.01$ for Fisher's Exact Test, which compared the number of missing and non-missing values between the biological groups of interest. After filtering, missing values were replaced by an estimate of the limit detection (half the lowest value). Multivariate descriptive analyses were carried out for these processed data, such as PLS and Volcano plots (Goodacre *et al.,* 2007; Vinaixa *et al.,* 2012; Worley and Powers 2013; Schiffman *et al.,* 2019). $\Delta^{13}C$ and agronomic data were checked for homogeneity of variance (Levene test) and for normality (Shapiro Wilk test). $\Delta^{13}C$ data were then tested using Kruskall Wallis tests followed by a Dunn's *post hoc* test. For agronomic parameters, bud burst percentage was evaluated with Chi-square tests, and vigour and fertility parameters were evaluated using Mann Whitney two-sample tests.

RESULTS

Climate data

Recorded climate data are presented in Figure 1. Mean temperatures during summer were slightly higher in 2018 compared to the past 10 years, especially in July and August (respectively, 22.7 and 21.2°C in 2018, compared to 10 year means of 19.9 and 19.3°C). Precipitation was less in 2018 compared to the past 10 years, particularly in June, July and August.

Esca foliar symptom expression

Esca symptom expression is described in Table 1. Symptom expression was moderate in 2018, with 4.42% of clone 76 vines and 3.16% of clone 95 vines affected. In 2016, 7.69% of clone 76 vines and 6.97% of clone 95 were affected, and in 2017, 9.08% of clone 76 and 8.02% of clone 95 vines showed esca symptoms. There were no statistically significant differences between the two clones within each year.

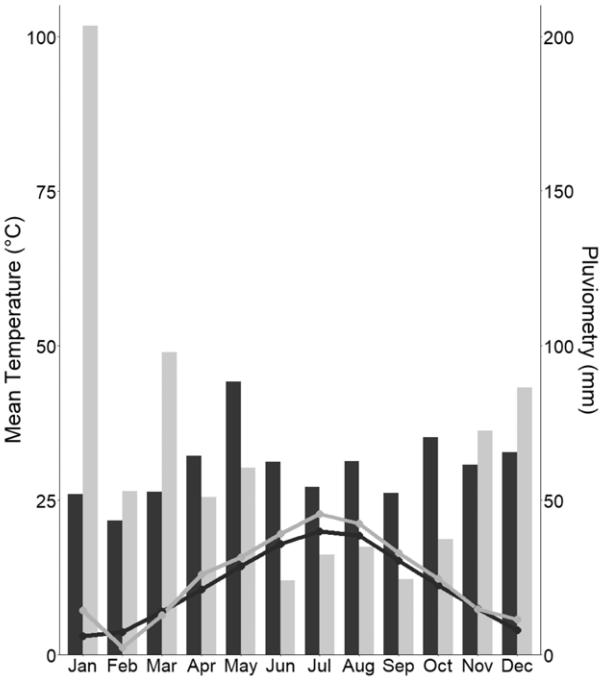

Figure 1. Ombrothermic diagram of monthly data relevant the study site showing rainfall (mm; histograms) and mean temperatures (°C, symbols connecting lines). Mean data for the 10-year period 2008-2017 are displayed in black, and 2018 data are displayed in grey.

Agronomic parameters

No statistically significant differences were found between clones 76 and 95 for the mean bud burst, fertility or vigour parameters (Table 2).

Water status

For both clones, $\Delta^{13}C$ values ranged from -22.82 to -23.75, which correspond to weak to moderate water stress (Van Leeuwen *et al.,* 2009). No significant differences were detected between the two clones, nor between the control or diseased vines of both clones (Table 2).

Table 1. Proportion (%) of grapevines displaying esca symptoms during August of eight years within the study plot, for Chardonnay clones 76 and 95. Statistically significant differences of expression ($P < 0.05$) between clones within each year are indicated by different letters.

Clone	Year							
	2009	2010	2011	2012	2015	2016	2017	2018
76	0.20a	0.40a	1.38a	2.39a	0.41a	7.69a	9.08a	4.42a
95	0.00a	0.79a	1.60a	3.82a	0.62a	6.97a	8.02a	3.16a

Table 2. Means and ranges of $\Delta^{13}C$, vigour, fertility and bud burst parameters obtained for the Chardonnay grapevine clones 76 and 95 from C (control) and D (esca-affected) leaf samples. Bud burst percentage = (number of shoots / number of buds remaining after Chablis pruning) × 100. Fertility = number of clusters/number of shoots. Vine vigour was determined by measurements of cane diameter between the first and second node of the third cane starting from the old part of the trunk (Chablis pruning). Statistically significant differences ($P < 0.05$) between clones are indicated by different letters.

Clone			$\Delta^{13}C$	Vigour	Fertility	Bud burst
Clone 76	C	Range	-23.03 to -22.92	0.48 to 1.19	1.11 to 2.42	87.50 to 100
		Mean	-22.97a	0.84a	1.85a	97.2a
	D	Range	-23.75 to -22.82	-	-	-
		Mean	-23.26ab	-	-	-
Clone 95	C	Range	-23.28 to -23	0.46 to 1.96	1.30 to 3.37	55.60 to 100
		Mean	-23.12ab	0.86a	2.06a	91.36a
	D	Range	-23.75 to -23.30	-	-	-
		Mean	-23.48b	-	-	-

Metabolite analyses

GC-MS analyses of leaf samples revealed 227 compounds, of 157 were identified (data not shown). Principal component analysis (PCA) showed overlapping groups for C, D- and D+ leaf samples from clones 76 and 95 (data not shown). PLS allowed separation of clone 76 D+ group compounds from those in clone 76 C and clone 95 C samples, whereas the compounds from the clone 76 D- group overlapped with both 76 C and D+. For clone 95, groups C, D- and D+ overlapped (Figure 2). Further analyses focused on C *versus* D+ comparisons. Volcano plots were produced to compare metabolite accumulation in C and D+ samples for each clone (Figure 3). Metabolites and their respective values are displayed in Table 3. For metabolites that were detected in several samples, after data processing with the different software packages, only the most relevant ones were selected and listed. Compounds with aberrant concentrations across samples (*i.e.* not constant during analyses and high variability in sample groups C, D or D+) were also not included in Table 3. For clone 76, only glycerate was more accumulated in C samples, whereas serine, and three other non-identified compounds were more accumulated in D+ samples. For clone 95, D-erythronic acid, myo-inositol-1-P, threonatelactone, and one unknown compound were more accumulated in C samples. In contrast, asparagine, galactosylglycerol, resveratrol, and four unknown compounds were more accumulated in the D+ samples.

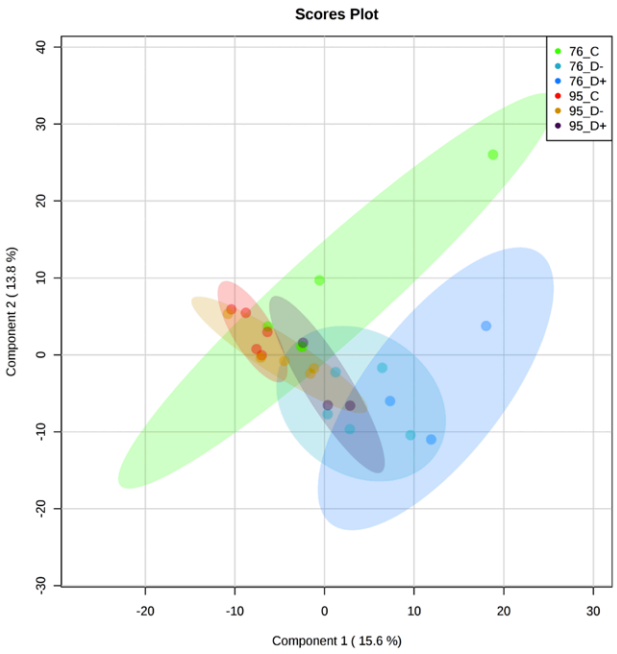

Figure 2. Partial Least Square (PLS) analysis of metabolite data obtained from GC-MS analyses of grapevine leaf extracts from different grapevine leaf samples of Chardonnay clones 76 and 95. For each group, individual samples are displayed as symbols and concentration ellipses are included. (C): green leaves sampled on heathy vines, as controls; (D-): green leaves sampled on asymptomatic canes of esca-affected vines showing partial apoplexy symptoms; (D+): green leaves sampled on symptomatic canes of esca-affected vines showing partial apoplexy symptoms.

DISCUSSION

In 2015, we studied a field plot planted with the two Chardonnay grapevine clones 76 and 95, to determine if esca foliar expression was clone dependent. The results highlighted that metabolites differentially accumulated between C and D+ leaves for both clones, and that there were differences in the metabolite profiles of C and D- leaves only for clone 95. Accumulation of some metab-

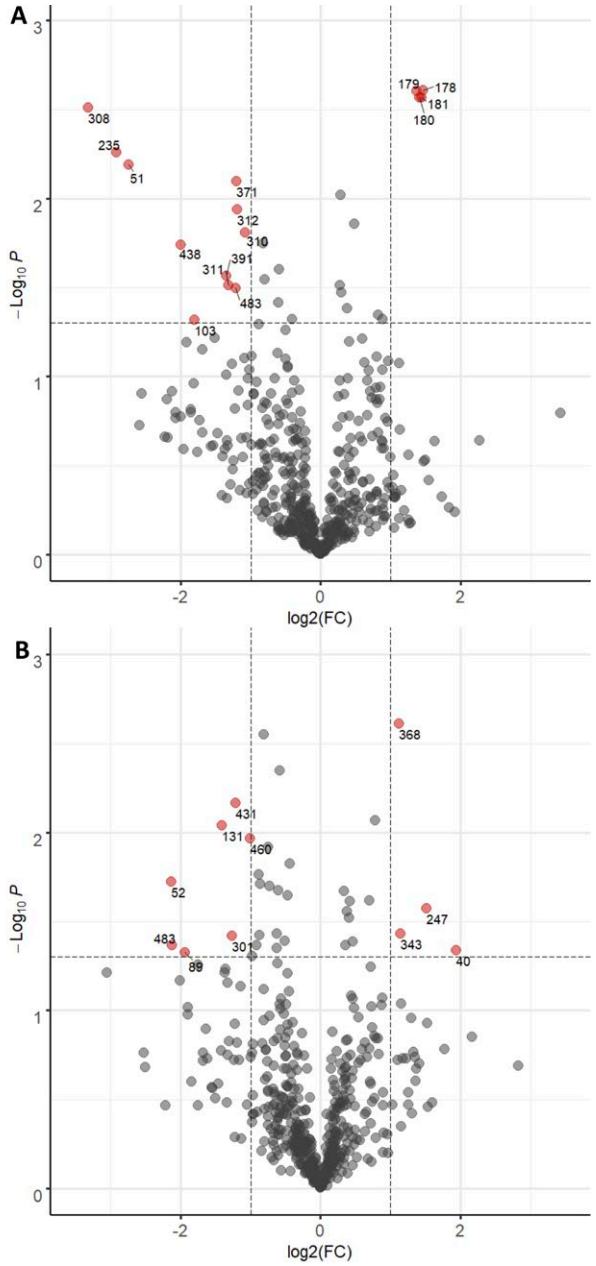

Figure 3. For each metabolite, fold change (C/D) and Student's T test p-value were calculated between C (control) and D (diseased) samples. The results are displayed on a Volcano plot, with $P < 0.05$ and FC > 2 thresholds. Each metabolite is displayed by a symbol, in red for $P < 0.05$, FC > 2, or in grey for $P > 0.05$, FC > 2. Only metabolites that had fold differences < 12 are displayed. Metabolites that were significant and validated the fold change threshold (red symbols) are annotated with their ID numbers. The panels are organized as A) clone 76 C *versus* D+, B) clone 95 C *versus* D+.

olites in opposite ways was also shown in D+ leaves of both clones, with lower amounts than in the control leaves of clone 76, and greater amounts for clone 95. The present study aimed to confirm these results for a second year (2018), where different environmental conditions applied to vines that were 3 years older.

Table 3. Metabolite analyses. Significant metabolite ID numbers, names, values and respective accumulations of significant metabolites ($P < 0.05$, fold change (FC) > 2) in grapevine leaf samples from Chardonnay grapevine clones 95 and 76. (C = greater accumulation in control leaf samples, D = greater accumulation in esca-affected leaf samples).

ID	Metabolite	*P*	FC	Accumulation	Clone
69	D-Erythronic acid	0.0099	1.05 10^2	C	95
245	Myo Inositol-1-P	0.0266	2.85	C	95
339	Threonatelactone	0.0371	2.20	C	95
364	U1579/306	0.0024	2.17	C	95
27	Asparagine	0.0134	0.02	D	95
129	Galactosylglycerol	0.0091	0.38	D	95
298	Resveratrol	0.0379	0.42	D	95
426	U2472.4/204	0.0068	0.43	D	95
436	U2565.5/179	2.84E-05	0.13	D	95
454	U2802.8/355	0.0108	0.50	D	95
476	U3142.4/202 Leu-Trp	0.0431	0.23	D	95
179	Glycerate	0.0027	2.70	C	76
308	Serine	0.0308	0.40	D	76
367	U1601.5/292	0.0080	0.43	D	76
432	U2540.8/204	0.0182	0.25	D	76
476	U3142.4/202 Leu-Trp	0.0319	0.43	D	76

In 2018, incidence of esca symptoms was low (4.42% for clone 76 and 3.16% for clone 95). This weak symptom expression can be explained by the relatively young age of vines (16 years). However, incidence was less than in 2016 (7.69% for clone 76 and 6.97% for clone 95) and 2017 (9.08% for clone 76 and 8.02% for clone 95). This can be partly explained by the particular climatic conditions that applied, especially during July, which is one of the most determinant months for expression of esca symptoms as affected by climatic conditions (Calzarano *et al.,* 2019). Temperatures were higher and precipitation was less in July 2018 compared to the average conditions of the previous 10 years. These elevated temperature and rainfall conditions during the growing season have been previously associated with high numbers of asymptomatic plants, whereas high rainfall and low temperatures may be correlated with increased symptom expression (Calzarano *et al.,* 2019; Serra *et al.,* 2019). Also, in July and August, rainfall was recorded on few days, with no rainfall precipitation for the rest of the months. In July, rainfall was recorded on 2 d, followed by 7 d of high temperatures 2 weeks later, and followed by 1 d of very high precipitation (20.5 mm). These climatic conditions led to moderate esca symptom expression and weak-to-

moderate water stress. Since the precise effects of precipitation on symptoms expression are not fully understood (Andreini *et al.,* 2014), and because expression and climatic data are variable, we could not directly correlate the two. Over the previous 8 years, esca has progressed with vine age, despite year-dependent expression. However, no statistically significant difference in expression between clones 76 and 95 was detected, indicating that these two clones do not have different susceptibilities to esca disease in the local French conditions.

Agronomic parameters were analyzed for control samples, to compare both clones in relation to the environmental conditions, independently of the disease. No statistically significant differences in vigour, bud burst or fertility parameters between clones 76 and 95 were detected. The differences observed for metabolite comparisons were therefore not due to clone-dependent physiological or agronomic factors.

PCA analyses of metabolite data showed overlapping groups, indicating that the analyses did not discriminate the different leaf sample groups analyzed, so there were no statistically significant differences when all the metabolites were considered. The deeper PLS analysis allowed discrimination of clone 76 D+ from C groups from both clones. For clone 95, the leaf sample groups C, D- and D+ overlapped. The overlapping area between C and D+ was small, indicating that, while the metabolites detected did not differ between these groups, some specific compounds were differentially accumulated. Volcano plots were therefore used to compare C and D+ samples from clones 76 and 95, and this highlighted different metabolite fingerprints for C and D+ for each clone. For clone 76, only glycerate was more accumulated in C samples, whereas serine was one of the compounds more accumulated in D+ samples. Beside photorespiration, serine can be synthesized in cell cytosol from 3-phosphoglycerate *via* glycerate as an intermediate (Igamberdiev and Kleczkowski, 2018). Despite being a minor pathway, this may play a role in highly specific stress conditions by regulating redox balance. For clone 95, resveratrol accumulated in D+ samples. This compound is a known grapevine phytoalexin and its accumulation in diseased organs is well documented (Chong *et al*., 2009;Adrian *et al.,* 2012). In our previous study, we observed accumulation of glycosylated resveratrol in D+ samples (Moret *et al.,* 2019). No other similarities in the metabolite profiles between years 2015 and 2018 were detected.

The results obtained in 2018 were very different from those obtained in 2015, for esca incidence, climate conditions and discrimination between samples and clones. The discriminating metabolite compounds were also different. Metabolomics still allowed detection of a clone-dependent leaf response to esca. However, this response was year-dependent and differed qualitatively and quantitatively when discriminating compounds were compared. Several factors are likely to explain these differences. Environmental conditions can affect leaf metabolism, and also esca foliar symptom expression (Fischer and Ashnaei 2019; Songy *et al.,* 2019). In addition, metabolite differences could be influenced by environmental conditions, as suggested by Bettenfeld *et al.,* (2020). They could also be due to changes in pathogen metabolism from year to year, indirectly influenced by the climatic conditions. Since esca pathogens produce toxins (Masi *et al.,* 2018), it is possible that their release varies qualitatively and quantitatively over time, leading to subsequent differences in the leaf metabolism.

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