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ORIGINAL RESEARCH ARTICLE

The grapevine metabolite profile of phloem sap is modified by flavescence dorée

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ABSTRACT

Flavescence dorée (FD) has been recorded in French vineyards since the mid-1950s; it has rapidly become a major threat to vineyard survival in different European grape-growing areas. Infection by the phytoplasma '*Candidatus* Phytoplasma vitis' causes leaf chlorosis and disturbs primary and secondary core metabolic pathways; it can also occlude sieve plates, therefore affecting normal solute transport through the phloem. Thus, in the present study we hypothesised that the metabolomic fingerprint of the phloem sap changes in FDp-infected vines. The metabolome of the phloem sap collected by centrifugation from the bark of the stem of healthy and infected vines (cv. 'Loureiro') was profiled by ESI-HRMS at two snapshot time points: E-L 33 (grape berry still hard and green) and E-L 38 (grape berry at maturity). An untargeted analysis of the phloem sap enabled the identification of 476 metabolites, whereas the targeted analysis focused on eight main classes, namely acids, sugar and polyols, amino acids, phenolic acids, hormones, vitamins and N compounds, as well as on several minor classes like fatty acids, phenols or diols. Depending on the developmental stage of the vine, major differences were observed in the composition of cv. 'Loureiro' phloem sap in response to FD infection in terms of sugar, organic acid and amino acid content; furthermore, compounds involved in plant defense, such as salicylic acid, ABA, vitamin C and gallic acid also significantly changed. Overall, these results have contributed towards increasing knowledge about the complex interactions between vine and FD phytoplasma.

KEYWORDS: Flavescence dorée, phytoplasma, phloem sap, *Vitis vinifera*, metabolome, infected vines

INTRODUCTION

Flavescence dorée (FD) is a grapevine disease caused by '*Candidatus* Phytoplasma vitis' (FDp) (Belli *et al*., 2010; Bertaccini and Duduk, 2009), which results in severe damage to or even the death of grapevines (*Vitis* spp.) (Chuche and Thiéry, 2014; Oliveira *et al*., 2020; Rizzoli *et al*., 2022). In Portugal, FDp was first detected in the Portuguese DOC region of 'Vinhos Verdes' in 2006 (deSousa *et al*., 2010). The visible external symptoms of grapevines infected by the flavescence dorée phytoplasma are similar to other grapevine yellows (GY), usually emerging after one to three years of latency after infection (Caudwell, 1964; Dermastia *et al*., 2017). Different studies have reported harmful effects of the infection at both physiological and molecular levels, revealing modifications in the primary and secondary metabolism. This is the case of the isoprenoid biosynthetic pathways, which are repressed in response to infection, accounting for leaf yellowing, which is a hallmark of the disease (Teixeira *et al*., 2020). Other studies have revealed that the accumulation of sucrose and starch in the leaf mesophyll is accompanied by the up-regulation of key genes involved in their synthesis, including Sucrose synthase 4 (*VvSusy4*) (Hren *et al*., 2009; Margaria *et al*., 2014; Prezelj *et al*., 2016). Likewise, a large accumulation of proanthocyanidins and anthocyanins has been detected in infected cv. 'Barbera' leaves, and the steady-state transcript levels of genes of the flavonoid pathway and proanthocyanidin branches have been found to be higher in infected plants (Hren *et al*., 2009; Margaria *et al*., 2014; Prezelj *et al*., 2016).

Phytoplasmas are usually confined to the sieve elements, from where they release defence and stress-related effectors that can spread throughout the vascular system and affect the distal plant organs and tissues, altering their physiology and structures (Namba, 2019; Sugio *et al*., 2011; van Bel, 2003). For this reason, the most reliable methods for the diagnosis of the disease are based on the amplification by PCR of the ribosomal elongation factor (tuf) and the translocase (secY) genes of the phytoplasma that has infected the leaf midribs (Oliveira *et al*., 2020).

Recently, a detailed investigation of leaf midribs of FDp-infected grapevines has revealed anomalies at the ultrastructural level, with the accumulation of lipids in the chloroplasts of parenchyma cells (Oliveira *et al*., 2020), as well as a Ca^{2+} influx into the sieve tubes, which can lead to callose deposition, protein plugging and eventually to the occlusion of the sieve plates (Musetti *et al*., 2013). Furthermore, FDp infection has also been reported to have indirect consequences on the phloematic and xylematic structures of vines (Jelmini *et al*., 2021; Rizzoli *et al*., 2022).

Plants produce several metabolites within autotrophic tissues that are translocated to heterotrophic organs via the phloem to be used as carbon and energy sources. Primary and secondary metabolites have being identified in the phloem sap, including sugars (e.g., sucrose, glucose, fructose), oligosaccharides (e.g., raffinose, stachyose, galactinol), sugar alcohols (e.g., mannitol, sorbitol and myo-inositol), amino acids, organic acids (e.g., malate, succinate and citrate), inorganic ions (e.g., K^+, Na^+ and Ca^{2+}), nucleotides, fatty acids (e.g., stearic acid, palmitic acid, oleic acid, linoleic acid) or hormones (e.g., indole-3-acetic acid, abscisic acid, jasmonic acid) (Lohaus, 2022). In grapevine phloem, sugars and polyols, amino acids and organic acids, together with phenylpropanoids, phosphates, polyhydroxy acids and N-compounds have also been identified (Tedesco *et al*., 2021). In higher plants, small noncoding RNAs and large messenger RNA (mRNA) molecules are transported between cells and over long distances via the phloem (Kehr and Kragler, 2018). Long-distance transport has been demonstrated for many plant hormones, including auxins, abscisic acid (ABA), cytokinins, gibberellins (GAs), strigolactones and salicylic acid. The transportation of hormones within the transpiration stream involves their being loaded into the xylem and unloaded at the target cells. Similarly, the transportation of hormones within the phloem can also involve loading and unloading steps (Park *et al*., 2017). Thus, the role of phloem in source-sink relationships does not only rely on assimilate transport; a rapid information transfer over long distances is also enabled via the phloem transport tubes that pervade the complete plant and thus connect even the most distant organs (Kehr and Buhtz, 2008).

Given the molecular, biochemical and ultrastructural modifications induced by '*Candidatus* Phytoplasma vitis' infection, which occur mainly in phloem vessels, in the present study we aimed to validate the following hypothesis: (i) the phloem sap composition of cv. 'Loureiro' changes substantially in response to infection, not only in terms of key photoassimilates but also in terms of signalling and defence molecules, and (ii) the metabolomic fingerprint of phloem sap resulting from infection is dependent on the plant developmental stage/severity of the infection. To address these hypotheses, the metabolome of the phloem sap from healthy and infected vines (cv. 'Loureiro') was profiled by HPLC-ESI-HRMS at two snapshot time points: at E-L 33 (grape berry still hard and green) and E-L 38 (grape berry at maturity). 'Loureiro' is the second most cultivated variety in the Demarcated Region of Vinhos Verdes, where the incidence of FD disease is critical (deSousa *et al*., 2010). Overall, our work aimed to highlight the large extent to which key photoassimilates and informative molecules, as well as sugars, are altered in response to infection. Besides their scientific interest, the results are biotechnologically relevant, as they can contribute to the optimisation of agricultural practices aiming to mitigate the infection symptoms/spread of the disease.

MATERIALS AND METHODS

1. Plant material

Grapevine canes from the white vine cv. 'Loureiro', were collected in the 2020 season in a commercial vineyard of the Controlled Appellation (DOC) region of Vinhos Verdes in the

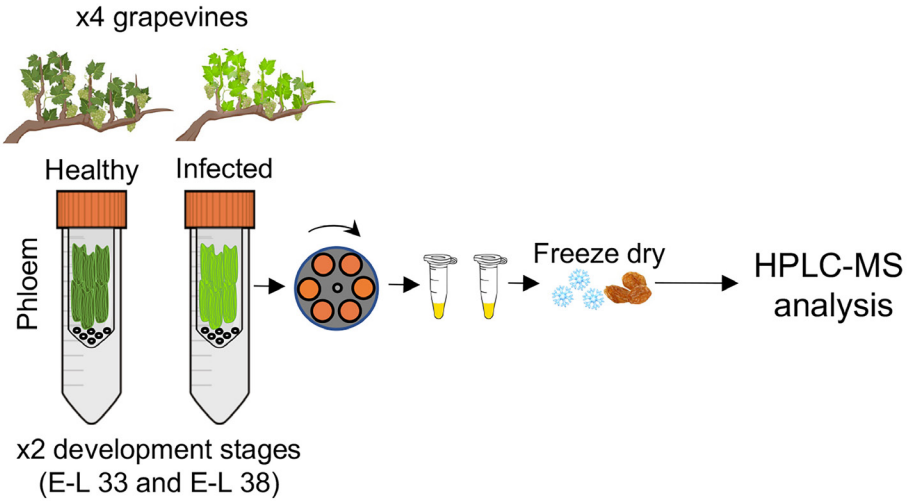

FIGURE 1. Schematic representation of phloem sap collection.

Healthy and flavescence dorée infected grapevine canes collected at two stages of development (E-L 33 and E-L 38) by the centrifugation method and subsequent metabolite analysis by HPLC-MS.

northwest region of Portugal (41°31'01.0"N; 8°12'56.1"W). This vineyard has a total area of 8 ha of planted vines, and the rows in which sampling was carried out have a northeast-southwest orientation. The rows of grapevine are located on a steep hill at around 300 m elevation. Vineyard management comprises no irrigation and standard cultural practices as applied by commercial farmers.

Two sampling time points were chosen during the season: E-L 33 (grape berry still hard and green) and E-L 38 (berries at mature stage) (Coombe, 1995). At each time point, four canes from four infected vines (hereafter referred to as being FDp-infected) were collected, the identification of the infected vines having previously been done by RFLP (Oliveira *et al*., 2020). Similarly, four canes from four healthy vines were collected for the control. The canes were transported to the laboratory in cooled containers and the phloem sap was collected by centrifuging the separated bark tissues of the canes according to Hijaz and Killiny, 2014 and Killiny, 2019. Briefly, the bark was cut into 2-cm pieces using a sterile razor blade and was separated from the xylem. The separated tissues were rinsed with distilled water and dried with disposable wipers. The bark tissues were vertically placed in a 15-mL falcon tube punctured with small holes; this tube was nested within a 50-mL falcon tube and the system was centrifuged at 12,000 *x g* for 15 min. (Figure 1). The phloem sap obtained using this method was immediately frozen in liquid nitrogen and freeze dried for five days in a Christ Alpha 2-4 LD Plus lyophiliser for use in the following metabolomics analysis based on HPLC-ESI-HRSM plantform.

2. Metabolomics analysis

Metabolites were extracted using 50 % (v/v) methanol, 0.1 FA, spiked with 5 µg/mL DL- formononetin as the internal standard, as reported in Frusciante *et al*. (2022). High resolution electrospray ionisation mass spectrometry (HPLC-ESI-HRMS) analysis was performed as reported in Noronha *et al*. (2022). For targeted metabolomics, literature resources were used to generate an ad hoc database for grape phloem sap; subsequently, it was possible to identify the metabolites on the basis of online absorption spectra (except for lipids) by comigration with authentic standards (when available), and by accurate masses obtained from the Pubchem database (http://pubchem.ncbi.nlm.nih.gov/) for native compounds or from the Metabolomics Fiehn Lab Mass Spectrometry Adduct Calculator for adducts. (http://fiehnlab.ucdavis.edu/staff/kind/Metabolomics/MS-Adduct-Calculator/).

The ion peak areas were normalised to the ion peak area of the internal standard (formononetin). Untargeted metabolomics were performed as previously described (Teixeira *et al*., 2020) using the SIEVE software (v2.2, ThermoFisher Scientific, Waltham, MA, USA). The FOLD IS quantification was based on the ion intensity of a given m/z and was calculated as the fold change of the Internal Standard ion intensity.

3. Multivariate analysis

Global polar compounds for untargeted metabolomics analyses were retrieved as previously described (Dono *et al.*, 2020), using the SIEVE software (v2.2, ThermoFisher Scientific, Waltham, MA, USA). Principal Component Analysis (PCA) and Partial Least Squares-Discriminant Analysis (PLS-DA) are multivariate statistical techniques used for dimensionality reduction, but PLS-DA takes into account both the variation in the predictor variables and the relationship between the predictor variables and the response variable. Both the PCA and the PLS-DA of the phloem sap metabolomics data were performed in R software version 4.1.0 using the mixOmics package v. 6.16.3 (Rohart *et al*., 2017). The same procedure was used on the targeted metabolites. Volcano plots were performed with significant values ($|\log_2 FC| > 1.0$; Log_{10} P-value ≤ 0.05) using ggplot2 package v.3.3.6 (Wickham *et al*., 2016). Bar charts were created using the highest values for each class of targeted metabolites.

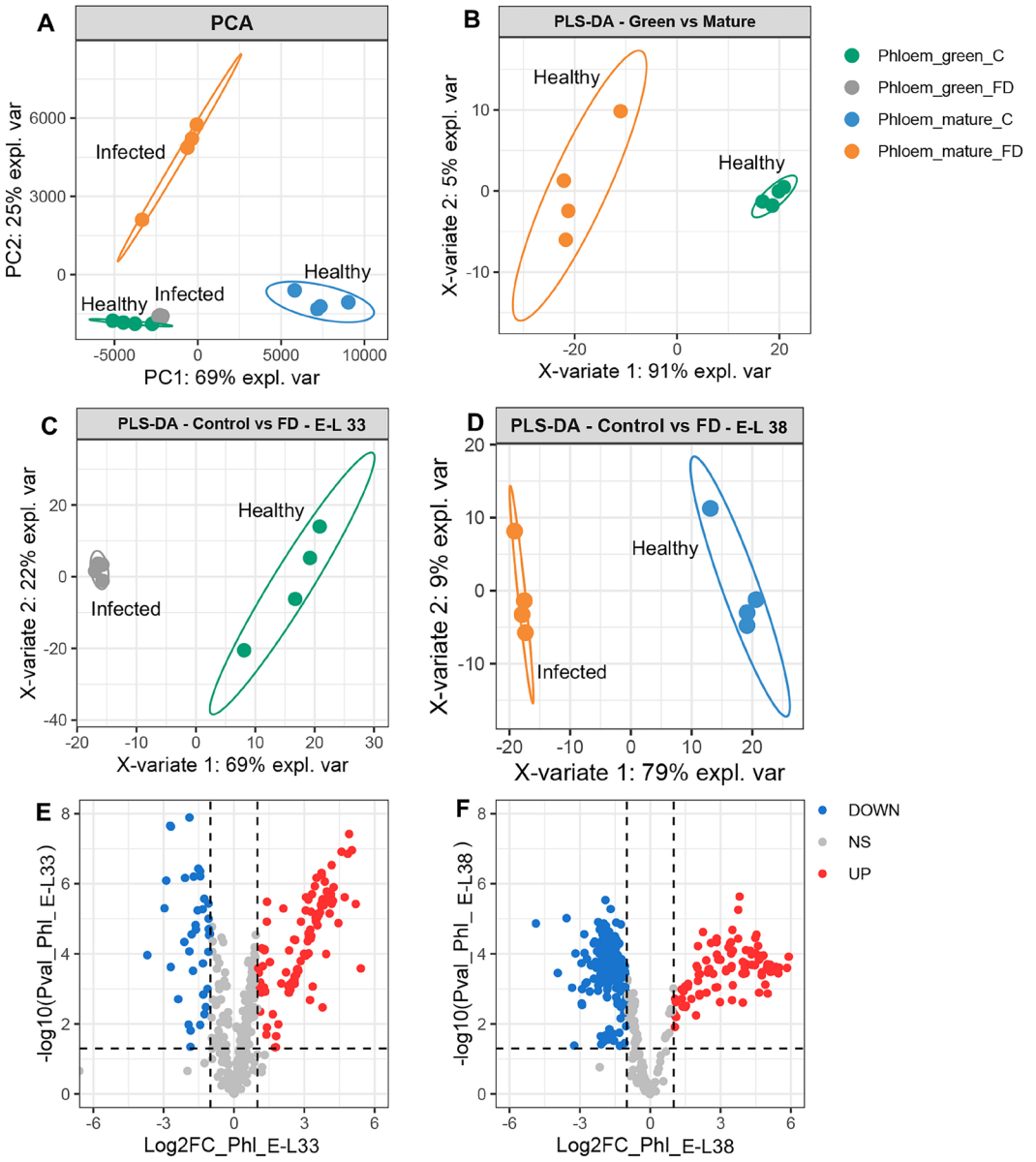

FIGURE 2. Untargeted metabolomics data of phloem sap from healthy and flavescence dorée infected grapevine canes collected at two stages of development (E-L33 and E-L38).

(A) Principal Component Analysis of data from healthy and infected grapevines at both development stages; (B) Partial Least-Squares Discriminant Analysis (PLS-DA) of data from healthy grapevines at both stages; (C, D) PLS-DA of data from healthy vs infected grapevines at E-L 33 and E-L 38 respectively; (E, F) Volcano plots with fold-change significant values (|log2FC| > 1.0; Log10 P-value ≤ 0.05) of data from healthy vs infected grapevines at E-L 33 and E-L 38 respectively.

A heatmap was obtained with the ComplexHeatmap package v. 2.9.3, (Gu *et al.*, 2016) using the mean of significantly different metabolites: the $\log_2$ ratio of fold change (Log_2 FC) between E-L 38 / E-L 33 stages, infected *vs.* healthy at E-L 33 stage and infected *vs.* healthy at E-L 38 stage. An analysis or variance (ANOVA) of all metabolomic data was performed to evaluate the variance within the groups, but no differences were found. Differences in metabolite amounts between healthy and infected grapevines were determined using Student's t test and marked with asterisks to denote significance levels: *P ≤ 0.05; **P ≤ 0.01; ***P ≤ 0.001.

RESULTS

1. Characterisation of cv. 'Loureiro' phloem sap composition

The untargeted analysis of the phloem sap resulted in the identification of 476 metabolites (Table S1). The unsupervised Principal Component Analysis (PCA) of the untargeted data (Table S1) represented 94 % of the total variation on the two first components, and it clearly separated phloem saps from healthy and infected grapevines, particularly at the E-L 38 stage (Figure 2A). To maximise the covariance between the independent variables X (sample

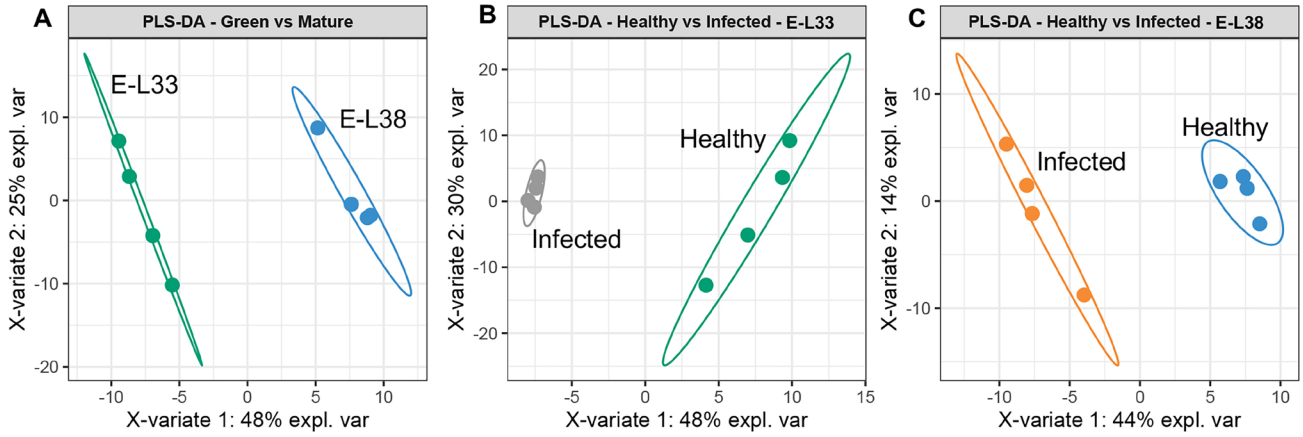

FIGURE 3. Multivariate dimensionality-reduction.

Partial Least-Squares Discriminant Analysis (PLS-DA) of targeted metabolites from phloem sap from healthy and infected grapevines at two stages of development. (A) E-L 33 vs. E-L 38 (B) infected vs. healthy at E-L 33, and (C) infected vs. healthy at E-L 38.

readings and the metabolomics data) and the corresponding dependent variable Y (samples), a supervised Partial Least Squares-Discriminant Analysis (PLS-DA) was performed. This approach showed a great variation in the metabolites of the phloem saps from healthy grapevines at E-L 33 and E-L38 (91 % variation PC1 x-axis; Figure 2B). In contrast, the supervised analysis comparing phytosanitary conditions displayed a variation of 69 % between healthy and FD-infected canes at E-L 33 (PC1-*x* axis, Figure 2C), which increased to 79 % at the E-L 38 developmental stage (Figure 2D). This increase in variation reflects the increase in metabolites, with the significant difference of 148 metabolites at E-L 33 and 317 metabolites at E-L 38 (Figures 2E and F); this indicates that the metabolome profile of canes changes with the progression of infection.

A targeted analysis was then performed to more accurately determine the diversity classes of metabolites present in the phloem sap at both of the grapevine developmental stages. The PLS-DA analysis of targeted metabolites (154) showed a 48 % variation in the phloem saps from healthy grapevines at the E-L 33 and E-L 38 stages (PC1 *x*-axis; Figure 3A), which is similar to the variation observed at E-L 33 in the phloem saps from healthy and infected grapevines (PC1-*x* axis; Figure 3B). At the E-L 38 developmental stage, a variation of 44 % (PC1-*x* axis; Figure 3C) was observed, indicating that the metabolome profile of key metabolites present in the phloem sap is shaped by flavescence dorée infection.

The identified metabolites were distributed between eight main classes comprising acids, sugar and polyols, sugar and polyol derivates, amino acids, phenolic acids, hormones, vitamins and N compounds, and several minor classes comprising fatty acids, phenols or diols (Figure 4-7; Table S2). Sugars and organic acids were, by far, the most abundant compounds in the phloem sap of cv. 'Loureiro'. Among the sugars, monosaccharide galactose, disaccharides sucrose and galactinol were the most abundant at the E-L 33 stage, but other pivotal sugars were also found (including glucose/fructose) and were more abundant at the E-L 38 stage than at the E-L 33 stage. Trisaccharide raffinose was also found in smaller amounts, and its concentration in the phloem sap increased with berry maturation (as from E-L 33 and E-L 38). While polyols, such as mannitol, sorbitol and galactinol, are usually relatively abundant in the grape berry (Conde *et al*., 2007a), in the present study they were found to be underrepresented (except for galactinol) in the phloem sap of cv. 'Loureiro' (Figure 4A). At E-L 33 and E-L 38, the levels of polyols derivatives in the phloem sap were relatively similar (Figure 4B). In addition, of the 19 organic acids identified in the phloem sap at E-L 33 and E-L 38, tartaric, citric and isocitric acids were the three most abundant, followed by lactic and glutaric acid. Notably, malic acid, which together with tartaric acid is the most abundant organic acid in the berry, was not identified (Figure 4C). The glucose derivative gluconic acid was also relatively abundant in the phloem sap of grapevine cv. 'Loureiro', together with D-glucoheptonic acid-1,4-lactone (Figure 4B). Key amino acid building blocks of proteins were also identified in the phloem sap of cv. 'Loureiro' at E-L 33 and E-L 38, with a series of noteworthy ones, namely proline, phenylalanine, glutamic acid and tryptophan, although in much lower relative amounts than the sugars and organic acids (Table S1). The amino acid L-glutamine and its derivative L-pyroglutamic acid were the most abundant amino acids at both developmental stages.

Interestingly, the phloem sap of cv. 'Loureiro' contained a diverse array of phenolic compounds, including phenolic acids, flavonoids and stilbenes, with a relative abundance equivalent to that found for the amino acids (Figure 5). Phenolic acids included ferulic, gallic and quinic acids, which predominated in the phloem sap in both developmental stages. The stilbene resveratrol and the flavonoids luteolin and naringenin were also detected at even lower amounts.

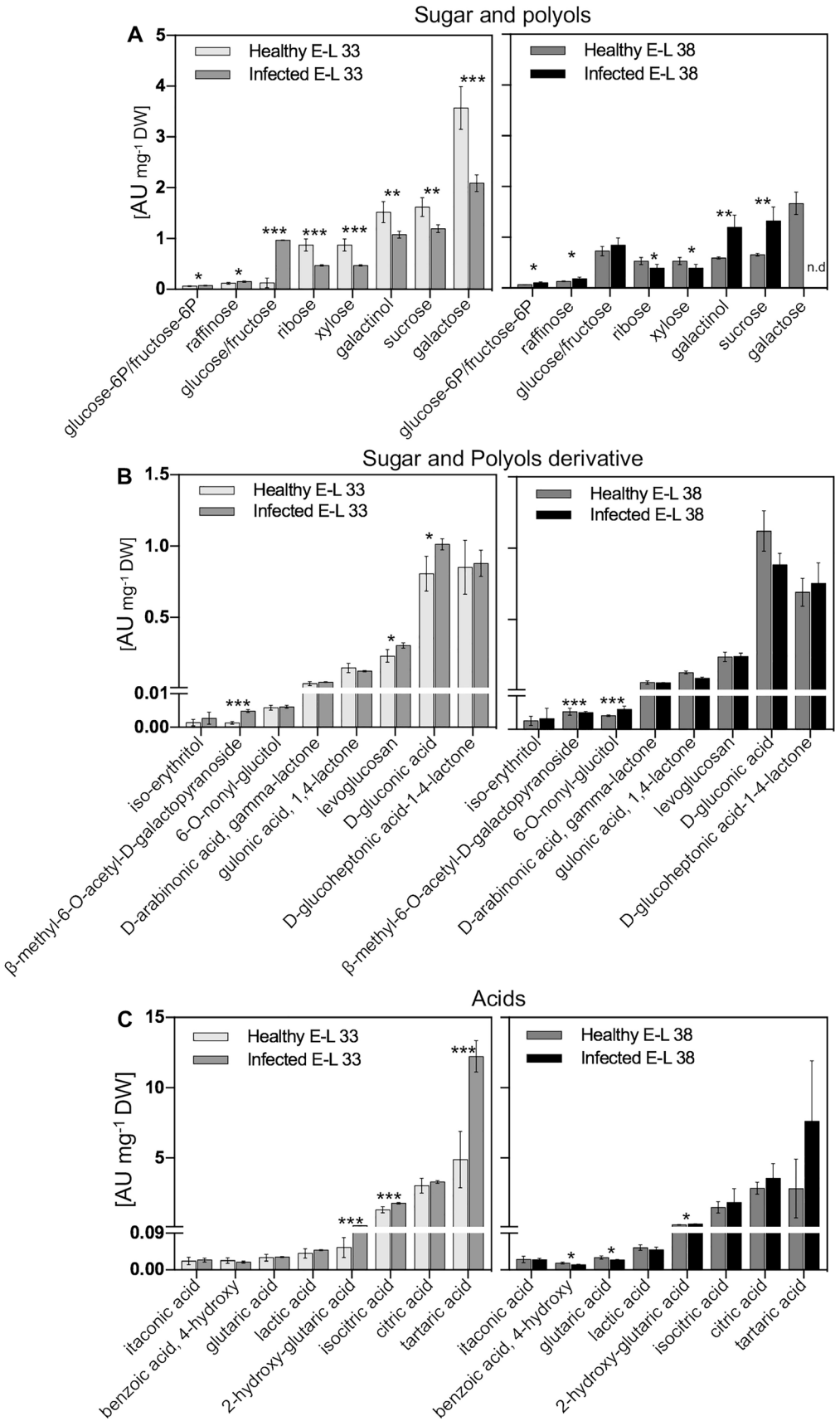

FIGURE 4. Main metabolites present in phloem sap from healthy and infected grapevines at two stages of development (E-L 33 and E-L 38).

(A) sugar and polyols, (B) sugar and polyols derivative, and (C) acids. Asterisks indicate statistical significance between healthy and FD-infected canes in each developmental condition according to the Student's t-test: *P ≤ 0.05; **P ≤ 0.01; ***P ≤ 0.001.

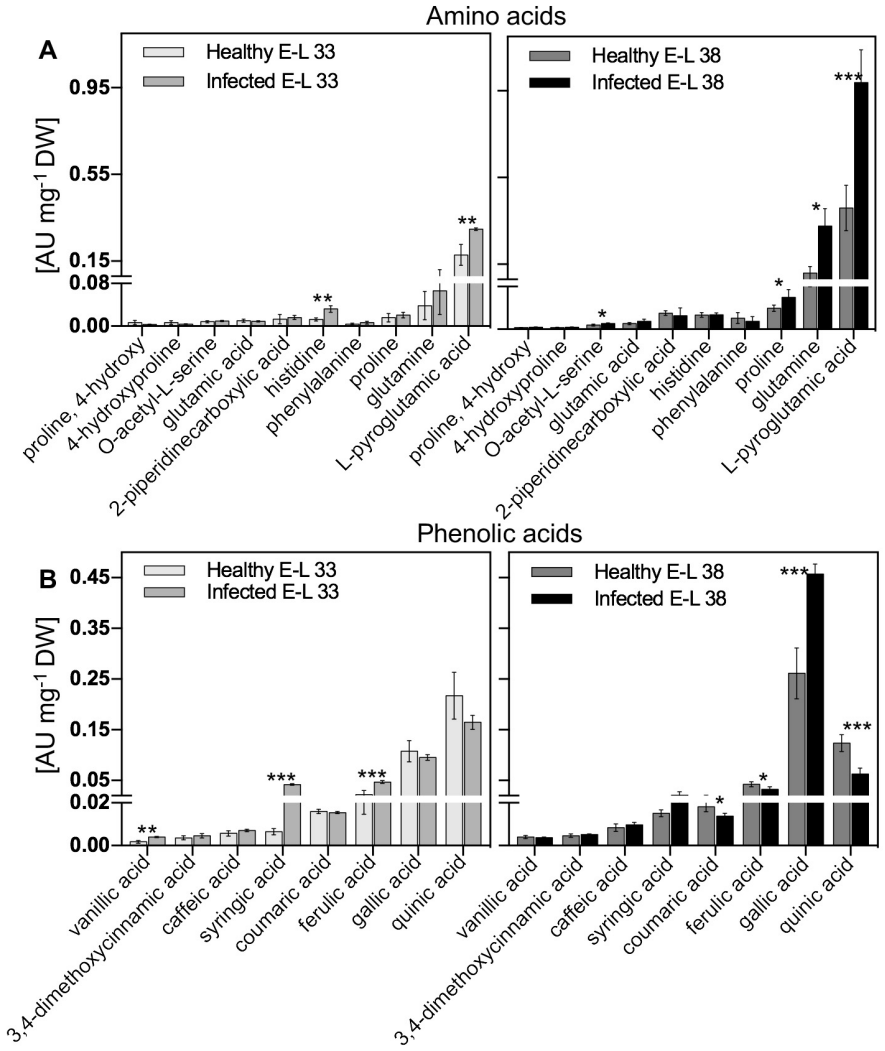

FIGURE 5. Main metabolites present in phloem sap from healthy and infected grapevines at two stages of development (E-L 33 and E-L 38).

(A) Amino acids and (B) Phenolic acids. Asterisks indicate statistical significance between healthy and FD-infected canes in each developmental condition according to the Student's t-test: *P ≤ 0.05; **P ≤ 0.01; ***P ≤ 0.001.

More specifically, resveratrol levels in the phloem sap increased 4-fold from E-L 33 to E-L 38 (Figure 7; Table S1).

Key plant hormones, including salicylic acid and abscisic acid, were found in the phloem sap of cv. 'Loureiro' at both developmental stages. More specifically, their levels either increased (O-glucosyl-zeatin, ABA-GE, abscisic acid and methyl-jasmonate) or slightly decreased (phaseic acid and salycilic acid) depending on the developmental stage between E-L 33 and E-L 38. Other metabolites, including vitamins and N compounds like nicotinic acid and adenine, were also detected in the present metabolomic analysis and their relative amounts also changed during development and in response to infection (Figure 7, Table S1).

2. Changes in the composition of cv. 'Loureiro' phloem sap in response to Flavescence dorée infection

At the E-L 33 stage, phytoplasma infection led to a general decrease in the levels of the most abundant sugars in the phloem sap, including galactose, sucrose and galactinol, whereas the levels of glucose/fructose increased (Figure 4B). However, at E-L 38, some considerable changes were also observed in response to FD infection: while monosaccharide galactose had completely disappeared, the contents of galactinol and sucrose increased by 101 and 102 % respectively. The levels of trisaccharide raffinose (galactose, glucose and fructose) were characterised by higher values at both the developmental stages in response to the infection.

Flavescence dorée infection was associated with an increasing trend in the levels of the most abundant organic acids in the phloem sap of cv. 'Loureiro' at both the E-L 33 and E-L 38 developmental stages (Figure 4A). In particular, tartaric acid levels in the phloem sap were boosted by 151 % at E-L 33 in response to phytoplasma infection. At the same developmental stage, the levels of the less abundant 2-hydroxy glutaric acid increased even more; i.e., by 216 %. However, at E-L 38, some organic acid levels, including 4-hydroxy benzoic and glutaric acid, were reduced in response to FD infection.

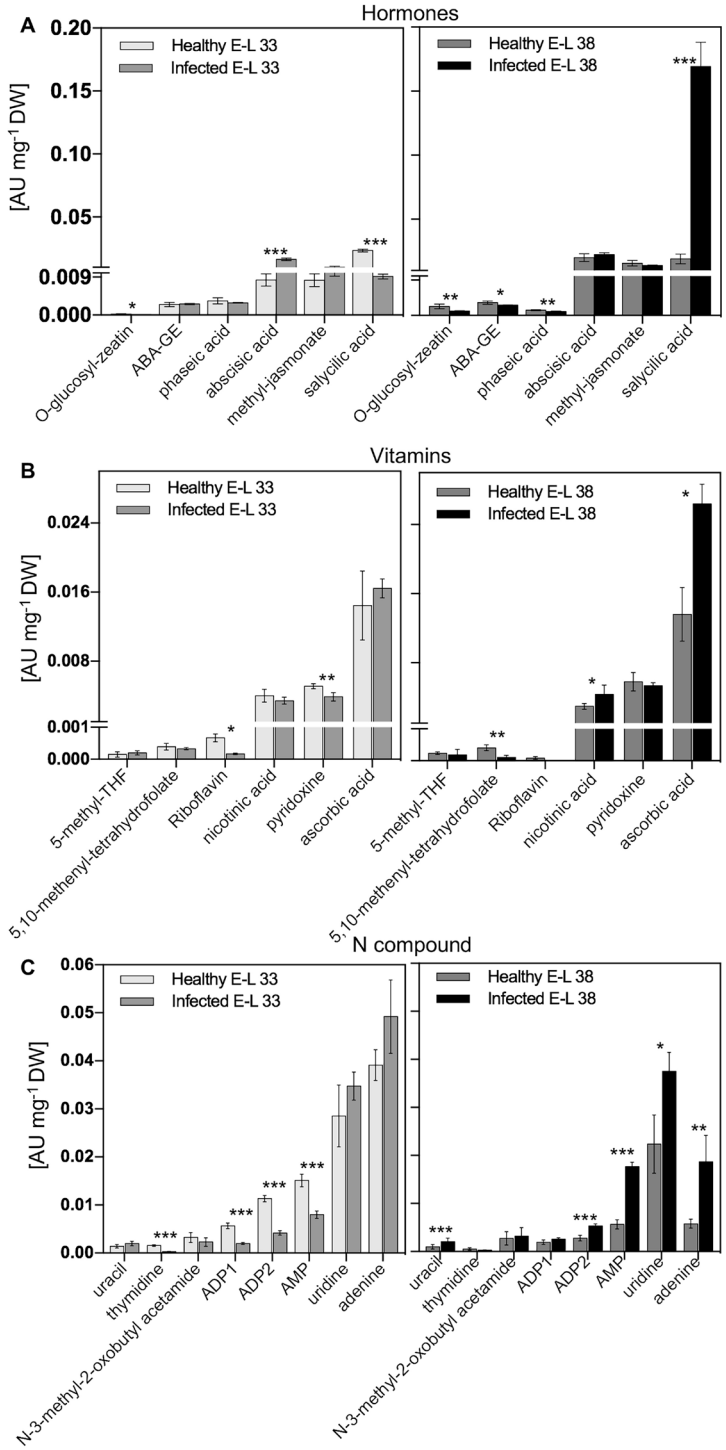

FIGURE 6. Main metabolites present in phloem sap from healthy and infected grapevines at two stages of development (E-L 33 and E-L 38).

(A) Hormones, (B) Vitamins, and (C) N compounds. Asterisks indicate statistical significance between healthy and FD-infected canes in each developmental condition according to the Student's *t*-test: *P ≤ 0.05; **P ≤ 0.01; ***P ≤ 0.001.

The heatmap in Figure 7 highlights the modifications to minor metabolites present in the phloem sap of healthy and infected grapevines at E-L 33 and E-L 38; it can be seen that erythronic and malonic acids significantly decreased in the phloem sap in response to infection at E-L 38, while five of the remaining acids incremented significantly (including pyruvic and 2-oxo glutaric acid).

In addition, the infection by FD led to a general increase in the amino acid content in the phloem sap levels at both of the developmental stages (Figure 5A). This was particularly evident for L-pyroglutamic acid (the cyclic lactam of glutamic acid), whose levels increased by 141 % in the phloem sap at the E-L 38 stage. The content of proline also increased by 52 % in the phloem sap in response to infection at the E-L 38

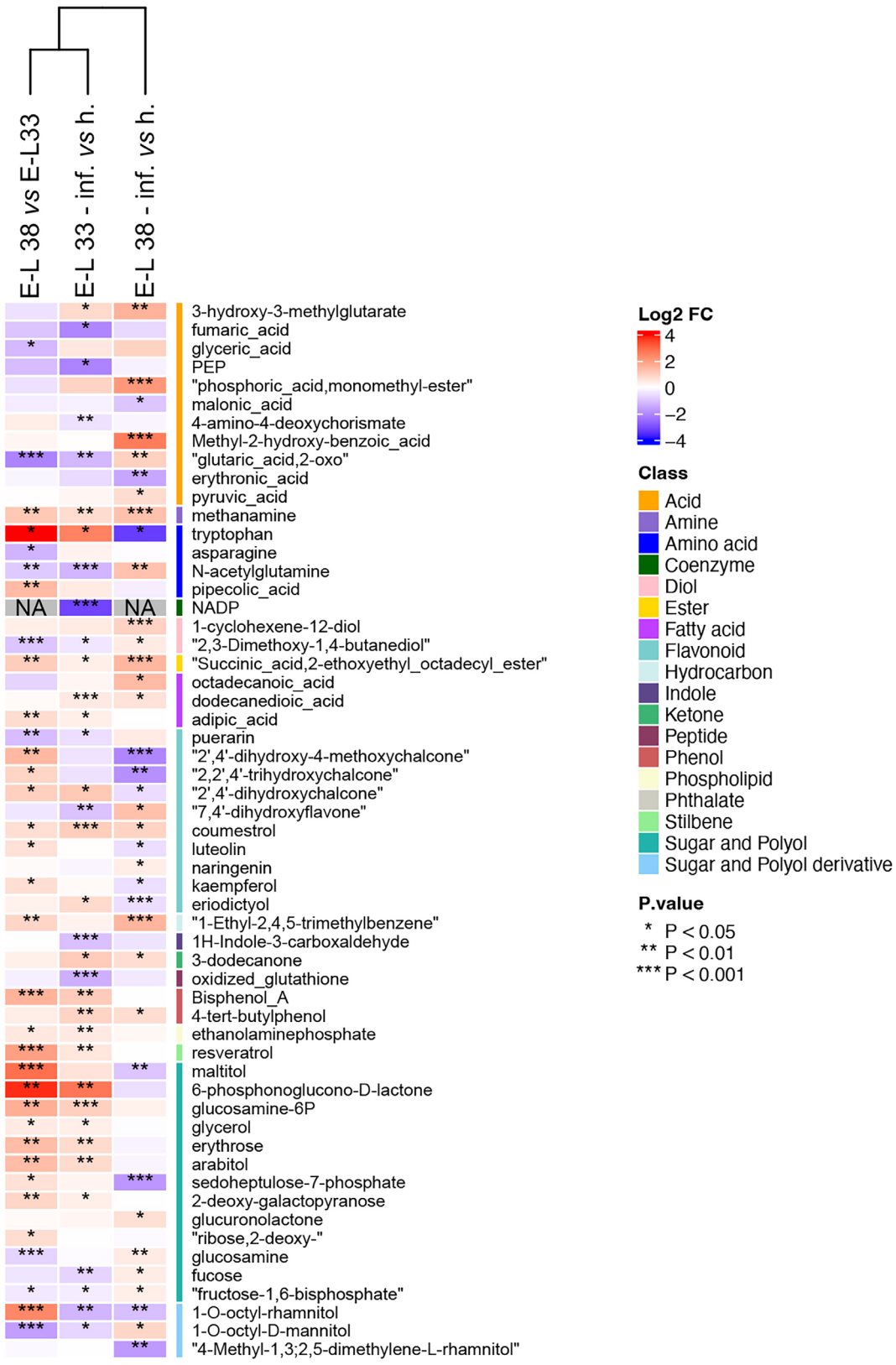

FIGURE 7. Heatmap of the modifications observed in minor metabolites present in phloem sap from healthy and infected grapevines at two stages of development (E-L 33 and E-L 38).

Each row represents a metabolite and each column represents the logarithm of the fold change (log_2 FC) between healthy samples at E-L 33 / E-L 38, infected / healthy at E-L 33 and infected / healthy at E-L 38. Classes of metabolites were labelled according to their position in the biosynthetic pathways. Asterisks indicate statistical significance between each sample type following Student's *t*-test: *P ≤ 0.05; **P ≤ 0.01; ***P ≤ 0.001; ****P ≤ 0.0001. Samples with no statistical significance in any of the three conditions were excluded.

stage. However, at the same developmental stage, the levels of the less represented tryptophan (whose relative amount increased during development - from E-L 33 to E-L 38) were reduced by 90 % in the phloem sap of the infected plants (Figure 7, Table S1).

The effect of the phytoplasma infection on the pattern of the eight phenolic acids identified in the phloem sap of cv. 'Loureiro' also differed between E-L 33 and E-L 38 (Figure 5B). In more detail, the most evident difference was in terms of the levels of gallic acid, which were not affected at E-L 33, but increased by 75 % at E-L 38. Additionally, syringic, coumaric and quinic acid levels in the phloem sap were also modified upon infection. Finally, resveratrol levels increased in the phloem sap of infected plants at E-L 33, but no significant changes were observed at E-L 38 (Figure 7, Table S1).

The most evident hormonal response to infection occurred at E-L 38, when the concentration of salicylic acid increased 9-fold (Figure 6A). At E-L 33, ABA levels increased by 97 % (but remained unchanged at E-L 38), while salicylic acid levels decreased by 61% in the infected grapevines; this corroborates the hypothesis that plant hormonal response to infection is dependent on the developmental stage and/or on the spreading of the disease. Additional metabolic changes included a significant increase in ascorbic acid levels (by 57 %) in the phloem sap at E-L 38 in response to infection (Figure 5B), and key N compounds (i.e., uracil, ADP, AMP, uridine and adenine), which levels changed in the phloem sap of infected grapevines, indicating that basic metabolic processes are affected by the disease at both developmental stages (Figure 6C).

DISCUSSION

1. Flavescence Dorée infection differentially affects phloem sap composition at E-L 33 and E-L 38 developmental stages

In the present study, grapevine phloem sap was collected by centrifugation after manually removing the stem bark, following the method of Hijaz and Killiny (Hijaz and Killiny, 2014; Killiny and Hijaz, 2015), which enabled the metabolite profile of phloem sap from healthy and FD-infected vines to be compared. The results of this comparison are relevant, because for many decades it has been a challenge for researchers to find appropriate methods for studying phloem sap composition, which is normally impaired by the rupture of the sieve elements when the phloem is cut, leading to the plugging of the pores of the sieve plates (Turgeon and Oparka, 2010). Classic methods include making incisions in the stem or cutting off petioles (Hall and Baker, 1972), EDTA-facilitated exudation (King and Zeevaart, 1974) or aphid stylectomy (Barlow and McCully, 1972; Fisher and Frame, 1984). More recently, phloem sap from Citrus sinensis was collected by centrifugation after manually removing the stem bark (Hijaz and Killiny, 2014; Killiny and Hijaz, 2015), which showed less artifact peaks than the K2-EDTA exudation (Killiny, 2019). Some studies on *Vitis vinifera* have also employed the EDTA-facilitated exudation method (Gourieroux *et al*., 2016; Tedesco *et al*., 2021). In a recent study by Tedesco *et al*. (2021) that aimed to evaluate the impact of metabolic scion x rootstock interaction on the composition of the phloem exudates, sugars (sucrose, mannose and acetol), polyols (myo-inositol, arabitol and threitol) and acids (tartaric, malic, succinic, shikimic, quinic, lactic and glycolic acids) were detected; the diversity and the relative abundance of the phloem metabolites identified in the present study in the stem bark of cv. 'Loureiro' are in line with these results.

Substantial changes in the phloem sap composition of grapevine cv. 'Loureiro' infected with FDp were also hypothesised in the present study, following consistent reports of phloem transport being impaired in FD-infected plants and considerable changes to carbohydrate metabolism; for example, *Catharanthus roseus*, periwinkle, coconut palm, maize, and *Vicia faba* (Gai *et al*., 2014; Hren *et al*., 2009; Junqueira *et al*., 2004; Maust *et al*., 2003; Santi *et al*., 2013). In this context, callose depositions have been observed in the sieve elements of *C. roseus*, *Euphorbia pulcherrina* (Christensen *et al*., 2005), *V. faba* and *V. vinifera* infected with FDp (Musetti *et al*., 2013; Oliveira *et al*., 2020; Prezelj *et al*., 2016). Moreover, the phloem metabolome was also altered in mulberry infected by *Candidatus* Phytoplasma asteris' (Gai *et al*., 2014) and apple infected by *Candidatus* Phytoplasma mali (Görg *et al.,* 2021).

The observed alterations in the phloem sap composition of cv. 'Loureiro' in response to infection are also likely related to metabolic changes promoted by the phytoplasma at leaf level. Indeed, our previous studies (Teixeira *et al*., 2020) have shown that the metabolism of isoprenoids in the leaves (in particular chlorophylls) is strongly affected, which is consistent with the severe FD-mediated repression of key genes involved in isoprenoid biosynthetic pathways. The concomitant accumulation of sucrose and starch in the leaf mesophyll of infected plants has been shown to be accompanied by the up regulation of key genes involved in their synthesis, including *Sucrose synthase 4* (*VvSusy4*) (Hren *et al*., 2009; Margaria *et al*., 2014; Prezelj *et al*., 2016). Furthermore, a limited leaf gas exchange has also been observed in grapevine cv. 'Nebbiolo' plants infected with FDp (Vitali *et al*., 2013).

The severity of the infection may explain, at least in part, some of the opposite effects that FD has on phloem sap composition at the E-L 33 and E-L 38 developmental stages. For instance, our results showed that FDp infection promoted the decrease in galactinol and sucrose at E-L 33, while at E-L 38 the amount of these sugars increased in response to infection. The same trend was observed for salicylic acid, which decreased at E-L 33, but increased abruptly at E-L 38. Furthermore, ferulic acid displayed higher levels at E-L 33 in FDp infected plants, but decreased at E-L 38. Indeed, it has been shown that the FDp titre (amount) increases during the development of cv. Nebbiolo and cv. Barbera, peaking at the mature stage of berry development (E-L 38), which was correlated with the severity of the

symptoms (Roggia *et al*., 2014). The developmental stage may also account for the observed differences in phloem composition in response to the infection. For instance, it is known that ABA peaks in the grapes as from just before véraison until the onset of ripening, thus it is conceivable that the effects of FD on their levels could be more pronounced at E-L 33. Nonetheless, several metabolites found at E-L 33 and E-L 38 showed similar patterns of variation in response to infection, such as L-pyroglutamic acid, syringic acid and galactose.

The results of the present study regarding grapevine at the earlier developmental stage is in agreement with the abovementioned study on mulberry, which reported a strong decrease in most of the sugars, sugar alcohols and sugar derivatives in the phloem sap of the two-week-old leaves of plants infected with *Candidatus* Phytoplasma asteris' (Gai *et al*., 2014). However, in our study, an increase in the levels of organic acids in response to FDp was observed, while the organic acids decreased in the mulberries in response to *Candidatus* Phytoplasma asteris infection, thus indicating the existence of only partly overlapping resistance mechanisms among different plant species.

Many organic acids, including glutaric, isocitric and tartaric acids, increased significantly in cv. 'Loureiro' bark at E-L 33 in response to FD-infection. The biological significance of this response requires further study. More specifically, tartaric acid was, by far, the most significantly affected organic acid, mainly at E-L 33, when its levels almost doubled in response to infection. The origin of tartaric acid lays outside the oxidative metabolism of sugars (Loewus and Stafford, 1958), and its biosynthesis begins with L-ascorbic acid (vitamin C) catabolism (Conde *et al*., 2007b). Notably, the Smirnoff–Wheeler pathway, in which vitamin C is synthesised from D-mannose and L-galactose (D-mannose/L-galactose pathway), represents the major route of vitamin C biosynthesis in plants (Dowdle *et al*., 2007; Smirnoff, 2018). Thus, the observed decrease in galactose levels in response to FD can be explained, at least in part, by the role of this sugar to fuel tartaric acid synthesis and vitamin C, as discussed below.

2. Flavescence dorée infection promotes the accumulation of compounds involved in plant defence pathways

The mobilisation of sugars and antioxidant compounds have been proposed as an effective strategy for counteracting FD pathogen spread and for preserving plant tissues from further oxidative damage (Pagliarani *et al*., 2020; Roggia *et al*., 2014). In this context, proline has also been associated with plant response to biotic and abiotic stress (Zarattini and Forlani, 2017); here, its levels increased by 52 % at E-L 38 in the phloem sap of cv. 'Loureiro' in response to FD. Notably, in cv. Modra frankinja infected by FD, proline levels in leaves also increased 3-fold (Prezelj *et al*., 2016). In parallel, the non-protein amino-acid derivative pyroglutamic acid, which has been reported as precursor of proline under stress conditions (Jiménez-Arias *et al*., 2019), increased abruptly at E-L38 in response to infection in the present study. The observation that the total amino acids content increased 2-fold in the phloem sap of cv. 'Loureiro' in response to FD needs further biological explanation. In mulberry (Gai *et al*., 2014), an increase in the amino acid content in response to phytoplasma infection has also been found in the phloem sap.

The observed increase in raffinose in the phloem sap of cv. 'Loureiro' following FD infection - albeit small compared to other responses - was consistent at both developmental stages. Raffinose and the subsequent higher molecular weight RFOs (stachyose and verbascose) are synthesised from sucrose by the subsequent addition of activated galactose moieties donated by galactinol, and are generally associated with the plant's response to drought, heat and cold stresses (Noronha *et al*., 2022). RFOs are characterised as compatible solutes involved in stress tolerance defence mechanisms, although evidence also suggests that they act as antioxidants (ElSayed *et al*., 2014; Peterbauer and Richter, 2001). Our results showed that, at E-L 38, the increase in raffinose was paralleled with higher levels of its precursor galactinol, which has also a recognised role in plant response to stress (Shimosaka and Ozawa, 2015).

It has been suggested that grapevine xylem size is reduced in FDp infected plants, a phenomenon generally associated with intense drought (Jelmini *et al*., 2021); it is thus tempting to speculate that some of the increases in osmolytes observed in the present study were produced in response to drought symptoms caused by FD-infection. In agreement, ABA content, which mediates drought stress responses, increased by 12 % in the phloem sap of infected vines at E-L 33. Furthermore, ABA induces stomatal closure, limiting water loss and restricting pathogen entry into the leaves (Bharath *et al*., 2021); interestingly, and in line with these results, it has previously been reported that ABA levels increased in leaves from FD-infected vines (Teixeira *et al*., 2020). Other reports have shown that increased hormone levels, including auxin, cytokinin and abscisic acid (ABA) are produced in plants in response to infection by phytoplasmas (Davey *et al*., 1981; Lee *et al*., 2000; Pertot *et al*., 1998; Tan and Whitlow, 2001). Moreover, in the present study, the phloem sap of infected grapevines at E-L 38 also contained increased levels of both salicylic acid (SA) and gallic acid. Salicylic acid (SA) is a hormone associated with plant defence against biotic stress (Rocher *et al*., 2006), which stimulates secondary metabolism, including the synthesis of gallic acid (Demirci *et al*., 2021), that can play an important role in plant defence.

Therefore, this study was a first step towards understanding the complex interactions between grapevine and the Flavescence dorée phytoplasma. Nonetheless, many questions remain unanswered regarding the biological significance of increased levels of specific compounds in response to infection. Some responses agreed with previous studies showing metabolic modifications in leaves in response to the infection, such as increased levels of ABA, while others can be related to the clogging of phloem vessels; for instance, when compounds like sugars were substantially reduced in

response to infection. Interestingly, we showed that several compounds, such as amino acids, were most abundant in the phloem sap of the infected plants; therefore, the blocking of the conducting vessels alone does not explain the observed differences between the metabolomes. Nonetheless, our study opens up promising avenues of research, particularly on the identification in phloem sap of specific pathogen-derived metabolites that can be considered diagnostic biomarkers of the disease, or the optimisation of agronomical strategies – like the exogenous application of gallic acid or SA – to mitigate FD symptoms.

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