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Research article

Seasonal changes in amino acids and phenolic compounds in fruits from hybrid cross populations of American grapes differing in disease resistance

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ABSTRACT

The production of wine grapes in upstate New York (USA) is limited by diseases that are promoted by the cool and sometimes rainy climate. A breeding program has been introducing disease resistance from related species into the cultivated stock. Previous work has indicated that such resistance may be based on biochemical reactions rather than on a hypersensitive reaction. We therefore undertook metabolic profiling of amino acids and phenolic compounds in berries from collections of susceptible and resistant hybrids over the course of berry development to determine whether any of these compounds could be causal in disease resistance. The most abundant amino acids were GLN, ARG, PRO and THR. The amount of amino acids in ripe berries was from 3 to 4.7-fold higher compared to earlier stages. The concentrations of total phenolics were variable through the season with no consistent trend between susceptible and resistant fruits. Notable changes in phenolic compounds, especially anthocyanins, were recorded, especially during the ripening phase, when phenolics and anthocyanins increased following veraison. The most abundant phenolic compounds were catechin and epicatechin; the most abundant anthocyanin was delphinidin-3-glucoside, which had a slightly greater concentration in resistant fruit at harvest, followed by malvidin-3-glucoside and petunidin-3-glucoside. The content of both amino acids and phenolic compounds in white-fruited parent cv. Horizon was equal to several-fold lower than the progeny plants, whether susceptible or resistant, depending on the harvest time. While no major differences between susceptible and resistant lines were found, multivariate analyses showed that it is possible to discriminate the susceptibility or resistance of grapes by analyzing their combined concentrations of amino acids, polyphenols and anthocyanins. Therefore, these compounds are influenced by the resistance capacity of grapes and could be used as a chemical fingerprint of this ability. However, it is likely that these are associations with disease resistance rather than their cause as no major consistent differences were noted.

1. Introduction

Grapes and wine are important agricultural products, as well as contributors to human health because of their content of many phytochemicals, especially antioxidant compounds such as stilbenes, including resveratrol, which function as antifungal compounds in the plants (Viret et al., 2018). The production of wine grapes in upstate New York (USA) is limited by diseases that are promoted by the cool and sometimes rainy climate. Especially important are powdery mildew (*Erisyphe necator*) (Wilcox, 2012) and downy mildew (*Plasmopora viticola*) (Wilcox, 2017), which infect both the leaves and developing fruit, and cause severe losses of grapes around the world. When highly susceptible cultivars are grown in New York, downy mildew can be a most serious disease causing complete defoliation and crop destruction (Kassemeyer et al., 2015). There is therefore much effort to develop disease resistant cultivars by introducing genes from disease-resistant

Abbreviations: A, August; CAT, catechin; t-CHALC, trans-chalcone; t-CINN, t-cinnamic acid; CYAN-3,5DG, cyanidin-3,5-diglucoside; CYAN-3G, cyanidin-3-glucoside; CYAN-CG, cyanidin-p-coumaroyl-glucoside; DELP-3G, delphinidin-3-glucoside; DELP-CG, delphinidin-p-coumaroyl-glucoside; L-EPIC, L-epicatechin; GA, gallic acid; J, July; LASSO, Least Absolute Shrinkage and Selection Operator procedure; LS, Late September; LDA, Linear Discriminant Analysis; MALV-3G, malvidin-3-glucoside; MALV-CG, malvidin-p-coumaroyl-glucoside; MYR, myricetin; NAR, naringenin; NER, Non-Error Rate; PEO-3G, peonidin-3-glucoside; PET-3G, petunidin-3-glucoside; PET-CG, petunidin-p-coumaroyl-glucoside; PROC-B2, procyanidin B2; S, September; t-RDE, trans-resveratroloside; t-RESV, trans-resveratrol; RUT, rutin

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wild species. However, the basis for the often-complex components of disease resistance is uncertain. The production of viniferins (oligomers of resveratrol) appears to contribute to the resistance of *V. riparia* (an American grape species) to the grapevine pathogens *Botrytis cinerea* (grey mold) and *Plasmopara viticola* (Langcake, 1981). The aim of this research was to analyze the range of chemical compounds in the fruits from grapes differing in disease resistance to determine if any changes in the global chemical composition can be found associated with disease resistance.

1.1. Amino acids in grapes

Several analyses of grape amino acids have been reported, with variation in the genotypes examined, climate (as determined by location), sample sources and method of analysis, leading to some variability in the results as might be expected (Gallander et al., 1969; Hernández-Orte et al., 2003; Huang and Ough, 1991; Soufleros et al., 2003; Vasconcelos and Neves, 1989). Most report the amino acids content of grape juice and/or the wine therefrom, but Gallander et al. (1969) analyzed crushed grapes. The older method used was by paper chromatography and ninhydrin colorimetry (Gallander et al., 1969), but more modern methods use HPLC with diode array or fluorescence detection with increasing detection and accuracy.

1.2. Phenolic compounds in grapes

Grapes are a rich source of phenolic compounds and numerous compilations have been recorded using different detection techniques. Grapevine resistance to pathogens is significantly dependent on the biosynthesis of secondary plant metabolites (Ehrhardt et al., 2014). Phenolic compounds are also of great importance for the quality of grapes and wine. Compounds such as anthocyanins, flavanols, and flavonols have a major impact on the characteristics, color and dryness of wine (Ehrhardt et al., 2014; Mattivi et al., 2006). Some grapevine phenolics, notably stilbenes, serve as phytoalexins that are induced in plants in response to stress. It is clear that the phenolic compounds vary in the fruit skin, pulp and seeds, and with genotype, climate, growing conditions and through the growing season; thus there is considerable variation in the relative amounts of the phenolics detected. Most experimental results use just the skins (e.g., (Mattivi et al., 2006; Ortega-Regules et al., 2006). Pinasseau et al. (2017) analyzed 96 phenolic compounds from the skins of ripe grape berries from 279 *V. vinifera* cultivars grown to assess the genetic variation for polyphenol composition and its modulation by irrigation. They found that different molecular families were affected either positively or negatively by drought varying with the cultivar. However, if the analysis runs from the earliest stages of berry development through the season, the results of the entire berries are usually recorded (Cuadros-Inostroza et al., 2016; Savoi et al., 2016).

The wild American grapes present a significantly different profile compared to cultivated *V. vinifera*. In a metabolomic comparison of wild American grape species with *V. vinifera* using UHPLC-QTOF-MS Narduzzi et al. (2015) noted a more complex content of anthocyanins and stilbenoids, together with hydrolysable tannins in the American grapes, whereas the vinifera berry skin and seeds had a much higher procyanidin accumulation in comparison to American berries. They also noted that the wild American grapes lacked aroma precursors (terpenoids, glycosides) that were present in the *vinifera* fruit. Diglucosides of anthocyanins are normally absent from *V. vinifera* but are found in small amounts in *Vitis* spp. native to America (De la Cruz et al., 2012). Liang et al. (2013) found 48 polyphenolic compounds, including 28 anthocyanins, 6 flavanols, 6 flavonols, 2 hydroxybenzoic acids and 6 hydroxycinnamic derivatives in ripe fruit of forty-eight accessions of *Vitis* hybrids preserved in the USDA-ARS *Vitis* germplasm repository in Geneva, New York. In an examination of the metabolomic profile of whole American grapes at maturity Ruocco et al. (2017) identified and quantified 124 selected metabolites including phenolics, proanthocyanidin and anthocyanin in five American species, two hybrids and two *V. vinifera* cultivars. There was a considerable variability in the metabolomic profiles with genotype. The proanthocyanidins of non-vinifera genotypes were mainly rich in oligomers and short-chain polymers. Most wild species contained both mono- and di-glucoside anthocyanins derivatives, but one hybrid and the vinifera cultivars contained only mono-glucoside anthocyanins.

1.3. Metabolic profiling of grapes

There have been several broader studies examining the metabolic profiles of grapes under different situations. Cuadros-Inostroza et al. (2016) investigated the metabolic profile of whole berries of Cabernet Sauvignon and Merlot cultivars during grape berry development during six stages from flowering to fully mature berries. One hundred and fifteen metabolites, including 19 amino acids, 4 fatty acids, 22 organic acids, 3 flavonoids, 19 sugars, were identified, with the relative levels in both cultivars. The analysis demonstrated changes in metabolic regulation as the maturity process progressed with both cultivars undergoing a highly coordinated shift of metabolite associated to primary metabolism during the stages involved in growth, development and ripening of berries. The profiles were characteristic for each stage, the most pronounced changes occurring at fruit setting and pre-veraison with sugars and amino acids levels showing an opposite trends as development proceeded. Multiple variations with regard to geographical location, vineyard location, including vineyard-specific variation, have been detected by metabolomic analysis (Anesi et al., 2015; Son et al., 2009), including differences in sugars, some amino acids, viniferins, stilbenes, anthocyanins and flavonoids.

Using proteomic analysis, metabolic profiling and multivariate analysis, Wang et al. (2017) have demonstrated the integration of protein and metabolite dynamics with their corresponding biochemical pathways over developmental time to show an energy-linked metabolism before veraison, with high abundances of amino acids and organic acids, and a subsequent shift to secondary metabolite synthesis. Anthocyanins were strongly accumulated after veraison whereas other flavonoids were in higher abundance at early developmental stages and decreased during the grape berry development. The rapid turnover of proteins involved in primary metabolism and growth in the photosynthetically active grapes appears to provide precursors for the production of protective secondary metabolites such as flavonols in the ripening stages of the berries (du Plessis et al., 2017).

Most studies on the metabolic profiles of grape fruits or leaves are aimed at comparisons between two or more growing conditions rather than an examination of the absolute contents of any class of metabolites. One of the first of these was by Pereira et al. (2006) who examined the influence of microclimate on 'Merlot' berries using NMR and HPLC, finding several discriminant phenolic, amino acid and sugar compounds between shaded and light-exposed berries. Continuing the climate theme Savoi et al. (2016) examined the influence of drought on compounds of the phenylpropanoid and terpenoid pathway in white grapes following whole berries through six development time points using UPLC-MS, reporting that drought increased concentrations of phenylpropanoids, monoterpenes, and tocopherols, while carotenoid and flavonoid concentrations varied with the developmental stage of the berries. Reshef et al. (2017) showed that sunlight affects the fruit metabolic profile of red cv. Cabernet Sauvignon in Israel, and the spatial pattern of compounds within grape clusters. Grape skin samples were analyzed using UPLC-QTOF-MS, and pulp primary metabolites by GC-MS. Sunlight affected the overall levels and patterns of accumulation of sugars, organic acids, amino acids and phenylpropanoids. Flavan-3-ol metabolites were reduced by sunlight exposure, whereas flavonols were highly induced. The overall levels of anthocyanins decreased with increased sunlight exposure, with malvidin anthocyanins and cyanidin-glucoside showing contrasting trends.

1.4. Metabolic profiling in relation to disease or disease resistance in grapes

Some metabolomic studies have given particular attention to the profiling of leaves or grape berries in relation to disease. Figueiredo et al. (2008) undertook the metabolic profiling of grape leaves in relation resistance to downy and powdery mildew. The resistance in cv. Regent, a red cultivar whose resistance derives from wild American species such as *Vitis rupestris*, *V. riparia*, and *V. lincecumii*, is considered to be based on biochemical reactions rather than on a hypersensitive reaction. The metabolic profile revealed an accumulation of compounds such as inositol and caffeic acid, which are known to confer resistance to fungi. Several metabolites differed between cv. Regent and susceptible cv. Trincadeira including glucose, inositol, succinic acid, alanine, glutamate and caffeic acid. In esca disease, caused by a complex of fungi, Lima et al. (2010) noted an increase in phenolic compounds along with alanine and gamma-aminobutyric acid in diseased leaves, accompanied by a decrease in carbohydrates, suggesting that disease causes a rerouting carbon and energy from primary to secondary metabolism.

Changes in grape fruits have also been reported in response to fungal infection. Degraded phenylpropanoids, flavonoid compounds, and sucrose together with increased glycerol, gluconic acid, and succinate, were found to be associated with the growth of *B. cinerea* on berries of *V. vinifera* cv. Chardonnay (Hong et al., 2012). Agudelo-Romero et al. (2015) examined metabolite changes in dark colored cv. Trincadeira berries upon infection with *Botrytis* using GC-MS. They noted an infection-associated reprogramming of carbohydrate and lipid metabolism towards an increased synthesis of secondary metabolites involved in plant defense, such as trans-resveratrol and gallic acid. Malic acid and tartaric acid declined and glucose and fructose increased especially post veraison. *Botrytis*, however, has a double face, depending on conditions. *Botrytis* as “noble rot” induces metabolic processes, namely the biosynthesis of anthocyanins, in white grape berries (cv Sémillon) normally seen only during the ripening of red-skinned grapes (Blanco-Ulate et al., 2015). The biosynthesis of terpenes and fatty acid aroma precursors also increased during noble rot. An intensive study of phenolic levels in five cultivars of disease-resistant of white and red grapes (including cv. Regent) at commercial maturity in two very different European locations was made using UHPLC-MS/MS by Ehrhardt et al. (2014), though they did not include a comparison with disease susceptible grapes. They quantified a large array of compounds including 55 phenolics: stilbenes, flavonols, flavanols, and anthocyanins.

The aim of this research is to analyze the range of chemical compounds of intact fruits over the course of fruit development from grapes differing in disease resistance to determine if any changes in the global chemical composition can be found associated with disease resistance. This metabolic profiling included phenolic compounds (by HPLC and diode array detection), which are often related to disease resistance and amino acids (by HPLC of fluorescence derivatives). The analyses also aimed at determining if specific phytochemical molecules could be associated with disease resistance. This would enable a determination of whether such molecules are part of the disease resistance or susceptibility mechanisms, and also whether a rapid analysis for such a compound could be used as a selective marker early in a breeding program.

Fig. 1. Grape bunches collected at early September sampling time. From left to right Horizon, resistant grape, susceptible grape affected by powdery mildew.

2. Material and methods

2.1. Plant materials

The metabolomic profiling was carried out on a range of hybrid grapes that differ in disease resistance, produced at the New York Agricultural Experiment Station. The grape plants were derived from a cross of grape cultivar 'Horizon' with Illinois 547-1 that were segregating for disease resistance. ‘Horizon’ is vigorous high-yielding, cold-tolerant white-wine cultivar derived from a cross between 'Seyval' (a cross of a European grape with American grape species) and 'Schuyler' (a cross between *V. vinifera* Zinfandel x Ontario [a vinifera x *V. labrusca* hybrid]) cultivars, originally made in 1945 and commercially available since 1982 (Reisch et al., 1982, 1983).[1] The Illinois 547-1 parent is a hybrid between *Vitis rupestris* B38 and *Vitis cinerea* B9, made at the University of Illinois by Dr. H. Barrett. (Ill.) 547-1 is a male-flowered genotype that is presumed to be genetically pigmented if berries were produced, and is highly resistant to powdery mildew and downy mildew. Eighteen sequence tagged site (STS) markers from 179 seedlings from Horizon x Illinois (Ill.) 547-1 correlated with segregation for downy mildew resistance in this population (Mahanil et al., 2007) (Fig. 1). The *V. cinerea* parent of Ill. 547-1 was also determined to harbor *Ren2*, a locus for resistance to powdery mildew (Dalbó et al., 2001; Feechan et al., 2015).

Mature vines from a cross between grape genotypes 'Horizon' x Illinois 547-1, plus the cv. Horizon, were field grown in a vineyard of the New York State Agricultural Experiment Station in Geneva, New York (Supplementary Table S1). The vines were grown under complete fungicide-free conditions, adjacent to susceptible vines, so that inoculum levels were likely to be high and the leaves and berries challenged by disease pressure (primary downy and powdery mildews) throughout the growing season. Fruit of ten susceptible, ten resistant lines and of the ‘Horizon’ mother plant were sampled four times during fruit development, from early fruit formation on July 9th (named day 0), growth in August 6th (day 29), veraison in early September 2nd (day 56), to almost fully ripe in late September 24th (day 78). One bunch was harvested from the main branch of each plant. Fruits were frozen in liquid nitrogen within two hours of harvest. Frozen grape berries were ground in liquid nitrogen to a fine powder with a pestle and mortar using 6–10 fruit per each sample; in susceptible grapes, berries were taken at random regardless of whether they were affected by disease. The frozen berry sample was transferred to 50 mL liquid N_2-pre-cooled plastic tubes. Frozen 0.5g sub-samples were transferred into Eppendorf tubes, with all tubes and implements precooled in liquid N_2. All tubes were stored in a −80 °C freezer. Samples were extracted with appropriate solvents and analyzed for their content of flavonoids and amino acids.

2.2. Amino acids analysis

One mL of cold 20 mM HCl was added to 0.5 g of ground grape berry powder. Ten μL of 25 mM norleucine was added as an internal quantitative standard. The extract was stirred for 20 min at room temperature and centrifuged at 13,000 g for 10 min. The supernatant was passed through a 0.4 mm filter tube into an Eppendorf tube and left at −20 °C overnight. The supernatant was re-centrifuged to separate

[1] For varietal parentage see http://www.hort.cornell.edu/reisch/grapegenetics/nyreleases.html.

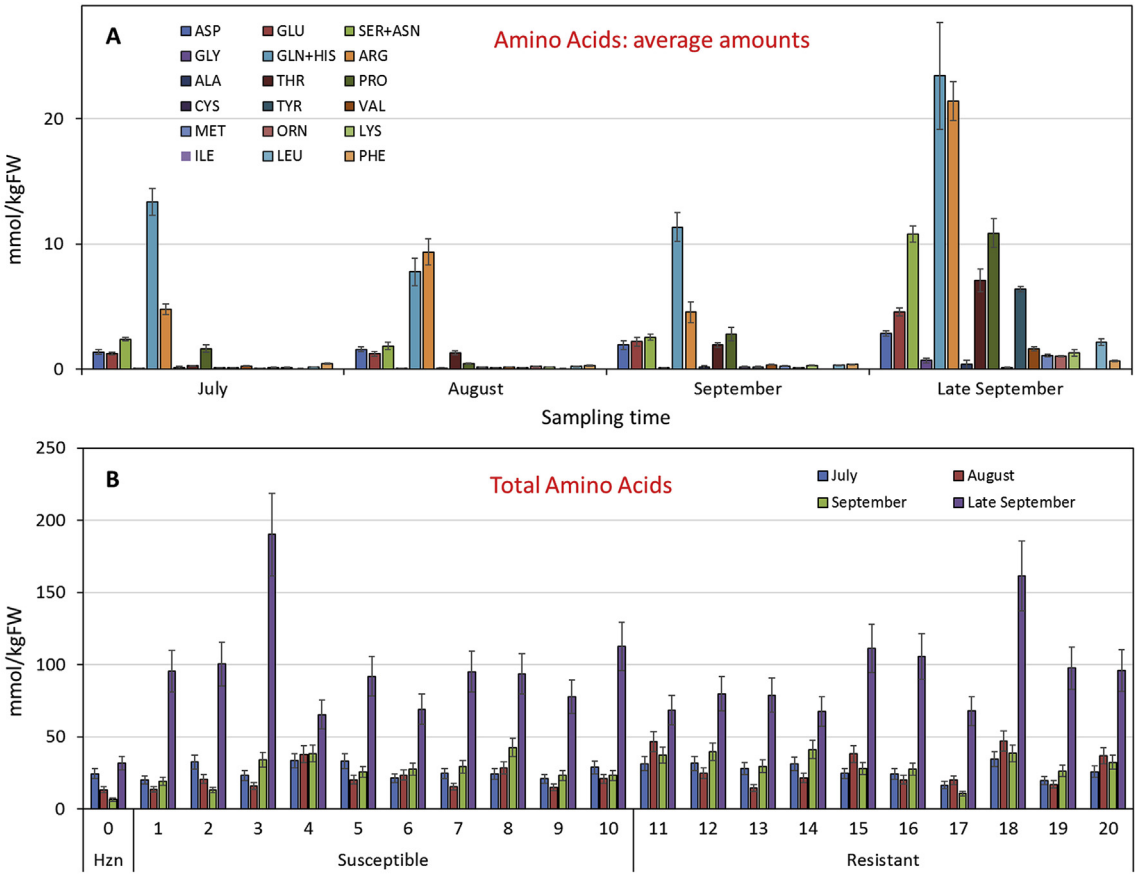

Fig. 2. (A) The average content of each amino acid in all 20 red grape lines from each harvest time (± SE, n = 40). (B) Total amino acids levels in berry samples of Horizon (mother plant, 0), susceptible (1–10) and resistant (11–20) plants collected at the four sampling times. (Data are the mean of 2 replicates ± SE). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

sugars and placed into a fresh Eppendorf tube.

Derivatization for amino acids was as per the recommended procedure using a Waters (Milford, MA, USA) AccQ·Fluor reagent kit. The Waters AccQ·Tag amino acid analysis method is a precolumn derivatization technique for amino acids. The AccQ·Fluor reagent converts both primary and secondary amino acids to stable, fluorescent derivatives. To 5 µL of extraction solution in an Eppendorf tube 35 µL of 0.2 M borate buffer, pH 8.8 and 10 µL of reconstituted 10 mM AccQ·Fluor reagent (6-aminoquinolyl-N-hydroxysuccinimidyl carbamate in acetonitrile) was added, with vortexing for several seconds at each step. After 1 min at room temperature the tubes were heated in a block at 55 °C for 10 min.

Amino acids were analyzed according to Cohen and Michaud (1993) with some modifications, using a Beckman 110B HPLC system with a Waters AccQ·Tag amino acid analysis column (4.0 µm Nova-Pak C18 column, 4.0 µm particle size, 3.9 mm × 150 mm) preceded by a Waters Sentry guard column. 5 µL of the above derivatized mixture was subjected to HPLC. The amino acids were eluted at a flow rate of 1.0 mL/min at a column temperature of 38 °C. Mobile phase A consisted of 140 mM sodium acetate and 17 mM triethylamine with the pH adjusted to 5.6 with phosphoric acid and mobile phase B was 60% acetonitrile in water (v/v). The HPLC gradient used was (as % B), 2%–7% over 5 min, 10% over 12 min, 33% over 19, hold 2 min, 80% over 9 min, 100% over 2 min hold for 5 min and return to 2%. Detection was by fluorescence (Perkin Elmer, model 650-LC, Norwalk, CT, USA) with excitation at 250 nm and emission at 395 nm, with quantitation by an EZChrome data system. R_ts were confirmed using standards. In was not possible to separate the peaks of SER from ASN and of HIS from GLN therefore data reported refer to the sum of the amounts of both couples of peaks (ASN + SER and GLN + HIS).

2.3. Phenolic compound analysis

1.5 mL of extraction solution (MeOH:H_2O:formic acid 70:28:2) was added to 0.5 g of grape powder at 4 °C, vortexed and then shaken for 30 min at 30 °C, centrifuged, and the supernatant microfiltered through a 0.45 µm centrifugal filter at 3000g. Analysis was by HPLC (HP1100 Liquid Chromatograph, Palo Alto, USA) with detection by a diode array detector (Agilent Technology, Palo Alto, CA, USA). The HPLC column used was an Inertsil ODS-3 column; (5.0 µm particle size, 4.6 mm × 250 mm) (GL Sciences Inc., Tokyo, Japan) with an Inertsil ODS-3 guard column (5.0 µm particle size, 4.0 mm × 10 mm) at a flow rate of 1.0 mL/min at 30 °C. 20 µL of the filtered supernatant was injected and run in a gradient of solvent A: 10% formic acid in water and solvent B: 10% of 88% formic acid dissolved in acetonitrile (as % B): 5%–15% over 25 min, 22% over 17 min, 36% over 18 min, returning to 5% over 5 min, and held for 10 min before rerunning. Detection wavelengths (band width 20 nm) used were: anthocyanins: 520 nm; flavones and flavonols: 365 nm; hydroxycinnamic acids: 320 nm; hydroxybenzoic acids, flavan-3-ols, and dihyrochalcones: 280 nm. R_ts and spectra were confirmed using standards.

2.4. Heat-maps and multivariate analysis

The starting dataset was composed of 39 variables (belonging to three typologies: amino acids, phenolic compounds and anthocyanins) and 168 objects, corresponding to two replicate measurements of 84 samples. Four samples (8 objects) correspond to the mother plant cv. Horizon, each of them sampled at a different time period (July (J), August (A), early September (S) and Late September (LS)). The remaining 80 samples (160 objects) belong half to the “susceptible” class

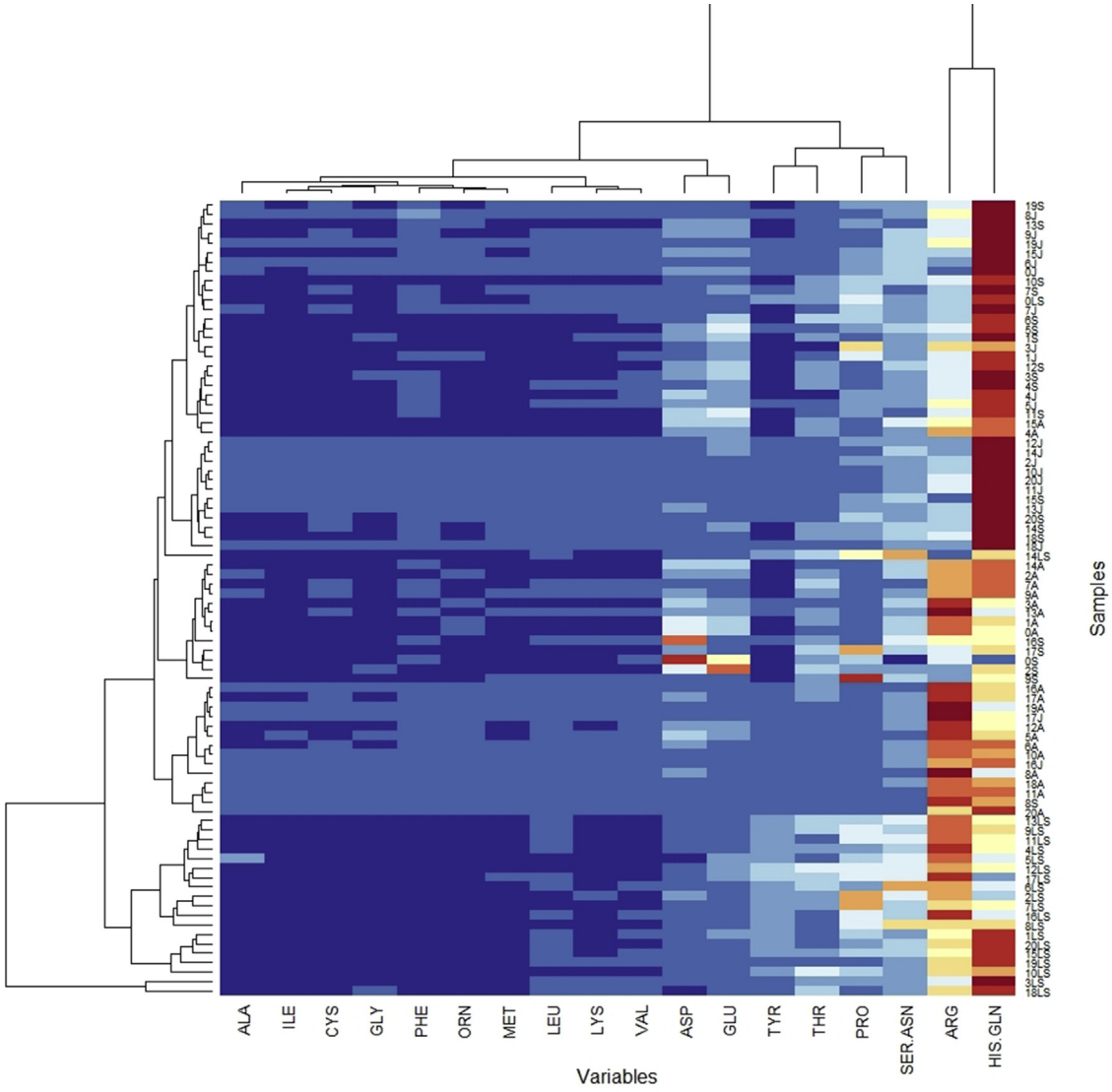

Fig. 3. Heat-map of the dataset containing amino acids variables. Red colour indicates higher concentration, blue colour indicates lower concentration. Numbers in the right part of the graph indicate the plant number/sampling time codes. July (J), August (A), September (S), Late September (LS). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

and half to the "resistant" class. They again represent 20 grape genotypes sampled at the same four periods of the year (Supplementary Tables S1 and S2). In order to show the behavior of the variables, two *heat-maps* were created by the software *R* version 3.3.3 (R Core Team, Vienna, Austria). A heat-map is a representation of data in the form of a map or diagram in which data values (i.e. contents of different metabolites) are represented as colours. In the present paper red colour indicates higher concentration, blue colour indicates lower concentration. A heat-map is useful for making a rapid check of the original data (for example, to look for groups of samples or variables that have a similar behavior), before performing any computation. To make the heat-maps clearer, the two replicates of all the variables of each sample were averaged, and the dataset was split in two sub-sets: the first one containing only amino acid variables, the second one containing phenolic compounds and anthocyanins. Therefore, the *amino acid heat-map* is representative of 84 samples and 18 amino acids, while the *phenolics plus anthocyanins heat-map* is representative of the same 84 samples and 21 phenolic compounds and anthocyanins.

In addition, the full metabolomic profiles were used to discriminate susceptible and resistant plants by a multivariate chemometric approach (Madsen et al., 2010). Linear Discriminant Analysis (LDA) (McLachlan, 2004) was applied to the metabolomics dataset. LDA is a chemometric tool that allows to classify a sample into a specific class. In order to create a LDA model, a training-set is needed. The training-set is a dataset formed by objects whose *a priori* class is well known. In this case the *a priori* classes are: "susceptible" or "resistant" or "mother plant" classes. Once the LDA model has been created and validated, the classification of unknown objects can be obtained. From the mathematical point of view, LDA rotates the space spanned by the original variables into a new subspace in which the distances between class centroids (the points defined by the mean of all variables of each class) are maximized. LDA-space is defined by a number of dimensions equal to the number of classes less one, each dimension being identified by a versor called Linear Discriminant (LD). Starting from the first one, each dimension is associated with a decreasing percentage of information (i.e. explained variance). Thus, it is possible to visualize the behavior of samples by projecting them onto the most informative two or three-dimension plot (discriminant plot). New samples are projected onto the LDA-space, and are assigned to the class for which the Mahalanobis distance from the class centroid is lower. Mahalanobis distance is a mathematical way for computing the distance between two objects in a multivariate field, taking into account the structure of data, rather than

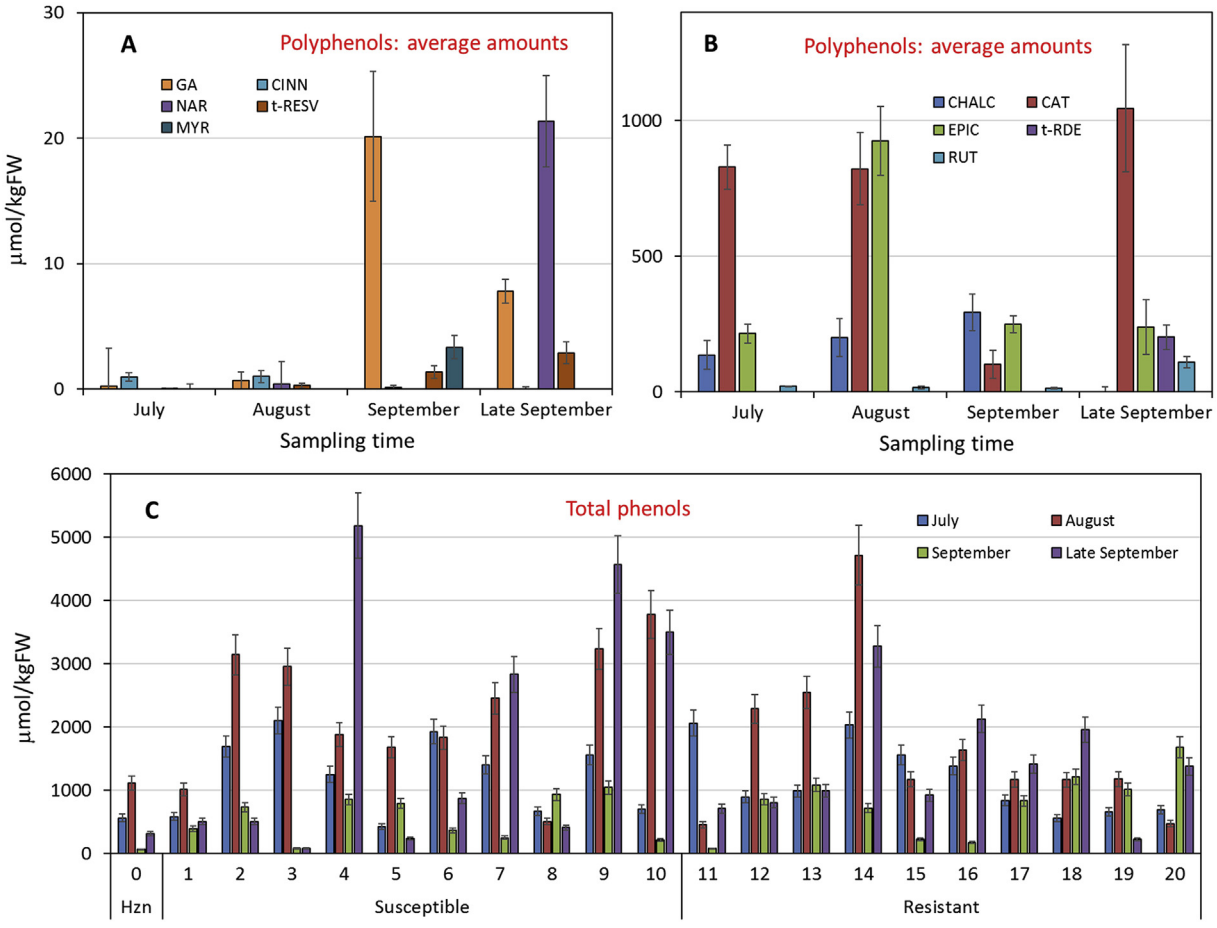

Fig. 4. (A, B) The average content of each polyphenol in all 20 red grape lines from each harvest time (± SE, n = 40). (C) Total phenol levels in berry samples of Horizon (mother plant, 0), susceptible (1–10) and resistant (11–20) plants collected at the four sampling times. (Data are the mean of 2 replicates ± SE). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

the simpler Euclidean distance between two points. Mathematical details of the Mahalanobis distance are reported by De Maesschalck et al. (2000). LDA models were validated by Leave-one-out (LOO) cross-validation (Stone, 1974). This method leaves one object (sample) of the dataset at a time out from the computation, and calculates the model with the others, then the left-out object is projected onto the model. At the end of the process, the percentage of correct classifications, Non-Error Rate (NER) is calculated. NER is an evaluation of the model performance; its value varies between 0 and 100%, where 100% indicates a perfect model. In order to improve NERs, a statistical selection of the most informative variables was carried out by the Least Absolute Shrinkage and Selection Operator (LASSO) procedure. Mathematical details of LASSO are described by Tibshirani (1996). The purpose of variable selection is to reduce analytical noise and spurious information by excluding non-informative or redundant variables from the chemometric analysis. All chemometric analyses were performed by the software *R* version 3.3.3 (R Core Team, Vienna, Austria), LDA were computed with the package *MASS* (Venables and Ripley, 2002), and LASSO with the package *glmnet* (Friedman et al., 2010).

3. Results and discussion

Free amino acids and phenolic compounds from ten susceptible (samples 1–10) and ten disease resistant (samples 11–18) hybrids, as well as the susceptible parent Horizon, were analyzed as to content and concentration over a three month period from early development in July (J), to veraison in early September (S), and to fully ripe in late September (LS). As expected, there was considerable variation amongst the lines, as well as changes during berry development and ripening, both in amount of single compounds and in the total content of amino acids (Fig. 2), phenolics and anthocyanins (Figs. 4 and 5). Heat-maps were created from the amino acid (Fig. 3) and phenolics data (Fig. 6).

3.1. Amino acids

The average amino acid content from all lines over developmental time from early berry development in July to fully ripe in late September is shown in Fig. 2A and the total amount of amino acids for all plants at the four sampling times is shown in Fig. 2B. The average amount of amino acids was from 3 to 4.7-fold higher in individual samples of LS berries with respect to earlier collected samples. Overall the most abundant amino acids were GLN + HIS (most likely GLN from the results of others (Gallander et al., 1969; Huang and Ough, 1991)) and ARG, with both showing the highest levels in the ripe fruit at LS, with means of 23.4 and 21.4 mmol/kgFW respectively (and maximum amounts of 82.4 mmol/kgFW in sample 3 LS and 37.6 mmol/kgFW in sample 18 LS, respectively). PRO and THR were other abundant amino acids especially at LS when their mean concentrations were 10.9 and 7.1 mmol/kgFW (17.3 mmol/kgFW in sample 18 LS and 23.5 mmol/kgFW in sample 2 LS respectively). The mean concentrations of total amino acids in susceptible and resistant fruits, respectively, were 26.2 and 26.7 mmol/kgFW in July (J), 21.1 and 28.6 mmol/kgFW in August (A), 27.6 and 31.1 mmol/kgFW in early September (S), and 99.1 and 93.4 mmol/kgFW in late September (LS). Overall the content of amino acids in Horizon mother plant was equal (J) or up to 4.6-fold lower (S) than the average of other plants, whether susceptible or resistant.

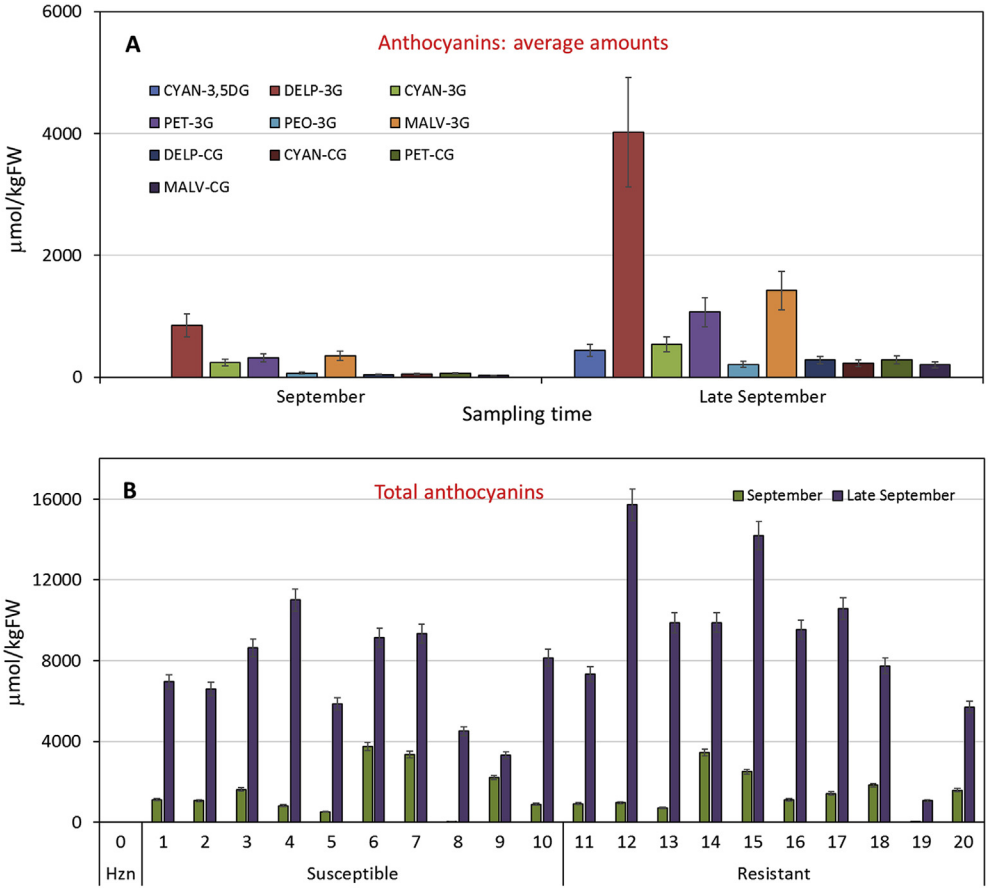

Fig. 5. (A) The average content of each anthocyanin in all 20 red grape lines from the September harvests (± SE, n = 40). (B) Total anthocyanin levels in berry samples of Horizon (mother plant, 0), susceptible (1–10) and resistant (11–20) plants collected at the two September sampling times. (Data are the mean of 2 replicates ± SE). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

In all cases previously, where multiple genotypes were tested (Gallander et al., 1969; Huang and Ough, 1991; Vasconcelos and Neves, 1989), substantial differences in amino acids have been noted. Allowing for differences in analytical techniques it appears that the predominant amino acids are PRO and ARG, with which amino acid is predominant and the ratios between them being varietally determined (Huang and Ough, 1991). The levels of amino acids reported by Huang and Ough are similar to considerably lower than our results (e.g., 16 mmol/L PRO in 'Cabernet Sauvignon' and 5 mmol/L ARG in 'Pinot noir'), but their data represent different species and location from our samples, which at least in part, could account for the differences. In contrast to Huang and Ough, Gallander et al. (1969), examining three American (*V. labrusca*) and five American-French hybrids, found ALA and SER were the most prominent amino acids using paper chromatography, but also noted PRO (0.2–4.5 mmol/L) and ARG (0.5–3 mmol/L) as prominent, but the levels varied considerably by genotype.

Fig. 3 represents a *heat-map* of the levels of 18 amino acids in 84 samples (based on Supplementary Table S2). At the top and at the left of these graphs, the dendrograms of cluster analyses based on variables and objects are respectively shown. In a dendrogram, the y-axis represents the Euclidean distance and the horizontal lines between two vertical lines represent the distance between two (or two groups of) objects (or variables). Thus, the higher is the position of a vertical line, the higher the distance between two objects, the lower is the similarity between them. Therefore, the *heat-map* is useful to investigate the behavior of objects and variables in the dataset. A group of amino acids (ALA, ILE, CYS, GLY, PHE, MET, LEU, LYS and VAL), in the left part of Fig. 3, have a lower concentration in all samples and their concentration does not significantly vary during the ripening period. By contrast, the other amino acids, in the right part of the *heat-map*, have, in general, a higher concentration, especially for ARG and GLN + HIS. It is also interesting to note that whereas TYR has a low overall concentration, comparable to the one of the first group of amino acids, it considerably increases in the samples harvested in late September (lower part of the *heat-map*).

3.2. Phenolic and anthocyanin compounds

The average content of phenolic compounds from all lines over developmental time from early berry development in July to fully ripe in late September is shown in Fig. 4A and the total amount of phenolic compounds for all plants at the four sampling times is shown in Fig. 4B. Overall the most abundant compounds were CAT and EPIC, respectively at 823 and 925 µmol/kgFW in August. In July and in late September CAT, at 829 and 1045 µmol/kgFW respectively, was much higher than EPIC, while at the beginning of September both were much lower, possibly because of diversion of catechins to the production of tannins. NAR was notable for appearing only during ripening and having a maximum concentration only in late September, albeit at a much lower concentration of 21.3 µmol/kgFW. The mean concentrations of total phenolics in susceptible and resistant fruits respectively, were 1233 and 1167 µmol/kgFW in July (J), 2250 and 1680 µmol/kgFW in August (A), 568 and 789 µmol/kgFW in early September (S), and 1870 and 1383 µmol/kgFW in late September (LS). The levels of CAT and EPIC were, on average 710 and 485 µmol/kgFW (206 mg/kg and 141 mg/kg) in susceptible plants and 690 and 328 µmol/kgFW (200 mg/kg and 95 mg/kg) in resistant plants. Overall the content of phenols in the 'Horizon' parent was from 2 (A) to 8.3-fold lower (S) than in susceptible plants and from 2 (J) to 11.5-fold (S) lower than in resistant plants.

The changes in phenolics and anthocyanins compounds were especially noticeable following veraison. Stilbenes t-RESV and t-RDE started to be synthesized after veraison, reaching average concentrations of 2.9 and 201 µmol/kgFW respectively in the red genotypes in late September (Fig. 4A). As expected, anthocyanins were detected only

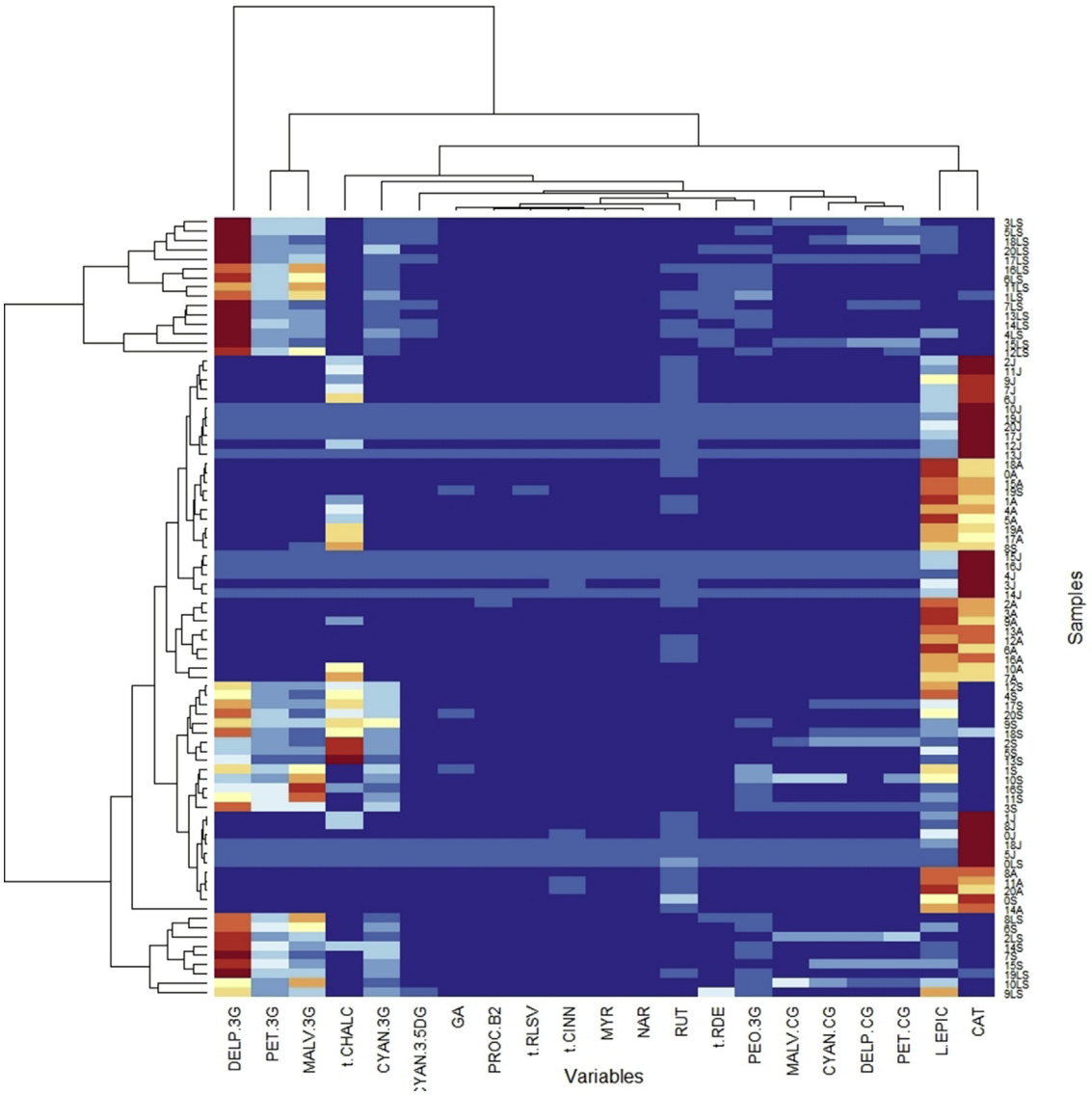

Fig. 6. Heat-map of the dataset containing phenol and anthocyanin variables. Red colour indicates higher concentration, blue colour indicates lower concentration. Numbers in the right part of the graph indicate the plant number/sampling time codes. July (J), August (A), September (S), Late September (LS). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

after veraison at the September (S) and Late September (LS) sampling times (Fig. 5A), and their total amounts are shown in Fig. 5B. Delphinidin-3-glucoside (DELP-3G) was the predominant anthocyanin reaching a mean concentration of 4017 μmol/kgFW, followed malvidin-3-glucoside (MALV-3G) at 1421 μmol/kgFW and petunidin-3-glucoside (PET-3G) at 1067 μmol/kgFW. Pelargonin 3-glucoside was not present in any of the samples. The mean concentrations of total anthocyanins in susceptible and resistant fruits respectively, were 1539 and 1456 μmol/kgFW in September, and 7350 and 9164 μmol/kgFW in late September. As the mother plant Horizon was a white grape, no anthocyanins were detected. DELP-3G had an LS average content of 3478 and 4774 μmol/kgFW (1739 mg/kg and 2387 mg/kg) respectively in susceptible and resistant plants.

There are numerous reports of the levels of phenolic compounds in grape fruits but the striking point is the differences in levels reported, sometimes as wide as an order of magnitude. The most useful comparisons are those of Liang et al. (2013) and Ruocco et al. (2017) as they analyzed full berries, as contrasted to solely the berry skins (Mattivi et al., 2006; Narduzzi et al., 2015; Ortega-Regules et al., 2006; Pinasseau et al., 2017; Reshef et al., 2017), and they included native American species and American hybrids as well as *V. vinifera*, though they only analyzed fruits at the fully-ripe stage. Even then there was a great variability in different genotypes and vintages. As with our results, Liang et al. (2013) found that anthocyanins were the main polyphenolic compounds in colored hybrid accessions accounting for 78% of the total polyphenols with a mean content of 3.4 g/kgFW. Delphinidin-glucosides accounted for 36% of the total anthocyanins in the hybrid grapes with malvidin-glucosides also prominent. Flavanols accounted for 33% of the total non-anthocyanin polyphenols and were comprised of procyanidin B1 (46%), catechin (26%), and epicatechin. Ruocco et al. (2017) reported the sum of all phenolic compounds in *V. vinifera* cultivars ranged from 445 to 1426 mg/kgFW, and from 182 to 3695 mg/kgFW in the wild genotypes. In the wild genotypes the phenolic acid content ranged from 20 to 265 mg/kgFW, and 18–133 mg/kgFW in *V. vinifera*. The total flavonol content ranged from 14 to 1032 mg/kgFW; flavan-3-ols, ranged from 335 to 1148 mg/kgFW in *V. vinifera* cultivars, and from 61 to 3120 mg/kgFW in wild genotypes; and stilbenes ranged from 10 to 180 mg/kgFW, with the highest level in *V. californica*. *V. californica* and one of the American hybrids had the highest and lowest total anthocyanin content within wild genotypes

Table 1
The detected amino acid and phenolic compounds are listed. The relative levels are indicated by + signs; * = present at a very low level; − = rarely present at a very low level. Variables selected by LASSO algorithm for each compound are indicated by **X**. Sampling period: J, July, A, August, S, September, LS, Late September.

Amino Acids					Phenolics and Anthocyanins				
Compound	J	A	S	LS	Compound	J	A	S	LS
ASP	++	++	++**X**	++	GA	***X**	***X**	+	+**X**
GLU	++	++	++	++	CAT	+++++**X**	+++++	++	+++++**X**
SER + ASN	++	++**X**	++**X**	+++**X**	L-EPIC	++**X**	+++++**X**	++**X**	++**X**
GLY			**X**	+	t-CHALC	++**X**	++**X**	+++	*
GLN + HIS	++++**X**	++++	++++**X**	+++++**X**	t-CINN	+**X**	+**X**	***X**	*
ARG	+++**X**	++++**X**	+++**X**	+++++**X**	RUT	+**X**	+	+**X**	++**X**
ALA	+**X**	+	+**X**	+**X**	NAR	−	*	−	**X**
THR	+	++**X**	++**X**	+++	t-RDE	−	−	−	++
PRO	++**X**	+**X**	++	++++**X**	t-RESV	−	***X**	***X**	***X**
CYS	+**X**	+	+	+	MYR	*	−	−	−
TYR	+**X**	+**X**	+**X**	+++**X**	PROC-B2		−		
VAL	+	+**X**	+	++	CYAN-3,5DG			−	+**X**
MET	+	+**X**	+	++	DELP-3G			+	+++++
ORN	+**X**	!**X**	+**X**	++**X**	CYAN-3G			+	+**X**
LYS	+**X**	+	+**X**	+	PET-3G			+	++
ILE	−**X**	−**X**	−**X**	−	PEO-3G			***X**	+**X**
LEU	+	+**X**	+	++	MALV-3G			+	++
PHE	+**X**	+	+**X**	++**X**	DELP-CG			***X**	+**X**
					CYAN-CG			*	+
					PET-CG			*	+
					MALV-CG			***X**	+

(5244 and 735 mg/kgFW respectively), whereas *V. vinifera* cultivars such as 'Cabernet Sauvignon' and 'Pinot noir' had a lower mean amount of anthocyanins (1045 and 437 mg/kgFW respectively). Given the wide variation, our results are in the same range as other reports.

A second *heat-map* was designed in order to represent the same 84 samples and 21 phenolic compounds and anthocyanins (Fig. 6) (based on Supplementary Table S3). Fig. 6 shows that the anthocyanin DELP-3G has an anomalous behavior compared to all other variables, and there are two binary clusters of variables which deviate from the general trend: the first one is composed by PET-3G and MALV-3G (both anthocyanins), the second one by EPIC and CAT (polyphenols). It is also interesting that DELP-3G and CAT have an opposite trend: when the concentration of one of them increases, the concentration of the other decreases. Samples can be divided into three clusters, mainly due to DELP-3G and CAT: the first and third ones, in the lower and higher part of the graph, are composed of samples with a high value of DELP-3G, while the second one, in the center, includes samples with a low value of DELP-3G. Also in this case, there is a group of variables (from CYAN-3,5DG to PET-CG, in the central part of Fig. 6) that have a similar trend and, in general, a low concentration in all samples.

3.3. Multivariate analysis

The behavior of each single variable in both heat-maps seems not to be influenced by the presence or absence of a "resistance factor", but is mainly due to the harvest sampling time. This means that it is difficult to find a single variable that perfectly discriminates susceptible and resistant plants. However such resistance-based information can be obtained by considering the metabolomic profile as a whole. Therefore the data were then subjected to multivariate analysis to tease out the finer changes and differences. "Multivariate analysis" is any mathematical processing that takes into account more than one independent variable at a time. The aim is to extract most (if not all) of the information carried by the data, and to create a mathematical model that describes the data in the simplest possible way (for example with a graph or with some significant numbers). Multivariate analysis has been used in the analysis of metabolites in grape fruits by Wang et al. (2017) in relation to the development of *V. vinifera*, and by Ruocco et al. (2017) for the comparison of American grape species with *V. vinifera* and hybrids. Principal Component Analysis has also been used to characterize the amino acids in wine to the original grape varieties (Vasconcelos and Neves, 1989).

A Linear Discriminant Analysis (LDA) model was computed on the starting dataset (168 objects x 39 variables). The aim of LDA is to discriminate the three classes "susceptible", "resistant" and "mother plant" on the basis of the full metabolomic profile. This first model (LDA1) gave a 64.3% of Non-Error Rate (NER), meaning that 64.3% of the objects of the dataset were correctly classified into their corresponding class. As generally in chemometrics a NER is only considered to be meaningful when higher than 75–80%, this result is non-definitive, but is considered interesting such that a refinement of the chemometric processing is worthy of further investigation to give more informative results. The dataset was then divided into 4 sub-groups of samples, based on the sampling period: July (J), August (A), September (S), Late September (LS). LDA models were computed on each of this sub-groups, giving the following NERs: 73.8% for J, 88.1% for A, 59.5% for S and 59.5% for LS. Therefore, the obtained results are good for the July and August groups, but poor for S and LS; this could mean that chemical composition is more important early in fruit development rather than later when the fruits are starting to ripen.

In order to improve the NERs, a Least Absolute Shrinkage and Selection Operator (LASSO) procedure was applied to each group. LASSO is a mathematical processing (or multivariate analysis) that has the aim of selecting, in the original dataset, a subset of independent variables that are the most informative for describing the dependent one. In this case, the dependent variable is the resistance class ("resistant", "susceptible" or "mother plant"), so that LASSO looks for that subset of variables that mostly discriminate these classes. LASSO selected subsets of the variables looking for the most informative ones with respect to the classification target. Then, for each group, a new LDA model (LDA2) was computed with the LASSO-selected variables only. The new NER obtained are: 88.1% for J (16 variables), 71.4% for A (15 variables), 88.1% for S (19 variables), 83.3% for LS (18 variables). Table 1 shows the LASSO selection for each group. The discriminant plots of these models for each harvest time are reported in Fig. 7. In these graphs, each point represents an object, the colors of which represent the prior class (black for "mother plant", green for "susceptible", red for "resistant"), while the symbols represent the posterior class (circle for "mother plant", cross for "susceptible" and triangle for "resistant"); the filled circles represent the corresponding

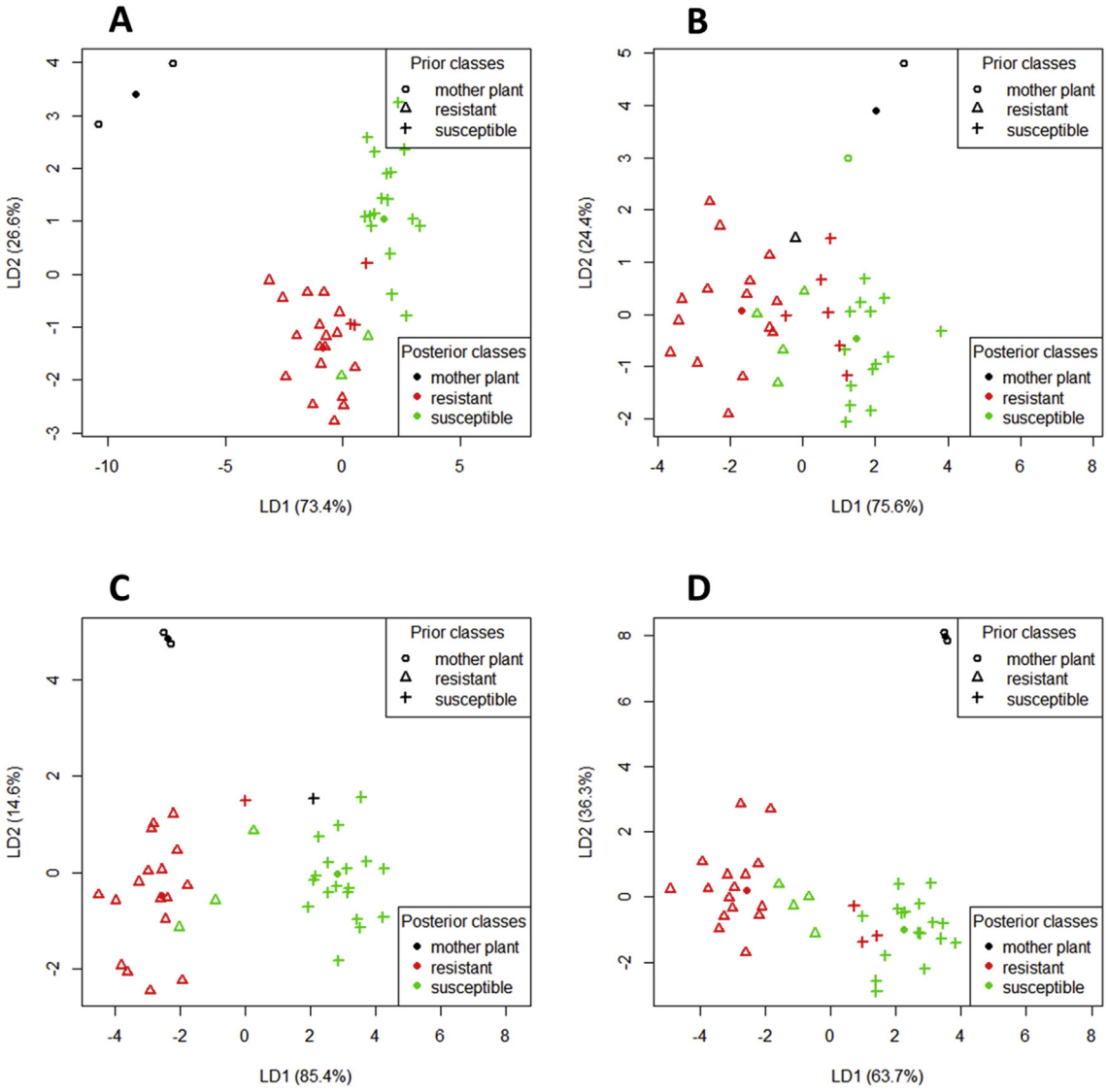

Fig. 7. LDA discriminant plots of the four groups, on the LASSO-refined dataset (LDA2 model): July (A), August (B), September (C), Late September (D). LD1 and LD2 are the versors calculated by LDA algorithm, which maximizes the inter-class discrimination. An explained-variance (i.e. percentage of total information) is associated to each LD and is reported between parenthesis.

prior class centroids. Thus, a point for which color and symbol correspond to the same class indicates a well-classified characteristic, whereas differing color and symbols indicate misclassified objects. Classification was performed by computing the Mahalanobis distance of each point to each centroid, assigning the object to the class for which it is lower. In this analysis it is not necessary to see an object "nearer to" a centroid in the graph to assign it to that class. Plots shown in Fig. 7 give a visual representation of NER, namely the percentage of samples that are correctly classified by cross-validation, as described in the "Multivariate analysis" section, and is, therefore, an evaluation of the ability of the LDA model to discriminate the classes of the dataset. In Fig. 7 it can be seen that, for all sampling times, the Horizon "mother plant" class is well discriminated from the others (i.e. its centroid is distant from the other ones). This is not surprising given that Horizon represents a genome prior to the introduction of further germplasm from the other native American grape species, and has green fruit rather than the red fruit of the derived lines. "Susceptible" and "resistant" classes are also well discriminated because their centroids are sufficiently distant from each other. However, there are some samples that are misclassified (the ones for which color and symbol differ), and that is why NER never reaches 100%.

Comparing the NERs before and after the LASSO-variable selection it can be seen that there is a general improvement in classification abilities of the models, mostly for S and LS. The August time period is an exception in that it is slightly worse (and its plot in Fig. 7B also shows greater overlap between "susceptible" and "resistant" classes). This is due to the fact that some variables, as for example DELP-3G, that seemed the most important for clustering in Fig. 5 but are always discarded by LASSO, may contain spurious information, with consequent negative effects on classification. From Table 1 it can be seen that, for each group, a different pattern of variables was selected. This is probably due to the differential fruit ripening: compounds that are useful for the discrimination in July, when the fruit is still developing, may no longer be useful in September, when the fruit is ripe. For example, no anthocyanins at all were found in J and A fruits, but some of them were considered important variables by LASSO for S and LS. These results are in agreement with those of Wang et al. (2017) (even if obtained with different statistical tools), who stated that the amino acids have different dynamics during grape development, and that anthocyanins are strongly accumulated after veraison, while EPIC, which is considered always important for discrimination by LASSO analysis, was also found to be an important marker for grapes by Pinasseau et al. (2017).

4. Conclusion

This work involves an analysis of amino acids and phenolic compounds in fruits of a hybrid population of grapes differing in disease resistance, throughout the growing season, to determine whether any of these compounds contributed to disease resistance. While LDA alone has only been used in a small number of plant research papers, we believe the combination of LDA (Linear Discriminant Analysis) and LASSO (Least Absolute Shrinkage and Selection Operator) to be unique in plant research. The nature and levels of amino acids and phenolic compounds showed considerable variation between different hybrids, but obvious changes occur with development especially between veraison and ripeness. Straight measurements of the concentrations of the levels of analyzed amino acid and phenolic compounds show no relationship to disease resistance but are mainly influenced by fruit development as determined by harvest date. However the multivariate analyses show that it is possible to discriminate the susceptibility or resistance of grapes by analyzing their combined concentrations of amino acids, polyphenols and anthocyanins. Therefore, these compounds are influenced by the resistance capacity of grapes and could be used as a chemical fingerprint of this ability. Moreover, the LASSO analysis shows that there is a different distribution of the most informative compounds related to resistance, depending on the ripening degree of the fruits: some compounds (as for example some anthocyanins) are informative only when the ripening is complete (in September), while some others (as some amino acids) are always informative. The analysis showed that the combination of LDA and LASSO techniques can distinguish these populations, but as no difference was unique to either population we believe that the overall difference is probably related to fungal infection rather than a factor in disease resistance. It has to be said that the concentrations of these compounds could be influenced by each other, so that a study performed on a single compound would be too simplistic to shed any light on the problem. What has still to be determined is how these variables are influenced by the capacity for resistance and why there are significant differences in concentrations of these between resistant and susceptible grapes. It seems unlikely, given the non-definitive nature of the chemical profiles, that resistance in these grapes is determined by any of these compounds, but rather that the noted differences are a consequence of other factors that are more important in determining resistance or susceptibility.

CRediT authorship contribution statement

Annalisa Tassoni: Formal analysis, Writing - original draft. **Alessandro Zappi:** Formal analysis, Writing - original draft. **Dora Melucci:** Supervision. **Bruce I. Reisch:** Conceptualization, Writing – review & editing. **Peter J. Davies:** Project administration, Resources, Writing - review & editing, Writing - original draft.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.plaphy.2018.11.034.

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