RESEARCH PAPER

Metabolomics reveals simultaneous influences of plant defence system and fungal growth in *Botrytis cinerea*-infected *Vitis vinifera* cv. Chardonnay berries

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Abstract

*Botrytis cinerea* is a fungal plant pathogen of grape berries, leading to economic and quality losses in wine production. The global metabolite changes induced by *B. cinerea* infection in grape have not been established to date, even though *B. cinerea* infection is known to cause significant changes in chemicals or metabolites. In order to better understand metabolic mechanisms linked to the infection process and to identify the metabolites associated with *B. cinerea* infection, 1H NMR spectroscopy was used in global metabolite profiling and multivariate statistical analysis of berries from healthy and botrytized bunches. Pattern recognition methods, such as principal component analysis, revealed clear metabolic discriminations between healthy and botrytized berries of botrytized bunches and healthy berries of healthy bunches. Significantly high levels of proline, glutamate, arginine, and alanine, which are accumulated upon plant stress, were found in healthy and botrytized berries of botrytized bunches. Moreover, largely degraded phenylpropanoids, flavonoid compounds, and sucrose together with markedly produced glycerol, gluconic acid, and succinate, all being directly associated with *B. cinerea* growth, were only found in botrytized berries of botrytized bunches. This study reports that *B. cinerea* infection causes significant metabolic changes in grape berry and highlights that both the metabolic perturbations associated with the plant defence system and those directly derived from fungal pathogen growth should be considered to better understand the interaction between metabolic variation and biotic pathogen stress in plants.

Key words: berry, *Botrytis cinerea*, champagne, chardonnay, metabolomics, NMR, *Vitis vinifera*.

Introduction

*Botrytis cinerea* is a filamentous plant pathogen that can be easily found anywhere in the world and that causes necrotic lesions on a wide range of plants and fruit. In particular, *B. cinerea* seriously damages grape berries between the onset of ripening and harvest, through the secretion of extracellular enzymes that degrade the plant cell wall for pathogen penetration and for nutrient consumption, which include aspartic protease, laccase, pectin methyl esterases, and polygalacturonases (Wubben et al., 1999;
Valette-Collet et al., 2003; ten Have et al., 2004; Cilindre et al., 2008). Grape berry infection by B. cinerea in the vineyard further leads to reductions in the yield and the quality of grapes and wine, as a consequence of significant modifications in the chemical composition of the grape berry itself (Bocquet et al., 1995).

Recently, such modifications of the global metabolite profiles were reported in champagne base wines when vinified with B. cinerea-infected grapes, thus revealing an inhibition of the wine fermentation and a subsequently quality loss (Hong et al., 2011). Therefore, studies of the overall effects of B. cinerea infection at the grape level are needed to improve the understanding of the physiological status and of the metabolic mechanisms associated with B. cinerea infection in grapes.

Today, state-of-the-art analytical approaches like metabolomics have emerged and a global understanding of whole-plant metabolic mechanisms is accessible. Metabolomics is a powerful method for a comprehensive investigation of metabolite variations in biological systems. It generally consists of global metabolic profiling and multivariate statistical analysis, and thereby provides efficient visualization and identification of the metabolites that depend on plant physiological conditions (Allwood et al., 2008). In particular, the 1H NMR-based metabolomics is widely used to better understand the physiology-related metabolic pathways in plants (Krishnan et al., 2005; Leiss et al., 2011). Recently, it has been demonstrated that metabolite levels of grape berries and wines are dependent on vintage, which is linked mainly to different climatic conditions prevailing at harvest time (Pereira et al., 2006; Lee et al., 2009). Although a number of studies through metabolite profiling or metabolomic studies in plants have reported that global metabolic perturbations help to gain insights into plant disease or stress responses (Choi et al., 2004; Krishnan et al., 2005; Abdel-Farid et al., 2009; Brechenmacher et al., 2010; Dai et al., 2010; Lima et al., 2010; Skirycz et al., 2010; Ward et al., 2011), few studies have focused on the global metabolic influences or the overall effects of biotic stress on grapevine Vitis vinifera (Ali et al., 2009; Lima et al., 2010). In particular, comprehensive investigation on metabolic changes as induced by B. cinerea infection in grape berries has not yet been clearly established. The aim of this study was to characterize and understand the comprehensive metabolic influence of B. cinerea infection in berries by means of a 1H NMR-based metabolomic approach.

Materials and methods

Chemicals

All chemical reagents were of analytical grade. The standard reagents, deuterium oxide (D2O, 99.0% 2H) and 3-(trimethylsilyl) [2,2,3,3–2H4] propionate (TSP, 98%), were purchased from Sigma (St Louis, MO, USA).

Samples

Five bunches of healthy and botrytized grape clusters (V. vinifera cv. Chardonnay) were randomly hand-harvested in the same vineyard in the Champagne area (France) in September 2010. All the intact berries were manually divided into skin and pulp, from which fungal mycelium on skin was carefully removed to exclude the effect of the mycelium itself on variations of pulp metabolites. Healthy skin and pulp were obtained from berries collected from healthy grape bunches (HH) but other samples of healthy skin and pulp were prepared from healthy berries of botrytized grape bunches (HB). Moreover, botrytized skin and pulp were obtained from botrytized berries collected from botrytized grape bunches (BB). HB did not have any contact with BB. All the skin and pulp samples were stored at –80 °C until extraction. Sample extraction was carried out according to Kim et al. (2010). Skins and pulps were separately ground with a pestle and mortar under liquid nitrogen and then freeze-dried for 48 hours. Freeze-dried samples (20 mg) were dissolved in a mixture of methanol-d4 (CD3OD, 400 µl), oxalate buffer (320 µl, pH 2.7, 200 mM, H2O/D2O 50:50), and deuterated water (80 µl, included 5 mM TSP) in a 1.5 ml Eppendorf tube. The mixture was sonicated to extract metabolites at 20 °C for 20 min and then centrifuged at 13,000 rpm for 15 min at 10 °C.

NMR spectroscopic analysis of skin and pulp extracts

The supernatant of each extract was transferred into 5 mm NMR tubes. D2O and TSP in the supernatant provided a field frequency lock and a chemical shift reference (1H, δ 0.00), respectively. 1H NMR spectra were acquired on a Bruker Avance 600 spectrometer (Bruker Biospin, Rheinstetten, Germany), operating at 600.16 MHz 1H frequency and a temperature of 300 K, using a cryogenic triple-resonance probe. A standard Bruker water presaturation pulse sequence (‘zgpr’) was used over a spectral width of 7194.24 Hz with 64 scans collected into 32 K data points after four dummy scans, using a 5 s relaxation delay and 5 s total acquisition time. A 0.3 Hz line-broadening function was applied to all spectra prior to Fourier transformation. Signal assignment for representative sample was facilitated by two-dimensional total correlation spectroscopy (TOCSY), heteronuclear multiple bond correlation (HMBC), heteronuclear single quantum correlation (HSQC), spiking experiments, and comparisons with a previous study (Hong et al., 2011). Furthermore, one-dimensional statistical TOCSY was also used for the signal assignment (Cloarec et al., 2005a).

Multivariate statistical analysis

All NMR spectra were manually corrected for phase and baseline distortions and then converted to ASCII format with full resolution. The ASCII format files were imported into MATLAB (R2008a, The Mathworks, Natick, MA). Probabilistic quotient normalization of the spectra using the median spectrum to estimate the most probabilistic quotient was carried out after total integral normalization to avoid dilution effects of samples and effects of metabolites in massive amounts on changes in the overall concentration of samples (Dieterle et al., 2006). After calibration of chemical shift to either glucose or TSP (Pearce et al., 2008), the spectra were further aligned by the isochrifthod method (Savorani et al., 2010). The full resolution of 1H NMR grape skin and pulp spectra without spectrum bucketing or binning was used for multivariate statistical analysis. Principal component analysis (PCA), an unsupervised pattern recognition method, was first performed to examine the intrinsic variation in the data set. Orthogonal-projection on latent structure discriminant analysis (OPLS-DA), a supervised pattern recognition method, was used to extract maximum information on discriminant compounds for the data. O-PLS-DA provides a way to remove systematic variation from an input data set X (compounds or metabolites) not correlated to the response set Y (discriminant classes) (Trygg and Wold, 2002) and Hotelling’s T2 regions, shown as an ellipse in the scores plot, define the 95% confidence interval of the modelled variation. The O-PLS-DA models were validated using a 7-fold cross-validation method and with a permutation test (200 permutations). To facilitate interpretation of the results, the O-PLS loadings or coefficients plots that revealed metabolites responsible for the metabolic discriminations between two classes, for example the skin or pulp samples from healthy and botrytized grape
bunches, were generated with a colour-coded correlation coefficient for each data point using MATLAB (The MathWorks) with scripts developed at Imperial College London (Cloarec et al., 2005b). The quality of the models is described by R^2_x and Q^2 values. R^2_x is defined as the proportion of variance in the data explained by the models and indicates goodness of fit, and Q^2 is defined as the proportion of variance in the data predictable by the model and indicates predictability.

**Gluconic acid determination**

\(\delta\)-Gluconic acid concentration was quantified using an enzymic analysis kit (R-Biopharm, Darmstadt, Germany) and expressed in mg (100 g dried sample)\(^{-1}\).

**Statistical analysis**

The Statistical Analysis System version 9.20 (SAS Institute, NC, USA) was used for data analysis by ANOVA and Duncan’s multiple-range test. In particular, the paired Student’s t-test was performed for the significance analysis of individual metabolite with integral area of NMR spectrum in pairs of the skin and pulp samples.

**Results and discussion**

**Metabolic profiling of skin and pulp from healthy and botrytized grape berries**

Fig. 1 shows the typical 600 MHz \(^1\)H NMR spectra of skin and pulp extracts from healthy and botrytized grape berries. A wide range of metabolites were assigned and provided complementary information on the global metabolic perturbations induced by *B. cinerea* infection. Assignment of metabolites was verified by using two-dimensional TOCSY NMR experiments (Supplementary Figs. S1 and S2, available at JXB online). Valine, isoleucine, leucine, alanine, threonine, lactate, arginine, proline, \(\gamma\)-aminobutyrate (GABA), glutamate, glutamine, succinate, citrate, malate, tartarate, glycerol, glucose, fructose, sucrose, \(\text{trans/cis-caffeic acid}, \text{trans-coumaric acid, quercetin-3-O-glu}

coside, kaempferol-3-O-gluoside, and trigonelline were identified in both skin and pulp, of which phenypropanoids, such as \(\text{trans/cis-caffeic acid and trans-coumaric acid, and flavonoid}

gluicosides of quercetin-3-O-glucoside and kaempferol-3-O-

gluoside were observed in skin but not in pulp (Fig. 1), and not observed in the skin obtained from BB (Fig. 2). These findings were consistent with previous reports in grape berry and wine (Ali et al., 2011; Hong et al., 2011; Mulas et al., 2011).

**Modelling for metabolic discrimination**

To provide comparative interpretations for the changes in the metabolite composition of grape berry as induced by *Botrytis* infection, a series of pattern recognition methods were employed. PCA, PLS-DA, and O-PLS-DA were applied to NMR spectral data to visualize metabolic discrimination between healthy and botrytized berries and find out discriminant metabolites or identify metabolites associated with *B. cinerea* infection.

Although skin extracts from HH, HB, and BB were partially overlapped in the PCA score plot (Fig. 3A), they were clearly differentiated in the O-PLS-DA score plots (Fig. 3B), demonstrating effective removal of systematic variation from the input data set (skin metabolites) not correlated to the response set (HH, HB, and BB) in the O-PLS-DA model, as described elsewhere (Trygg and Wold, 2002).

Moreover, pulp extracts were also clearly discriminated among HB, BB, and HH (Supplementary Fig. S3). To identify the metabolites responsible for the discriminations in the O-PLS-DA score plots, an O-PLS loading or coefficient plots in pairwise comparisons were generated (Fig. 4). The upper sections of the O-PLS coefficient plots represent metabolites that were higher in skins obtained from HB or BB than in skins from HH, whereas the lower sections reveal metabolites that were lower. In the present study, the correlation coefficients in the O-PLS coefficient plots were considered to be significant when >0.45, which corresponded to the critical value of the correlation coefficient at \(P < 0.05\) verified by Student’s t-test with integral areas of NMR spectra corresponding to individual metabolites.

In fact, although all models showed clear discriminations among skin from HH, HB, and BB in the O-PLS-DA score plots (Fig. 3) and also among pulp (Supplementary Fig. S3), the O-PLS models in the pairwise comparisons between HH and HB both for skin and pulp had weak predictability, as indicated by low \(Q^2\) values (Supplementary Table S1), and thus were not validated by permuted test. However, the models between skins from HH and BB were validated (Supplementary Fig. S4). The main reason of the weak predictability between the samples from HH and HB might be due to contributions of no significant differences in sugar levels that dominate in the \(^1\)H NMR spectra to the statistical significance in the O-PLS models (Fig. 4A). Therefore, when the models were regenerated after removing sugar regions from 3.0 to 5.3 ppm in the \(^1\)H NMR spectra, their predictability increased (Supplementary Table S1) and consequently the corresponding models were validated.

**Metabolic variations induced by *B. cinerea* infection**

\(\delta\)-Gluconic acid was only observed in BB samples and accumulated twice more in the skin rather than the pulp (Table 1).

Even though the O-PLS model of metabolic discrimination between the skins from HH and HB was not significant, the discriminant metabolites were identified in the corresponding O-PLS coefficient plot (Fig. 4A). Statistical significances in their differences are given in Table 1. Significantly increased levels of valine, isoleucine, leucine, threonine, arginine, and proline, together with decreased levels of sucrose, \(\text{trans/cis-caffeic acid, trans-coumaric acid,}

and quercetin/kaempferol-3-O-glucoside, were observed in the skin of HB compared to those in the skins of HH (Fig. 4A). Moreover, the skins of BB were characterized by higher levels of alanine, glutamate, succinate, fructose, and glucose than in the skins of HH, while sucrose levels were lower in the skins of BB than in those of HH (Fig. 4B). Interestingly, phenolic compounds such as \(\text{trans/cis-caffeic acid, trans-coumaric acid,}

and quercetin/kaempferol-3-O-glucoside were not detected in the skins of BB.

With regard to the pulp, valine, isoleucine, threonine, proline, glutamine, and glutamate levels were significantly higher in the pulp of HB, and succinate, arginine and GABA levels were higher in the pulp of BB, when compared to those of HH (Table 1). Furthermore, glycerol was highly increased in skins and pulps of BB, as shown in Fig. 5. These significant
Fig. 1. Representative 600 MHz $^1$H NMR spectra of grape skin (A–C) and pulp (D–F) obtained from healthy berry of healthy bunch (HH, A and D), healthy berry of botrytized bunch (HB, B and E), and botrytized berry of botrytized bunch (BB, C and F). MeOD, a residual deuterated methanol. Asterisks denote unknown compounds.
Metabolomics of V. vinifera berry infected with B. cinerea perturbations of metabolites in the skin and the pulp obtained from HB and BB demonstrate that B. cinerea affects the metabolome of healthy berries as well as botrytized berries from botrytized grape bunches. However, when compared with HH, the berries of HB and BB revealed markedly distinct metabolic differences from each other, likely because of indirect and direct effects, respectively, of the B. cinerea infection process. Statistical significances of the distinct metabolite changes and metabolic mechanisms in berries between HB and BB are summarized in Fig. 6.

Metabolites related to plant defence mechanisms

Marked accumulations of proline, arginine, alanine, and glutamate were found in the berries of HB and BB obtained from botrytized grape bunches. GABA, proline, arginine, alanine, and glutamate are known to be accumulated in plants under abiotic and biotic stresses, and some of them are converted to stress-related metabolites in plants to counteract these stresses (Choi et al., 2004; Allwood et al., 2008; Lima et al., 2010). Proline can be involved in cell-wall reinforcement in response to abiotic and biotic stresses in the plant through the accumulation of proline-rich proteins and hydroxyproline-rich glycoproteins, which are important structural components of plant cell walls (Cassab, 1998). Moreover, proline substantially contributes to the cytoplasmic osmotic adjustment under plant abiotic stress, resulting in its accumulation (Hare and Cress, 1997; Ashraf and Foolad, 2007; Verbruggen and Hermans, 2008). Indeed, it is well known that proline accumulation is related to plant susceptibility to pathogen infection, as proline antagonizes plant defences (Haudecoeur et al., 2009). These observations are consistent with the increased proline levels found in the berries of HB in the present study.

Arginine is a major nitrogen storage compound in higher plant and also acts as precursor of polyamines and is thus involved in a conjugation between polyamines and phenolic compounds.
Fig. 3. PCA (A) and O-PLS-DA (B) score plots derived from $^1$H NMR spectra of skins obtained from healthy berry of healthy bunch (HH), healthy berry of botrytized bunch (HB), and botrytized berry of botrytized bunch (BB), indicating clear metabolic differences among the samples (this figure is available in colour at JXB online).
Fig. 4. O-PLS coefficient or loading plots from $^1$H NMR spectra of skins obtained from healthy berry of healthy bunch (HH) and healthy berry of botrytized bunch (HB, A), and botrytized berry of botrytized bunch (BB, B), revealing the identification of metabolites contributing to the discriminations of HB and BB from HH. (C) a and b are expansions from panel B. Asterisks denote unknown compounds (this figure is available in colour at JXB online).
such as cinnamic acids, for example p-coumaric, ferulic, and caffeic acids (Walters, 2003; Alcázar et al., 2006). The resulting conjugates, hydroxycinnamic acid amides, have been shown to accumulate in plants during pathogen-induced defence response. Therefore, accumulation of arginine in the berries of HB is likely a consequence of B. cinerea infection in grape berries to synthesize polyamines that will be conjugated with phenolic compounds in order to fight with the infection.

Together with proline and arginine accumulation, glutamate accumulation in the berries of HB also reflects an activation of plant defence systems against B. cinerea infection since glutamate is a precursor of stress-related molecules such as proline, arginine, and GABA (Forde and Lea, 2007). Moreover, a high production of glutamate in pathogenic fungus-resistant grapevine cultivars has previously been reported (Figueiredo et al., 2008). In addition, constitutive accumulation of alanine in grapevine cultivars resistant to fungal infection has been reported, demonstrating its protective role against biotic stresses (Figueiredo et al., 2008; Lima et al., 2010). These reports pointed out that the exact function and mechanism of stress-induced alanine in the plant cell is still unclear. However, it would be likely that alanine accumulation reflects the pathogen infection status in plants, as shown in the present study (Fig. 6A). Alanine synthesis is increased in order to regulate cellular osmosis that is decreased by high cellular carbohydrate levels under anaerobic or hypoxic conditions in diseased plants, in which GABA catabolism by GABA transaminase leads to alanine accumulation (Reggiani et al., 2000; Fait et al., 2007; Miyashita and Good, 2008). Accumulation of alanine and GABA was also reported in esca disease-infected grapevine leaves (V. vinifera cv. Alvarinho), where alanine synthesis increases by 25% (Lima et al., 2010). In the present study, alanine levels in the berries of HB were increased by 250%, whereas GABA levels did not change. Therefore, the large conversion of GABA to alanine in the berries of HB, as observed in the present study, may explain why differences in GABA levels between the berries of HH and HB were not significant.

Considering that proline, hydroxyproline-rich proteins, and several other proteins serve as structural constituents of the plant cell wall (Cassab, 1998; Nanjo et al., 1999), it is likely that increased levels of amino acids, such as valine, threonine, and isoleucine, in the berries of HB would be a consequence of elevated amino acid synthesis of plant host during an active synthesis of cell-wall constituents in response to B. cinerea infection.

Phenylpropanoid compounds that are commonly accumulated in plants as a response to fungal infection (Lima et al., 2010; Lloyd et al., 2011) were clearly but partly degraded in the berries of HB and completely degraded in the berries of BB (Figs. 2 and 4 and Supplementary Table S2). Moreover, flavonoid glucosides such as quercetin-3-O-glucoside and kaempferol-3-O-glucoside

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### Table 1. Metabolites of skin and pulp obtained from healthy berry of healthy bunch (HH), healthy berry of botrytized bunch (HB), and botrytized berry of botrytized bunch (BB). All values were calculated from integral areas of corresponding NMR peaks after spectral normalization. Values with different letters are significantly different by Duncan’s multiple test at P < 0.05. a.u., Arbitrary units; n.d., not detected; –, metabolized or degraded completely.

<table>
<thead>
<tr>
<th>Metabolites (au × 10^{-3})</th>
<th>Skin</th>
<th>Pulp</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HH</td>
<td>HB</td>
</tr>
<tr>
<td></td>
<td>HH</td>
<td>HB</td>
</tr>
<tr>
<td>Valine</td>
<td>0.42 ± 0.08</td>
<td>0.92 ± 0.38</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>0.21 ± 0.05</td>
<td>0.38 ± 0.11</td>
</tr>
<tr>
<td>Threonine</td>
<td>2.00 ± 0.51</td>
<td>3.82 ± 0.13</td>
</tr>
<tr>
<td>Alanine</td>
<td>1.44 ± 0.25</td>
<td>3.74 ± 2.42</td>
</tr>
<tr>
<td>Arginine</td>
<td>3.36 ± 1.32</td>
<td>9.26 ± 4.56</td>
</tr>
<tr>
<td>Proline</td>
<td>2.45 ± 0.63</td>
<td>6.06 ± 2.10</td>
</tr>
<tr>
<td>Glutamine</td>
<td>1.29 ± 0.36</td>
<td>1.27 ± 0.27</td>
</tr>
<tr>
<td>Glutamate</td>
<td>1.63 ± 0.45</td>
<td>4.04 ± 2.50</td>
</tr>
<tr>
<td>Succinate</td>
<td>0.80 ± 0.15</td>
<td>0.45 ± 0.14</td>
</tr>
<tr>
<td>Malate</td>
<td>18.95 ± 7.07</td>
<td>15.83 ± 5.20</td>
</tr>
<tr>
<td>Citrate</td>
<td>4.73 ± 1.00</td>
<td>3.67 ± 0.78</td>
</tr>
<tr>
<td>Glycerol</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>Fructose</td>
<td>222.4 ± 20.6</td>
<td>243.8 ± 19.6</td>
</tr>
<tr>
<td>α-Glucose</td>
<td>152.2 ± 10.65</td>
<td>159.79 ± 14.63</td>
</tr>
<tr>
<td>β-Glucose</td>
<td>215.64 ± 17.03</td>
<td>225.88 ± 19.79</td>
</tr>
<tr>
<td>Succrose</td>
<td>20.42 ± 3.21</td>
<td>16.33 ± 2.56</td>
</tr>
<tr>
<td>GABA</td>
<td>1.74 ± 0.38</td>
<td>2.11 ± 0.49</td>
</tr>
<tr>
<td>trans-Caffeic acid</td>
<td>0.80 ± 0.26</td>
<td>0.21 ± 0.02</td>
</tr>
<tr>
<td>cis-Caffeic acid</td>
<td>0.76 ± 0.28</td>
<td>0.19 ± 0.06</td>
</tr>
<tr>
<td>trans-Coumaric acid</td>
<td>0.62 ± 0.24</td>
<td>0.16 ± 0.02</td>
</tr>
<tr>
<td>Quercetin-O-glucoside</td>
<td>0.42 ± 0.27</td>
<td>0.27 ± 0.14</td>
</tr>
<tr>
<td>Kaempferol-O-glucoside</td>
<td>0.34 ± 0.36</td>
<td>0.18 ± 0.24</td>
</tr>
<tr>
<td>d-Gluconic acid*</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
</tbody>
</table>

* d-Gluconic acid concentration in mg (100 g dried sample)^{-1} was determined by an enzymatic method.
Fig. 5. Comparisons of relative glycerol levels in skin (A) and pulp (B) obtained from healthy berry of healthy bunch (HH), healthy berry of botrytized bunch (HB), and botrytized berry of botrytized bunch (BB) as normalized intensities of raw $^1$H NMR spectra (this figure is available in colour at JXB online).
were also found to be partly degraded in the berries of HB. The partial degradations of phenolic and flavonoid compounds in the berries of HB indicated that *B. cinerea* infection had already induced slight metabolic variations in the berries of HB at the harvest time, together with an activation of the plant defence systems, even though infection was invisible to the naked eye on the berries of HB. It is well known that *B. cinerea* infection leads to a considerable accumulation of glycerol and gluconic acid, especially in the grape skin (Ribereau-Gayon et al., 2006), which is consistent with the present results on BB. In general, gluconic acid is directly derived from glucose by a glucose oxidase secreted by *B. cinerea* through the Embden–Meyerhof and hexose monophosphate shunt pathways and TCA cycle (Donèche, 1989). Interestingly, relative amounts of glucose in grape berries were markedly higher compared to relative amounts of sucrose, but a largely reduced sucrose content in the berries of BB demonstrated that *B. cinerea* preferentially used sucrose as a carbon source for growth rather than glucose (Supplementary Fig. S5 and Supplementary Table S2), which is consistent with

**Fig. 6.** Schematic metabolic perturbation derived by *B. cinerea* infection in grape berry, revealing a plant defence mechanism in healthy berry of botrytized grape bunch (HB) (A) and a direct influence of *B. cinerea* growth on metabolites of botrytized berry of botrytized grape bunch (BB) (B). Metabolites in bold were observed in the present study whereas metabolites in grey were not observed. Changes in metabolites with averages of integral areas corresponding to NMR peaks from both berries of HB and BB were compared with those from HH. Significant changes in the metabolites were assessed at *P* < 0.05 through a Student’s t-test (this figure is available in colour at JXB online).
recent reports that *B. cinerea* prefers sucrose as a carbon source to glucose and fructose in *B. cinerea*-inoculated sunflower cotyledons (Dulermo et al., 2009) and *B. cinerea*-cultured medium (Donèche, 1987). When one considers that *B. cinerea* mainly grows in the skin of grape berries and compares the perturbations in the levels of sucrose, glucose, and fructose in the skin, the skin of BB exhibited elevated glucose and fructose levels and reduced sucrose levels compared with the skin of HH (Table 1). This result is in good agreement with the finding that glucose and fructose accumulate in the leaf apoplast of tomato infected by *Cladosporium fulvum* as sucrose declines, before reaching the later stages of infection (Joosten et al., 1990), through cleavage of photosynthetic sucrose into fructose and glucose by plant and/or fungal invertases (Noeldner et al., 1994).

Although the level of GABA, one of the plant stress-associated compounds, was not changed in the berries of HB, maybe because of its large conversion to alanine by a GABA transaminase in response to *B. cinerea* infection, GABA was accumulated in the berries of BB (Fig. 6B). GABA accumulation in the berries of BB might result from lack of alanine synthesis by GABA transaminase during *B. cinerea* growth, because *B. cinerea* cannot use GABA as a nutrient source in the plant due to a lack of GABA transaminase, even though several fungi and yeasts secrete GABA transaminases (Solomon and Oliver, 2002; Brauc et al., 2011). Therefore, the GABA produced in the berries of HB consequently would remain in the berries of BB without further transformation into alanine. The observation that the concentrations of amino acids such as valine, threonine, and isoleucine were not significantly changed in the berries of BB but were accumulated in the berries of HB would indicate their use as a nitrogen source for the active growth of *B. cinerea*. Furthermore, the active growth of *B. cinerea* was proved in the berries of BB by the marked accumulation of succinate, one of the major TCA cycle intermediates, together with a degradation of malate, which was not observed in the berries of HB.

The variations or perturbations of the most common metabolites associated with plant biotic stresses and/or related to *B. cinerea* growth observed in the present study are in excellent agreement with the literature data. In particular, this study highlights that two distinct metabolic pathways, one associated with plant defence system and the other with *B. cinerea* growth in healthy and botrytized berries from *B. cinerea*-infected grape bunches are simultaneously involved in the metabolic perturbations observed in the botrytized grape bunches. Moreover, the results of this study, together with simultaneous and global metabolite profiling, would provide useful information and reveal biomarkers for the monitoring and management of pathogen infection of grape bunches at the early infection stage in the vineyard.

**Supplementary material**

Supplementary data are available at *JXB* online.
Supplementary Table S1. O-PLS-DA modelling results from pairwise comparisons in skin and pulp
Supplementary Fig. S1. Expansion of two-dimensional 1H-1H total correlation spectroscopy NMR spectrum corresponding to amino and organic acids’ regions obtained from HH

Supplementary Fig. S2. Expansion of two-dimensional TOCSY NMR spectrum corresponding to regions of phenylpropanoid and flavonoid glycoside compounds obtained from HH
Supplementary Fig. S3. PCA score plots derived from 1H NMR spectra of pulp extracts obtained from HH, HB, and BB
Supplementary Fig. S4. O-PLS-DA score plots and model validations in pairwise comparisons of skin extracts between HH and HB and between HH and BB
Supplementary Fig. S5. Comparisons of relative sucrose levels among skin samples obtained from HH, HB, and BB as normalized intensities of raw 1H NMR spectra

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**References**


