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TESI DI DOTTORATO DI RICERCA

Metabolomics and Transcriptomics: novel approaches to understand resistance in grape against *Plasmopara viticola*

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Ars longa, vita brevis, occasio praeceps, experimentum periculosum, iudicium difficile. (Hippocrates)

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Abstract

The grapevine is the most economically important fruit crop worldwide. Among the species of fungi considered to be the main grapevine pathogens, downy mildew is considered to be an extremely destructive disease of the grapevine, caused by the oomycete Plasmopara viticola (Berk. et Curt.) Berl. et de Toni. Grapevine research is directed towards better understanding of plant defence mechanisms and characterisation of the particular plant-pathogen interactions affecting the species. One of the most promising future strategies to ensure plant protection against disease is to stop the use of chemical compounds and focus on the selection of varieties showing durable specific resistance. Understanding plant-pathogen interaction is important for the future of the breeding; indeed grapevine species can be crosses, including resistant traits using conventional breeding techniques. In the last few years, comprehensive studies called omics have been applied to model plant study and these have contributed enormously to plant science. The project aims to decipher the mechanisms responsible for resistance in vine plants, since the molecular bases of the defence mechanism against *P. viticola* are still poorly understood. In particular, early responses to the pathogen, occurring within the initial 96 hours post inoculation, have been investigated in grape varieties using metabolomic and transcriptomic data. The use of leaf discs is widely adopted in experiments regarding the effect of different types of biotic stress on the biochemical response of the grapevine. Since there is little knowledge regarding mechanical wounding of grapevine leaves, we analyzed changes in phenolic, lipid and carotenoid content in Bianca grapevine leaves subjected to mechanical wounding (leaf discs), testing two different sizes of leaf discs (1.1 cm and 2.8 cm in diameter). One of the most well-known defence responses in vine plants is the production of defence compounds, mainly secondary metabolites also known as phytoalexins. Primary metabolism is also involved in plant defense with the participation of different molecules including carbohydrates, organic acids, amines, amino acids and lipids not only as a source of energy but also as a source of signaling molecules to directly or indirectly trigger defense response. We developed a rapid and versatile method for the extraction, identification and quantification of different classes of grape lipids using liquid chromatographic tandem

mass spectrometry (LC-MS/MS). We also validated a method for the identification and quantification of primary compounds belonging to different chemical classes: acids, amminoacids, amines/others and sugars using a GC-MS method of separation and identification, interesting in terms of elucidating the role of primary compounds in plantmicrobe interaction in future work. In this project the primary and secondary metabolism were investigated after P. viticola infection, in Bianca grapevine leaves with the aim of covering all the most important classes of plant metabolites. Our results gave a picture of plant metabolome perturbation. Several molecules were altered in Bianca leaf discs compared to the control after P. viticola infection, and they could act as potential biomarkers in Bianca variety after infection with P. viticola. Since plant resistance and plant-pathogen interaction are complex biological processes involving many signalling pathways, the multi omic approach is most suitable for examining these traits. An integrated metabolomic and transcriptomic approach was also applied to correlate variation in gene expression and metabolic perturbation in resistant Jasmine grapevine leaves, with the aim of discovering a specific and early stage biomarkers related to Downy mildew resistance.

Aim of the Ph.D. Project

The grapevine is the most economically important fruit crop worldwide. As for other crops, yield and quality are often affected by external factors in which fungal pathogens play a major part (Ferreira et al. 2004). For this reason grapevine research is directed towards better understanding plant defence mechanisms and characterisation of the particular plant-pathogen interactions affecting the species.

To ensure plant protection against disease, it is necessary to develop a new strategy against pathogen infections; one of the most promising future strategies is to stop the use of chemical compounds and focus on the selection of varieties showing durable specific resistance. Understanding plant-pathogen interaction is important for the future of the breeding; indeed grapevine species can be crosses, including resistant traits using conventional breeding techniques.

In the last few years, comprehensive studies called omics have been applied to model plant study and these have contributed enormously to plant science. Omics is a powerful approach in terms of identifying key genes for important traits, clarifying the mechanisms of physiological events and revealing unknown metabolic pathways in fruit trees.

The project aims to decipher the mechanisms responsible for resistance in vine plants, since the molecular bases of the defence mechanism against *Plasmopara viticola* are still poorly understood. In particular, early responses to the pathogen, occurring within the initial 96 hours post inoculation, have been investigated in different grape varieties using metabolomic and transcriptomic data.

The metabolomic approach enables the analysis of hundreds of putative biomarkers in different chemical classes, allowing better understanding of the defence response. One of the most well-known defence responses in vine plants is the production of defence compounds, mainly secondary metabolites also known as phytoalexins. In this project the primary and secondary metabolism were investigated after *P. viticola* infection, with the aim of covering all the most important classes of plant metabolites.

Since plant resistance and plant-pathogen interaction are complex biological processes involving many signalling pathways, the multi omic approach is most suitable for examining these traits.

An integrated metabolomic and transcriptomic approach was also applied to correlate variation in gene expression and metabolic perturbation in resistant grapevine leaves, with the aim of discovering a specific and early stage biomarker related to Downy mildew resistance.

The thesis is divided into six parts:

- (a) introduction (Chapter I);
- (b) development and validation of a new analytical method for targeted analysis of lipid compounds (Chapter II);
- (c) evaluation of mechanical wounding perturbation in the grapevine leaf metabolism (Chapter III);
- (d) study of metabolic perturbation in response to *Plasmopara viticola* infection in a resistant Bianca grape variety; development and validation of a method for primary compound quantification (Chapter IV);
- (e) multi-omic approach in a resistant Jasmine grape variety: metabolomics and transcriptomic analysis (Chapter V);
- (f) concluding remarks.

Chapter 1

Introduction

Plants are exposed daily to a large number of environmental stresses, such as drought, flooding, salinity, nutritional deficiency, intense sun light, adverse climatic conditions, pollutants, pathogens and phytophagous insects and animals (Harborne 1999). Since they do not have an immune system comparable to animals, plants have developed a stunning array of major strategies for counteracting adverse conditions, with one of the most significant being the synthesis of protective phytochemicals. These important phytochemicals are mainly secondary metabolites, not directly involved in basic processes like growth, development and reproduction, but involved in defence mechanisms (Bennett and Wallsgrove 1994; Dixon 2001).

The grapevine (*Vitis vinifera* L.) was among the first fruit species to be domesticated and today represents one of the main crop species around the world, cultivated mainly to produce wine but also juice, fresh fruit and raisins. With production of 7,6 thousand hectares (Kha) in vineyards worldwide in 2016, almost equivalent to that of 2015 (OIV; www.oiv.int), the grapevine plays an essential role in the economy of many countries. In 2016 global wine production (total for wine, including sparkling and special wine, but excluding juice and must), was to 267 million hectolitres (mhl), with a decline of 3% compared withthe previous year. Italy (51 mhl) confirms its place as the leading world producer, with slightly higher than average production, followed by France (43.5 mhl) and Spain (39.4 mhl) (from 'OIV Global economic vitiviniculture data'- 2017).

Unfortunately, viticulture is threatened by numerous pathogens causing severe harvest losses. Among the species of fungi considered to be the main grapevine pathogens, the three responsible for most damage in the vineyards are grey mould (*Botrytis cinerea*), downy mildew (*Plasmopara viticola*) and powdery mildew (*Erysiphe necator*). Downy mildew is considered to be an extremely destructive disease of the grapevine, caused by the oomycete *Plasmopara viticola* (Berk. et Curt.) Berl. et de Toni. It is an obligate biotrophic organism that depends exclusively on living plant cells for its growth and propagation (Heath and Skalamera 1997). All cultivated European *Vitis vinifera* cultivars are susceptible to *P. viticola*, which has caused enormous losses in Europe since 1870, when it first appeared, probably with the importation of American rootstocks resistant to Phylloxera (Viennot-Bourgin 1949). *P. viticola* infection occurs with penetration of the pathogen through the stomata cells. All green plant parts can be infested, but the pathogen usually colonises young leaves or young berries, reducing yield

and quality (Langcake and Lovell 1980; Gindro et al. 2003). Two symptoms can be observed: firstly yellow circular spots on the adaxial side of leaves, called "oilspots" as soon as 5 to 7 days post infection, while white downy fungal growth (sporangia) will appear on the abaxial leaf surface in correspondence with oilspots and in other infected plant parts, exclusively if climatic conditions are favourable for the pathogen development.(Blaeser M. 1978; Blaeser M. and H.C. Weltzien 1978; Blaeser and Weltzien 1979).

Even before any disease symptoms appear, the invading pathogen prevents the stomata from closing at night or in response to water deficit, so unrestrained transpiration may lead to water loss and wilting of infected leaves (Allègre et al. 2007). European V. vinifera cultivars, the most widely cultivated at global level, are highly susceptible to P. viticola, whereas some North American wild species have evolved host resistance (Munson T.V. 1909; Langcake and Lovell 1980; Merdinoglu et al. 2003). P. viticola has a tendency to colonise both susceptible and resistant varieties, however not all varieties adopt the same strategy against the pathogen. Following infection, grapevines rely on preformed and inducible resistance mechanisms for defence (Keller et al. 2003; Gabler et al. 2003). Susceptible varieties of V. vinifera allow the causal agent P. viticola to establish biotrophism at the expense of mesophyll cells and to complete their life cycle under most conditions. However, the development of the parasite is known to be inhibited by resistant cultivars mainly due to induction of specific stress related metabolites known as phytoalexins, as well as Phatogen Related (PR) proteins (Dercks and Creasy 1989a; Derckel et al. 1999; Slaughter et al. 2008; Ferri et al. 2009; Godard et al. 2009; Gessler et al. 2011). Stilbene phytoalexins of Vitaceae have been found to be involved in or associated with plant defence and are considered to be active compounds with antifungal activity against various pathogens, including P. viticola (Dercks and Creasy 1989b). The ability to accumulate stilbenes after P. viticola infection differs in Vitis species and stilbenes usually appear earlier and with a higher concentration in resistant varieties as compared to susceptible varieties. In some cases this accumulation does not happen, indeed Vitis cinerea and Vitis champinii are poor stilbene producers but still have resistance against pathogens (Keller 2015). This suggests that phytoalexins are not the only class of compounds involved in downy mildew resistance and that it is necessary to better investigate this mechanism. Early inducible responses include the deposition of new cell wall material, the release of reactive oxygen species (ROS) and hypersensitive cell death (HR) at the infection site, controlled by direct or indirect interaction between pathogen avirulence gene products and those of plant resistance genes, and can be the result of multiple signalling pathways (Heath 2000). The appearance of necrotic cells near invasion sites in resistant varieties is an example of localized programmed cell death that stops the pathogen, which as a biotroph depends on live host cells (Langcake and Lovell 1980; Busam et al. 1997; Kortekamp 2006).

Quantitative trait loci (QTLs) for resistance to downy mildew have been identified (Merdinoglu et al. 2003; Fischer et al. 2004; Welter et al. 2007; Marguerit et al. 2009; Bellin et al. 2009; Moreira et al. 2010) and named "Resistance to Plasmopara viticola" (Rpv). Studies of the V. vinifera genome have revealed that resistance genes and other genes involved in defence processes tend to be located on chromosomes 5, 7, 9, 12, 13, 18 and 19, in genomic regions associated with P. viticola resistance in wild grapevines (Di Gaspero and Cipriani 2003; Di Gaspero et al. 2007; Velasco et al. 2007; Moroldo et al. 2008). Since V. vinifera does not carry any resistance to downy mildew, the current strategy to control the disease in Europe relies on the repeated use of fungicides, with an adverse impact on the environment as well as negative effects on human health. Additionally, this crop protection strategy is increasingly less efficient over time, and fungicide resistance is frequently found in pathogen populations in commercial vineyards (Gómez-Zeledón et al. 2013) due to the development of new and more resistant strains of pathogen (Chen et al. 2007). Pesticides are still effective at the moment, but it is necessary to find alternative solutions to ensure protection of the environment and human health. The use of grapevine varieties showing durable resistance to downy mildew is a promising strategy to control the disease (Bisson et al. 2002), V. vinifera can be crossed with non-vinifera grapevine species to include resistant traits using conventional breeding techniques (Peixe et al. 2004; Eibach et al. 2007; Gessler et al. 2011).

Recent technical developments in genomics, transcriptomics, proteomics and metabolomics have become key tools in the development of systems biology. These new platforms of so-called "-omic" technologies allow detailed investigation of complex phenomena, and enable understanding of the molecular mechanism responsible for the phenotype of organisms during development or in response to the environment. In the last two decades comprehensive omics studies have been applied to model plant study and have contributed enormously to plant science (Shiratake and Suzuki 2016). Genomics refers to large-scale molecular analysis of multiple genes, gene products or regions of genes. Transcriptomics and proteomics refer respectively to the study of the entire set of RNAs and proteins derived from genome. Metabolomics represents the study of the metabolome, the totality of small molecules formed by a cell, tissue or organism under certain conditions. Recently, combination and integration of several omics has been performed on a single sample or material, and these are called multi-omics or integratedomics. The advances in omics studies are supported by the invention and improvement of analytical instruments, including the next generation DNA sequencer (NGS) and mass spectrometer (MS). Omics data are analysed using bioinformatics, and various important genes, proteins, metabolites and metabolic pathways have been identified with these approaches. The grapevine genome was sequenced in 2007, was the first among fruit trees and the fourth among flowering plants by the French-Italian Public Consortium (Jaillon et al. 2007) and by the Italian-American Collaboration (Velasco et al. 2007). Many transcriptomics studies of the grapevine have focused on the response to pathogens, including fungi, oomycetes, viruses and phytoplasma (Malacarne et al. 2011; Giraud et al. 2012; Abbà et al. 2014; Almagro et al. 2014; Gauthier et al. 2014; Li et al. 2015). To better understand the interaction between the grapevine and Botrytis cinerea, multi-omics approaches were recently adopted; Agudelo-Romero et al. (2015), observed changes in the transcriptome and metabolome, providing evidence of reprogramming of carbohydrate and lipid metabolisms towards synthesis of the secondary metabolites involved in V. vinifera cv. Trincadeira berries following infection with Botrytis cinerea, including resveratrol (Agudelo-Romero et al. 2015). The integration of transcriptomics and proteomics suggests that cell wall strengthening, accumulation of Pathogenesis Related proteins (PR) and excretion of lytic enzymes are important molecular mechanisms in the defence of the grapevine against Botrytis cinerea (Dadakova et al. 2015). Metabolomics studies have been reported for the grapevine and some of them investigated differences between grapevine cultivars (Mulas et al. 2011; Gika et al. 2012; Teixeira et al. 2014; Degu et al. 2014). Studies have been carried out and others are still underway to decipher the mechanisms responsible for resistance in vine plants. It is still necessary to investigate defence mechanisms against P. viticola in the grapevine. Multiomics information from different cultivars could be useful in order to proceed with the best breeding strategies in the future.

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Lipids are critical components of plant cell membranes and provide energy for metabolic activities. In recent years, increasing evidence has shown that lipids also function as mediators in many plant processes, including signal transduction, cytoskeletal rearrangement and membrane trafficking (Wang 2004). These processes are crucial both for cell survival, growth and differentiation and for plant responses to water, temperature, salinity, pests and pathogens.

The hydrophobic nature of lipids and the relative instability of some products of lipid metabolism have limited our understanding of the involvement of lipids in plantmicrobe interaction. However, studies with the model plant *Arabidopsis thaliana* have expedited efforts to understand the role of lipids and proteins involved in lipid metabolism and signalling in plant-microbe interaction. Although grape lipids are a very important class of plant metabolites, knowledge about them is still very limited to date, with the exception of those located in seeds. Lipids and lipid metabolites released from the membrane work as signal molecules in activating the plant defence response (Shah 2005).

The study of lipids has been complicated by their structural diversity and complexity. Following the LIPID MAPS classification (<u>http://www.lipidmaps.org</u>), lipids can be divided in eight categories: fatty acyls (FA), glycerolipids (GL), glycerophospholipids (GP), shingolipids (SP), sterol lipids (ST), prenol lipids (PL), saccharolipids (SL) and polyketides (PK). Recent advances in liquid chromatography coupled to mass spectrometry have paved the way for faster analysis of lipids with minimal sample preparation.

The aim of this study was to develop a rapid and versatile method for the extraction, identification and quantification of different classes of grape lipids: fatty acids, sterols, glycerolipids, glycerophospholipids and sphingolipids using liquid chromatographic electrospray ionization tandem mass spectrometry (LC/ESI-MS/MS), interesting in terms of elucidating the role of lipids in plant-microbe interaction in future work. We were able to set up instrumental conditions to obtain very good class separation on the basis of retention time using liquid chromatography (LC). Structural information

to confirm lipid identity was obtained with a preliminary fragmentation study, showing a characteristic MS/MS fragmentation pattern for each class of compounds. The method was validated for 33 lipids, with the linearity range expressed as R² from 0.95 to 1.00; the limits of quantification (LOQ) were different for each compound and were in the range of 0.003-14.88 ng/mL. Intra-day and inter-day repeatability were evaluated by calculating the coefficients of variation (CV%). The linearity data were used to assess the percentage of matrix effect (ME%), which was calculated as (1-slope in solvent/slope in matrix), expressed as a percentage. We obtained relative recovery ranges over 90% for 12 compounds, between 80% and 90% for 8 compounds, between 70% and 80% for 5 compounds, between 60% and 70% for 6 compounds and below 60% for 1 compound.

The method was successfully applied for the analysis of 18 healthy grape samples (10 red grape and 8 white grape varieties) from 4 different genetic groups: *Vitis vinifera*, *Vitis non-vinifera*, Muscat and hybrid (Emanuelli et al. 2013). Preliminary observations suggest the existence of diversity in the composition of grape lipids according to the cultivar, which requires further confirmation. With regard to differences in the lipid profile or concentration linked to grape colour, we did not notice any particular trend in these samples. The method can easily be extended to other plant tissues and to include further compounds. We believe it is a starting point for analysis of the lipid profile in different grape tissues, an essential goal for better understanding the role of lipids in grape physiology.

My personal contribution to this work mainly concerned the setting up of the instrumental conditions: identification of mass transitions (MRM) and optimization of the instrumental parameters for each metabolite for analysis with UHPLC–ESI-MS/MS. Moreover, I was involved in method validation according to the European pesticide guidelines (European Commission, Document No. SANCO/12495/2011 Validation and quality control procedures for pesticide residues analysis in food and feed, (2011)) I was also involved in sample preparation and analysis of the first samples for the initial application of the method using grape berries. Finally, I participated in writing the manuscript as first co-author, and by managing the comments and improvements to the text by other authors.

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Chapter II

A rapid LC–MS/MS method for quantitative profiling of fatty acids, sterols, glycerolipids, glycerophospholipids and sphingolipids in grapes

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A rapid LC–MS/MS method for quantitative profiling of fatty acids, sterols, glycerolipids, glycerophospholipids and sphingolipids in grapes



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ABSTRACT

The abundance of lipids in plants is influenced by genotype and phenotype. Despite being a very important class of plant metabolites, knowledge of grape lipids is still very limited to date, with the exception of those located in seeds. Few investigations of grape lipids have shown that their profile depends on grape maturity, the variety and their location in the berry. Recent advances in liquid chromatography coupled to mass spectrometry have paved the way for faster analysis of lipids with minimal sample preparation. Here we describe a validation method for the extraction, identification and quantification of different classes of grape lipids: fatty acids, sterols, glycerolipids, glycerophospholipids and sphingolipids using liquid chromatographic electrospray ionization tandem mass spectrometry (LC-ESI-MS/MS). The method was validated for 33 lipids, with linearity range (R^2 =0.95-1.00), LOQ (0.003-14.88 ng mL⁻¹) and intraday and interday repeatability being evaluated for each lipid. The lipid profiling method developed was successfully applied to the analysis of 18 grape samples (10 red grape and 8 white grape varieties) from 4 different genetic groups: *Vitis vinifera*, *Vitis* non-*vinifera*, Muscat and hybrid; 33 lipids were identified and quantified. This method, which can be easily expanded to include further compounds and other plant tissues, is the starting point for analysis of the lipid profile in different grape tissues, an essential goal for better understanding the role of lipids in grape physiology.

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1. Introduction

Lipids have an essential role in all plant cells, in terms of structure and organization, signalling events, protein regulation, metabolic transformation and trafficking [1]. The vegetative cells of plants contain from 5% to 10% of lipids (dry weight), and almost all of this weight is found in the membranes [2]. Although each square centimetre of a plant leaf may contain only 0.2 mg of lipids, the lipid membranes are the main barriers delineating the cell and its compartments and they form the sites where many essential processes occur, including light-harvesting and the electron transport reactions of photosynthesis [2]. Epidermal cells produce cuticular lipids that coat the surface of plants, providing the crucial hydrophobic barrier that prevents water loss and also providing protection against pathogens and other environmental stresses [3]. Furthermore, lipids and lipid metabolites released from membranes work as signal molecules in activating the plant defence response [4]. However, the study of lipids has been complicated due to their structural diversity and complexity. Following the classification proposed by LIPID MAPS (http://www.lipidmaps.org), lipids can be classically divided in eight categories, namely fatty acyls (FA), gly-cerolipids (GL), glycerophospholipids (GP), sphingolipids (SP), sterol lipids (ST), prenol lipids (PL), saccharolipids (SL) and polyketides (PK) [1]. Furthermore, each of these categories includes distinct classes and sub-classes, once again increasing the complexity of this family of compounds. The most abundant types of lipids in plant cells are those deriving from fatty acid and glycerolipid biosynthetic pathways; for example, small amounts of fatty acids are important as precursors for the hormone jasmonic acid and in the acylation of certain membrane proteins. Other classes of lipids derive from the isoprenoid pathway, such as sterols [2]. Glycerophospholipids are the main constituent in the cellular membrane and understanding lipid changes is useful for understanding cell function [5].

Little research has been conducted on grape lipids, with the exception of grape seed lipid composition, and therefore knowledge of grape lipid composition is still very limited and there are only a few studies on the topic. The relationship between the evolution of grape constituents during berry development and ripening was studied by Rubio et al. [6]. Roufet et al. and Bauman et al. studied the effects of maturation on fatty acid content in grapes, Le Fur et al.

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studied the evolution of phytosterols in grape berry skins during the last stages of ripening, while Barron et al. found a relationship between triglycerides and grape ripening indices [7–9]. Gallander and Peng studied six different grape varieties observing that *vinifera* varieties contained high levels of unsaturated fatty acids when compared to other varieties [10]. Studies related to the fatty acid composition of different tissues in grapes have shown that linolenic acid is predominant in leaves, while linoleic acid is most abundant in pericarps and phospholipids are present in larger quantities in the skin and pulp [11]. The composition of lipids has been analysed in different tissues, such as the leaves, pericarp, skin and seeds of Cabernet Sauvignon grapes, and the fatty acid profile of *Vitis labrusca* and *Vitis vinifera* grapes was analysed in different components [11,12].

The plasma membrane and microsomal fraction in grape leaves have been isolated because of their importance as an indicator of environmental changes, while sterol, phospholipid and sphingolipid composition have been discussed in relation to membrane fluidity in grapevine leaves [13]. Grape seed oil is rich in unsaturated fatty acids such as linoleic and oleic acid and thus offers many advantages for human consumption; the food, pharmaceutical and cosmetic industries have shown great interest in grape seed oil due to its exceptional antioxidant properties [14]. Because of its nutritional and therapeutic properties, grape seed oil has been analysed and the main components in terms of oil, fatty acids, vitamin E, active compounds and phytosterol composition quantified in several varieties [15,16]. Fatty acids, sterols and the triacylglycerol composition of grape seed oil has been evaluated in grapes from different countries (France, Italy and Spain), making it possible to distinguish the origin of the oil based on lipid profiling using GC-MS analysis [17]. Matrix-assisted-laser-desorption-ionization mass spectrometry (MALDI-MS) was used by De Marchi and colleagues [18] in order to characterise the triacylglycerol (TAG) composition of grape seed oils, in a very short run time, obtaining useful information about grape varieties and processing conditions. The polyunsaturated fatty acids alpha-linolenic and linoleic acid are the substrates of the well characterized enzyme system, including both lipoxygenase and hydroperoxide lyase activities, which forms the C6-aldehydes and alcohols, which are responsible of the "green flavour" [19]. Reduced glutathione has been suggested to conjugate to the C6-aldehyde hexenal, leading to accumulation in grapes of the precursors of tropical flavours S-(3hexan-1-ol)-glutathione and S-(3-hexan-1-ol)-L-cysteine [20]. These reactions have taken place already during the early phases of grape processing, such as machine harvesting and transportation, leading to the generation of C-6 volatiles and of accumulation of the precursors of tropical flavours of wines [21].

Grape lipids are important factors in oenology since they are capable of modulating the yeast metabolism. This is of major importance in the case of white and rosé winemaking, where lipids can be a limiting factor due to the short contact with the grape skins. Grape lipids are essential during fermentation in order to limit the production of excessive amounts of acetic acid from acetic acid metabolism, favouring the penetration of amino acids into the yeast cell [22-24]. Ergosterol, other sterols and certain long-chain fatty acids such as oleanolic acid and oleic acid are known to act as "growth factors", increasing the yeast population and accelerating the fermentation speed under complete anaereobiosis. Moreover, they have been shown to act as "survival factors", since yeasts well supplemented in these grape lipids are more viable and are thus capable of prolonging their fermentation activity [24]. In other words, grape lipids can induce nutritional limitation of yeast activities, a complex factor which is more severe in musts with a concomitant deficiency in assimilable nitrogen [25]. In conclusion, our current understanding of yeast physiology shows the need for a metabolite profiling method capable of quantitatively screening for grape lipids [25,26].

Several analytical methods using numerous techniques have been developed to investigate lipids, including thin-layer chromatography, gas chromatography and HPLC-UV, but because of the complexity of this family of compounds, recently mass spectrometry has become the leading technology for rapid lipidomic analysis [27], due to its good sensitivity, specificity and dynamic range. Recent developments achieved in liquid chromatography, such as ultra high performance liquid chromatography (UHPLC) and the availability of new versatile stationary phases, has made this technique suitable for lipid profiling. Thus UHPLC-MS based approaches can help to expand the number and classes of lipids that can be analysed by offering new standards in terms of sensitivity and selectivity [28]. Electrospray ionization (ESI) is the most widely used ionization technique for the analysis of lipids: in positive mode, the observed MS spectra are dominated by protonated molecules, $[M+H]^+$ or other ionic species, due to the high tendency of lipids to form adducts with sodium, potassium and ammonium; in negative mode, the deprotonated molecule [M-H]⁻ and some acetate and/or formate adducts are generally observed [29,30]. The use of high resolution shotgun lipidomics allows the identification of different triglycerides in seed oils [31]. Finally, an interesting approach for the study of subcellular lipidomics is MALDI imaging [32].

To our knowledge, reports on extensive method validation for lipid profiling analysis using LC–MS/MS in complex grape matrices are limited, and the data published to date are only available for grape seed oil composition [16,18]. Here we propose a sensitive and accurate LC–tandem-MS-based method for the simultaneous determination and quantification of multiple classes of lipids such as fatty acids, sterols, glycerolipids, glycerophospholipids and sphingolipids in grape samples. Application of this method could greatly enhance knowledge of the role of grape lipids for different technological and biological questions regarding grape growing and winemaking.

2. Experimental method

2.1. Chemicals and materials

Standards were purchased from Sigma-Aldrich (Sigma-Aldrich, Milan, Italy) and Avanti Polar Lipids (Alabaster, AL). The chemicals acetonitrile (ACN, LC–MS grade), 2-propanol (IPA), methanol (CH₃OH, LC–MS grade) and chloroform (CHCl₃) were purchased from Sigma-Aldrich. Formic acid (HCOOH) and ammonium formate (NH₄COOH) additive for LC–MS were also from FLUKA Sigma-Aldrich. All aqueous solutions, including the HPLC mobile phase, were prepared with water purified using a Milli-Q system (Millipore, Vimodrone, Milan, Italy).

The following chemical standards of lipids were purchased from Aldrich-Fluka-Sigma S.r.l. (Milan, Italy): oleoyl-L-carnitine hydrochloride, palmitoyl-L-carnitine hydrochloride, desmosterol, ergosterol, lanosterol, uvaol, arachidic acid, behenic acid, *cis*-11-eicosenoic acid, erucic acid, heptadecanoic acid, margaric acid, lignoceric acid, linoleic acid, linolenic acid, myristic acid, myristoleic acid, oleanolic acid, oleic acid, *cis*-vaccenic acid, palmitic acid, palmitoleic acid, stearic acid, 1,2,3-tripentadecanoylglycerol 1-linoleoyl-rac-glycerol, 1-monopalmitoleoyl-rac-glycerol, 1-oleoyl-rac-glycerol, glyceryl trioleate, glyceryl tripalmitoleate, 1,2-dilinoleoyl-sn-glycero-3-phosphocholine, 1,2-dioleoyl-sn-glycero-3-phosphocholine, 1,2-dioleoylsn-glycero-3-phospho-rac-(1-glycerol)sodium salt, 1-palmitoyl-snglycero-3-phosphocholine, ceramide. Docosahexaenoic acid and cholesterol bought from Aldrich-Fluka-Sigma S.r.l. (Milano, Italia), were chosen as internal standards (IS).

Table 1

Compounds with relative	e molecular formula,	Chemspider ID an	d molecular weight.
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Class	Compound	Chemspider ID	Molecular formula	Molecular weight (MW)
Carnitines	Oleoyl-L-carnitine hydrochloride	21403150	C25H47NO4	425.4
	Palmitoyl-L-carnitine hydrochloride	16737192	C23H46CINO4	435.3
Sterols	Cholesterol (IS)	5775	C27H46O	386.4
	Desmosterol	388662	C27H44O	384.3
	Ergosterol	392539	C28H44O	396.3
	Lanosterol	216175	C30H50O	426.4
	Uvaol	83774	C30H50O2	442.4
Fatty acids	Arachidic acid	10035	C20H40O2	312.3
	Behenic acid	7923	C22H44O2	340.3
	cis-11-Eicosenoic acid	4445895	C20H38O2	310.3
	Docosahexaenoic acid (IS)	393183	C22H32O2	328.2
	Erucic acid	4444561	C22H42O2	338.3
	Heptadecanoic acid	10033	C17H34O2	270.3
	Lignoceric acid	10724	C24H48O2	368.4
	Linoleic acid	4444105	C18H32O2	280.2
	Linolenic acid	444437	C18H30O2	278.2
	Myristic acid	10539	C14H28O2	228.2
	Myristoleic acid	4444564	C14H26O2	226.2
	Oleic acid+ <i>cis</i> -vaccenic acid	393217	C18H34O2	282.3
	Palmitic acid	960	C16H32O2	256.2
	Palmitoleic acid	393216	C16H30O2	254.2
	Stearic acid	5091	C18H36O2	284.3
Glycerolipids	1,2,3-Tripentadecanoylglycerol	3496974	C48H92O6	764.7
	1-Linoleoyl-rac-glycerol	4446589	C21H38O4	354.3
	1-Monopalmitoleoyl-rac-glycerol	8059588	C19H36O4	328.3
	1-Oleoyl-rac-glycerol	4446588	C21H40O4	356.3
	Glyceryl trioleate	4593733	C57H10406	884.8
	Glyceryl tripalmitoleate	7822939	C51H92O6	800.7
Glycerophospholipids	1,2-Dilinoleoyl-sn-glycero-3-phosphocholine	4450312	C44H80NO8P	781.6
	1,2-Dioleoyl-sn-glycero-3-phosphocholine	4941669	C44H80NO8P	785.6
	1,2-Dioleoyl-sn-glycero-3-phospho-rac-(1-glycerol)sodium salt	17347228	C42H79NaO10P	796.5
	1-Palmitoyl-sn-glycero-3-phosphocholine	405288	C24H51N07P	495.3
Sphingolipids	Ceramide	4446678	C36H71NO3	565.5
Prenols	Oleanolic acid	10062	C30H48O3	456.4

Butylated hydroxytoluene (BHT), provided by Aldrich-Fluka-Sigma S.r.l. (Milano, Italia), was used as an antioxidant during the extraction steps.

2.2. Preparation of lipid standards

All analytical standards used for method development are listed in Table 1 with the relative molecular formula and Chemspider ID.

Standard stock solutions of lipids were prepared individually at a concentration range from 1 to 10 mg mL⁻¹ in a CHCl₃/CH₃OH mixture (2:1 v/v) in dark glass tubes. A working standard solution of each analyte was prepared from the stock solution by appropriate dilution in ACN/IPA/H₂O mixture (65:30:5 v/v/v) and employed for development and validation of the method.

A combined standard spiking solution of all analytes was prepared by diluting the respective stock solutions in ACN/IPA/H₂O (65:30:5 v/v/v) and this was diluted again in order to obtain 21 points with decreasing concentrations of each analyte, in the range of concentrations reported in Table 2.

IS solutions of docosahexaenoic acid and cholesterol were prepared at concentrations of $100 \ \mu g \ mL^{-1}$ and $1 \ \mu g \ mL^{-1}$ respectively and used to check sample preparation efficiency. The stock of standard solutions and the spiking solutions were stored at $-20\ ^\circ\text{C}.$

2.3. Sample collection

Grapes samples were from the "Fondazione Edmund Mach" grape germplasm collection (ITA362), located in San Michele all'Adige, Italy (46°18'N, 11°13'E). All plants were grafted onto the rootstock Kober 5BB in five replicates and trained using the Guyot system. Samples of healthy grapes from 10 red and 8 white grape varieties (Table S1) were harvested at technological maturity in the same year (2010) and were analysed using the method developed.

2.4. Sample preparation

Grape powder was prepared as previous described by Gika et al. [33] and kept at -80 °C. Lipids were extracted according to the Folch's method [34]. A precise amount of 0.5 (\pm 0.005) g from each sample was weighed and 1.5 mL of CH₃OH were added, vortexed for 30 s, 3 mL of CHCl₃ containing butylated hydroxytoluene (BHT 50 mg L⁻¹) were added, followed by the addition of 10 µL of internal standard (IS) (docosahexaenoic acid 100 µg mL⁻¹). Samples were

Table 2

Calibration parameters. Linear dynamic range (LDR), coefficient of determination (R²) limit of quantification (LOQ), limit of detection (LOD) and matrix effect, obtained using solutions of standards addition in grape matrix.

Class	Compound	Linearity range (µg mL ⁻¹)	Water calibration curves Equation	R ²	Grape calibration curves Equation	R ²	Matrix effect (%)	LOD (ng mL ⁻¹)	LOQ (ng mL ⁻¹)
Carnitines	Oleoyl-L-carnitine hydrochloride Palmitoyl-L-carnitine hydrochloride	0.0002–5.8 0.00002–4.36	y = 1,781,6884x + 1,170,800 $y = 39,651,340x + 78,003$	1.00 1.00	y = 14,144,865x + 666,169 $y = 29,621,992x + 3023$	1.00 1.00	-26.0 -33.9	0.1 0.01	0.3 0.03
Sterols	Desmosterol Ergosterol Lanosterol Uvaol	0.09–3.845 0.09–1.985 0.05–1.0675 0.005–1.105	y = 295,822x + 65,554 y = 100,137x - 12 y = 107,149x - 4434 y = 2,507,521x + 86,572	0.95 0.99 1.00 0.99	y = 274,231x + 108,744 y = 87,502x - 3004 y = 85,316x - 4259 y = 2,295,751x + 49,898	0.90 1.00 1.00 0.98	- 7.9 - 14.4 - 25.8 - 9.2	19.3 49.6 21.3 2.2	57.9 148.8 63.9 6.7
Fatty acids	Arachidic acid Behenic acid <i>cis</i> -11-eicosenoic acid Erucic acid Heptadecanoic acid Lignoceric acid Linoleic acid Linoleic acid Myristic acid Oleic acid + <i>cis</i> -Vaccenic acid Palmitic acid Stearic acid	0.003-31.3 0.008-17.1 0.07-15.5 0.001-33.9 0.01-27.1 0.003-9.2 0.007-28.0 0.05-57 0.05-56.5 0.02-28.3 0.1-32 0.02-25.4 0.002-28.4	y = 1971,102x - 83,094 y = 2,607,531x + 130,259 y = 7673x + 1005 y = 4,874,309x - 636,823 y = 531,131x + 728,127 y = 2,712,524x + 683,788 y = 2,563,516x + 5,753,986 y = 821,382x + 828,911 y = 365,484x + 96,999 y = 391,868x - 15,488 y = 1,975,170x + 9,760,208 y = 19,724x + 12,835 y = 601,474x + 285,142 y = 1,476,286x + 1,248,339	1.00 1.00 0.99 0.99 0.98 0.97 0.99 1.00 1.00 0.96 1.00 0.99 0.99 0.99	y=2,046,868x+264,544 y=2,628,503x-52,941 y=6317x+1060 y=4,949,227x-656,680 y=486,268x+460,828 y=2,841,008x+337,484 y=2,170,119x+8,184,061 y=842,024x+806,833 y=334,746x+185,754 y=368,574x-36,527 y=1,777,976x+3,723,146 y=17,856x-19,368 y=534,259x+284,641 y=1,506,209x-142,367	0.99 1.00 0.99 0.99 0.98 0.94 0.99 1.00 1.00 0.97 0.98 0.99 0.99	$\begin{array}{r} 3.7 \\ 0.8 \\ -21.5 \\ 1.9 \\ -9.2 \\ 4.5 \\ -18.1 \\ 2.5 \\ -9.2 \\ -6.3 \\ -11.1 \\ -10.5 \\ -12.6 \\ 2.0 \end{array}$	1.6 3.4 31.1 0.8 11.3 1.9 7.0 2.8 22.8 11.3 14.1 32.0 12.7 1.4	4.7 10.2 93.3 2.5 33.9 5.6 21.0 8.4 68.4 33.9 42.3 128.2 38.1 4.3
Glycerolipids Glycerophospholipids	1.2.3-Tripentadecanoylglycerol 1-Linoleoyl-rac-glycerol 1-Monopalmitoleoyl-rac-glycerol 1-Oleoyl-rac-glycerol Glyceryl trioleate Glyceryl tripalmitoleate 1.2-Dilinoleoyl-sn-glycero-3-phosphocholine 1.2-Dioleoyl-sn-glycero-3-phosphocholine 1.2-Dioleoyl-sn-glycero-3-phospho-rac-(1-glycerol)	0.00007-0.8 0.02-14.2 0.02-52.5 0.003-17.9 0.03-7.1 0.003-3.5 0.01-15.6 0.006-12.6 0.01-12.8	y = 119,296,649x + 6,912,932 y = 139,154x + 1410 y = 1,270,229x - 135,275 y = 1,951,698x - 242,429 y = 7,274,665x + 2,401,909 y = 34,292,391x + 1,504,691 y = 59,249,626x + 21,763,259 y = 60,549,785x + 10,796,759 y = 41,360,769x + 1,615,101	0.96 1.00 1.00 0.96 1.00 0.98 0.99 0.99	y = 120,666,830x + 3,263,178 y = 116,890x - 10,601 y = 1,051,850x - 109,119 y = 1,799,361x - 506,911 y = 6,816,032x + 1,467,346 y = 35,000,163x - 39,599 y = 58,952,735x + 19,159,586 y = 63,008,040x + 11,502,248 y = 41,001,805x + 3,811,341	0.97 0.97 1.00 0.97 1.00 0.98 0.99 0.97	$ \begin{array}{r} 1.1 \\ -19.1 \\ -20.8 \\ -8.5 \\ 6.7 \\ 2.0 \\ -0.5 \\ 3.9 \\ -0.9 \end{array} $	0.04 14.2 10.5 1.8 14.1 1.4 6.3 3.1 6.4	0.1 42.6 31.5 5.3 42.3 4.3 18.8 9.4 19.2
Sphingolipids Prenols	1-Palmitoyl-sn-glycero-3-phosphocholine Ceramide Oleanolic acid	0.01–19.9 0.0002–14.9 0.04–4.6	y = 14,155,899x + 678,383 $y = 14,453,621x + 5,731,780$ $y = 1,604,598x + 474,133$	1.00 0.97 0.97	y = 11,959,790x + 2,361,447 $y = 15,128,604x + 6,550,053$ $y = 1,469,483x + 592,518$	0.99 0.96 0.95	- 18.4 4.5 - 9.2	8.0 0.1 22.8	23.9 0.4 68.4

Class	Compound	Ionization mode	Precursor ion	Q1 Product ion	DP	ЕР	CE	СХР	Q2 Product ion	DP	EP	CE	СХР	t _R	Q2/Q1
Carnitines	Oleoyl-L-carnitine hydrochloride Palmitoyl-L-carnitine hydrochloride	$[M+H]^+$ $[M+H]^+$	426.3 400.3	367.0 341.0	120 173	11 10	26 24	17 14	265.0 239.0	120 173	11 10	30 28	13 14	6.5 6.2	39.0 33.8
Sterols	Cholesterol (IS) Desmosterol	$[M+1-H_2O]^+$ $[M+NH_4]^+$	369.5 402.4	147.0 367.2	140 146	8 10	35 13	25 20	135.0	140	8	35	23	15.7 14.1	0.6
	Ergosterol Lanosterol Uvaol	$[M+H]^+$ $[M+NH_4]^+$ $[M+NH_4]^+$	397.3 444.3 460.4	69.3 409.5 443.4	6 51 100	10 10 10	33 13 11	14 32 22	379.4 191.3 191.4	6 51 100	10 10 10	17 21 21	22 16 10	14.9 16.9 7.6	150.1 38.3 40.5
Fatty acids	Arachidic acid Behenic acid	$[M+HCOO]^-$ $[M+HCOO]^-$	357.2	311.4 339.4	-5 -60	- 10 - 11	-12 -15	-21 -10	45.1	-5 -60	- 10 - 11	-40 -47	-5 -16	14.5 16.0	46.7
	<i>cis</i> -11-Eicosenoic acid Docosahexaenoic acid (IS)	[M – H] ⁻ [M – HCOO] ⁻	309.4 373.3	291.5 327.3	- 167 - 60	- 11 - 10	-33 -10	- 19 - 21	155.0 283.4	- 167 - 60	- 11 - 10	-37 -20	- 10 - 20	12.7 8.4	16.2 0.1
	Erucic acid Heptadecanoic acid	[M+HCOO] ⁻ [M+HCOO] ⁻ [M+HCOO] ⁻	383.2 315.4 413.2	337.3 269.3 367.3	-95 -45	- 10 - 13	-12 -10	-23 -17	45.0 45.0	-95 -10	-10 -10	-44 - 32	-5 -5	14.6 11.0	35.6 64.6 35.9
	Linoletic acid Linolenic acid	[M+HCOO] ⁻ [M+HCOO] ⁻	325.2 323.3	279.2 277.2	- 60 - 65	-12 -6	- 10 - 8	-21 -17	261.0 259.3	- 60 - 65	-12 - 6	-35 -27	-23 -17	9.0 8.2	0.4 0.5
	Myristic acid Myristoleic acid	[M+HCOO] ⁻ [M+HCOO] ⁻	273.2 271.3	227.2 225.3	-5 -45	- 10 - 11	- 18 - 20	- 15 - 17	45.0 45.0	-5 -30	-10 - 10	-26 -26	-7 -7	8.4 6.6	99.2 87.6
	Palmitic acid Palmitic acid	[M+HCOO] [M+HCOO] ⁻ [M+HCOO] ⁻	327.2 301.2 299.3	281.2 255.3 253.3	- 50 - 55 - 55	- 10 - 10 - 10	- 14 - 16 - 18	- 17 - 23 - 15	45.1 44.9 44.7	- 50 - 55 - 55	-10 -10 -10	- 34 - 38 - 34	-5 -5 -7	10.8 10.1 8.5	48.4 80.2 37.6
Clucarolinide	Stearic acid	$[M + HCOO]^{-}$ $[M + NH_{2}]^{+}$	329.3	283.3	- 60	- 12	-9 31	- 22	265.5	- 60	-12	-41	- 16 17	12.7	0.3
oryceronplus	1-Linoleoyl-rac-glycerol 1-Monopalmitoleoyl-rac-glycerol	$[M+H]^+$ $[M+H]^+$	355.3 329.3	337.0 121.2	300 104	37	15 29	16 9	263.0 219.3	300 104	3 7	13 17.5	21 17	7.6 7.1	25.0 179.7
	1-Oleoyl-rac-glycerol Glyceryl trioleate Glyceryl tripalmitoleate	$[M+H]^+$ $[M+NH_4]^+$ $[M+NH_4]^+$	357.3 902.8 818.7	265.2 265.0 547.0	250 80 300	10 8 4	15 55 28	13 14 27	283.2 603.0 237.3	250 80 300	10 8 4	13 60 53	17 14 12	9.2 24.4 23.7	27.2 59.8 22.3
Glycerophospholipids	1,2-Dilinoleoyl-sn-glycero-3-phosphocholine 1,2-Dioleoyl-sn-glycero-3-phosphocholine	$[M+H]^+$ $[M+H]^+$	782.6 786.6	184.0 184.0	100 116	15 3	27 27	23 14	125.0 125.0	100 116	15 3	123 134	35 14	15.6 18.0	78.7 57.6
	1,2-Dioleoyl-sn-glycero-3-phospho-rac-(1-glycerol)sodium salt	$[M - Na + NH_4]^+$	792.5	603.6	50	6	35	15	339.3	50	6	45	14	16.3	2.9
Sphingolipids	Ceramide	$[M+H]^+$	490.4 566.6	478.4 548.6	100	8	18	14	264.0	100	14 8	35 41	14	19.8	54.7
Prenols	Oleanolic acid	$[M+NH_4]^+$	474.3	439.4	146	10	15	32	191.2	146	10	27	12	7.8	27.7

 Table 3

 Mass transitions (MRM) and instrumental parameters optimized for each metabolyte for the analysis by UHPLC-ESI-MS/MS.

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placed in an orbital shaker for 60 min. Afterwards 1.25 mL of water was added. After 10 min the samples were centrifuged at 3600 rpm for 10 min. The total lower lipid-rich layer was collected. The second extraction was performed using 2 mL of CHCl₃/CH₃OH/H₂O 86:14:1 v/v/v. The samples were centrifuged at 3600 rpm for 10 min, the total lower lipid-rich layer was collected. Both fractions were unified and evaporated to dryness under N₂. Samples were reconstituted in 300 µL of ACN/IPA/H₂O (65:30:5 v/v/v) containing the IS (cholesterol at a concentration of 1 µg mL⁻¹) before LC-MS/MS analysis. Five microliters of sample were injected into the LC-MS/MS system. Each sample was diluted 1:100 with ACN/IPA/H₂O (65:30:5 v/v/v) and reinjected for quantification of the most abundant lipids.

2.5. Instrumental conditions

Separation was performed using a UHPLC Dionex 3000 (Thermo Fisher Scientific Germany), with a RP Ascentis Express column (15 cm \times 2.1 mm; 2.7 μ m C18) purchased from Sigma (Milan, Italy). Column temperature was set at 55 °C using a Peltier effect column oven (Dionex Thermo Fisher Scientific Germany). Samples were injected using an autosampler (Dionex Thermo Fisher Scientific Germany) set at 10 °C. Separation was carried out following a 30 min multistep linear gradient as reported by Hu et al. [35]. From 0 to 1.5 min isocratic elution with 32% B; from 1.5 to 4 min increase to 45% B, then to 52% B in 1 min, to 58% B in 3 min, to 66% B in 3 min, to 70% B in 3 min, to 75% B in 4 min, to 97% B in 3 min, then 97% B was maintained for 4 min. From 25.0 to 25.1 min solvent B was decreased to 32% and then maintained for another 4.9 min for column re-equilibration. Total duration of the analysis was 30 min, including post-time. The flow-rate was $0.26 \text{ mL} \text{min}^{-1}$, mobile phase A consisted of ACN 40% in water, NH₄COOH 10 mM and HCOOH 0.1% and B consisted of IPA 90%, ACN 10%, NH₄COOH 10 mM and HCOOH 0.1%.

The UHPLC system was coupled directly to an API 5500 triplequadrupole mass spectrometer (Applied Biosystems/MDS Sciex, Toronto, Canada) equipped with a electrospray source. Analyst™ software version 1.6.1 (Applera Corporation, Norwalk, CT, USA) was used for instrument control and data acquisition. The transitions and spectrometric parameters were optimized individually for each standard by direct infusion of their solutions (10 μ g mL⁻¹) in water/ ACN (40:60 v/v) with NH₄COOH 10 mM and HCOOH 0.1% into a mass spectrometer at a flow rate of 10 μ L min⁻¹. The two most abundant fragments to use as quantifier and qualifier were identified for each compound. Declustering potential (DP) and entrance potential (EP) were optimized for each precursor ion and collision energy (CE) and Collision Cell Exit Potential (CXP) for each product ion. Table 3 shows the compound-specific instrumental parameters used in the analytical method. The presence of our metabolite of interest was confirmed using the q/Q ratio [36]. The spray voltage was set at 5500 V for positive mode and -4500 V for negative mode. The source temperature was set at 250 °C, the nebulizer gas (Gas 1) and heater gas (Gas 2) at 40 and 20 psi respectively (1 psi=6894.76 Pa). UHP nitrogen (99.999%) was used as both curtain and collision gas (CAD) at 20 and 9 psi respectively.

2.6. Method validation

The method validation assays were performed according to the currently accepted US Food and Drug Administration (FDA) bioanalytical method validation guide [37]. Validation assays were established on calibration standards and quality control (QC) samples prepared as a pool of grape samples and extracted according to the procedure described above. The standard mix was used for calibration curves and in order to establish the LOQ. The QC sample was used in order to evaluate the stability of samples during analysis, intra-day and inter-day variability and to evaluate the performance of the extraction method in the real samples.

2.6.1. Linearity, limit of detection, limit of quantification and matrix effect

Calibration curves were made in pure solvent (ACN/IPA/H₂O) and matrix extract (QC). To evaluate the percentage of matrix effect for each compound, calibration curves were compared. Matrix effect values were determined by comparing solvent and matrix-matched calibration curves in terms of slope ratios: %ME=100% x (1–slope solvent/slope matrix) as shown in Table 2 [38].

To assess linearity, three independent calibration curves were prepared by adding increasing concentrations of each lipid in different concentration ranges, as shown in Table 2. The limits of quantification (LOQ) and limits of detection (LOD) were evaluated at the concentration in which the quantifier transition presented a signal to noise (S/N) ratio of > 10 and > 3 respectively.

2.6.2. Intra-day and inter-day variability

To determine intra-day and inter-day variability, 10 replicates of a QC sample middle concentration level were extracted and injected on the same day and then re-injected for 5 consecutive days. Intra-day and inter-day variability were evaluated by calculating the coefficients of variation (CV%) as shown in Table 4. CV% should not exceed a value of 15% for intra-day assay and 20% for inter-day assay.

2.6.3. Recovery, precision and accuracy

The QC sample was used for method validation, by spiking it with known amounts of each lipid, corresponding to three different points (low, middle and high) in the calibration range for each analyte. The recovery test was estimated on 10 spiked grape samples and calculated as the average of the "measured value/ expected value" ratio (%) (Table 4).

Accuracy and precision were calculated by analysing 10 QC spiked samples, corresponding to a medium point. Precision was reported as the Relative Standard Deviation (RSD) between the replicate measurements in spiked QC samples, while accuracy was reported as the Relative Error (RE), which was calculated as the difference between the measured value and the theoretical value, divided by the theoretical value and expressed as a percentage (Table 4).

2.6.4. Application of the method to grape samples

The applicability of the proposed analytical method was tested by measuring the levels of standard lipids in 18 samples of grapes (10 red grape and 8 white grape varieties) from 4 different genetic groups [39]: *V. vinifera*, *Vitis* non-*vinifera*, Muscat and hybrid (Table S1). All samples were harvested at technological maturity and were analysed using the method developed.

3. Results and discussion

In contrast with the other main components of plants (proteins, carbohydrates and nucleic acids), lipids are defined on the basis of their physical properties, rather than their common chemical structure. Thus lipids are often loosely defined as compounds that are insoluble in water and that can be extracted from cells using non-polar organic solvents (such as chloroform). The most abundant types of lipid in most cells, however, are those deriving from the fatty acid and glycerolipid biosynthetic pathway, and these are the lipids we took into consideration in this analysis, together with sterols and glycerophospholipids [2].

Table 4

Intra-day ar	nd inter-day	(CV%),	recovery ((%)	accuracy	(%RE)	and	precision	(%RSD)	•
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Class	Compound	Intra-day CV (%)	Inter-day CV (%)	Recovery	Accuracy	Precision
Carnitines	Oleoyl-L-carnitine hydrochloride	1.40	14.28	88.77	- 11.23	3.07
	Palmitoyl-L-carnitine hydrochloride	2.49	13.49	83.29	- 16.71	4.12
Sterols	Desmosterol	5.21	17.41	60.04	- 39.96	20.14
	Ergosterol	3.22	7.93	82.73	- 17.27	41.81
	Lanosterol	3.99	19.89	95.86	- 4.14	11.52
	Uvaol	3.23	17.52	115.71	15.71	6.13
Fatty acids	Arachidic acid Behenic acid <i>cis</i> -11-Eicosenoic acid Erucic acid Heptadecanoic acid Lignoceric acid Linolenic acid Myristic acid Myristoleic acid Oleic acid+ <i>cis</i> -Vaccenic acid Palmitoleic acid Stearic acid	3.10 2.65 6.97 2.70 3.89 2.62 2.82 2.83 3.22 2.88 3.45 18.86 2.23 5.11	1.23 4.44 1.47 3.66 1.91 6.56 6.27 2.97 2.94 6.35 3.56 20.35 3.82 2.61	63.39 67.38 105.47 68.89 82.79 66.29 92.24 99.25 79.02 78.87 79.84 84.74 92.25 72.48	$\begin{array}{c} -36.61 \\ -32.62 \\ 5.47 \\ -31.11 \\ -17.21 \\ -33.71 \\ -7.76 \\ -0.75 \\ -20.98 \\ -21.13 \\ -20.16 \\ -15.26 \\ -7.75 \\ -27.52 \end{array}$	5.34 9.75 6.39 3.19 6.23 5.10 9.63 8.05 3.76 5.18 33.15 3.36 4.42 14.38
Glycerolipids	1,2,3-Tripentadecanoy[g]ycerol	0.96	5.23	80.63	- 19.37	6.80
	1-Linoleoyl-rac-g]ycerol	4.56	13.47	95.73	- 4.27	11.26
	1-Monopalmitoleoyl-rac-g]ycerol	3.07	5.27	82.06	- 17.94	5.86
	1-Oleoyl-rac-g]ycerol	2.71	3.95	92.01	- 7.99	5.82
	Glyceryl trioleate	1.07	8.89	32.54	- 67.46	1.99
	Glyceryl tripalmitoleate	0.76	4.80	71.35	- 28.65	3.04
Glycerophospholipids	1,2-Dilinoleoyl-sn-glycero-3-phosphocholine	1.48	6.50	82.01	- 17.99	5.68
	1,2-Dioleoyl-sn-glycero-3-phosphocholine	1.33	2.39	64.82	- 35.18	7.33
	1,2-Dioleoyl-sn-glycero-3-phospho-rac-(1-glycerol)sodium salt	1.55	6.33	90.37	- 9.63	1.82
	1-Palmitoyl-sn-glycero-3-phosphocholine	1.76	1.97	91.68	- 8.32	4.01
Sphingolipids	Ceramide	1.12	8.14	124.14	24.14	4.89
Prenols	Oleanolic acid	2.59	14.56	153.54	53.54	3.39

3.1. Reverse phase liquid chromatography (LC)

Lipid analysis takes different approaches into consideration: liquid chromatography mass spectrometry or fast infusion analysis for example, although the use of liquid chromatography is more useful for the quantification of low-abundance lipids because separation can enrich low-abundance molecular species and exclude the interaction of many lipid species, while chromatographically separating isobaric compounds. With these methods column separation facilitates the identification of isomeric species with identical fragmentation patterns, whereas shotgun lipidomics in this field encounter some difficulties. Many reviews have reported the application of LC-MS to the quantification of low-abundance lipids [40]. In Fig. 1 we show that the different classes of grape lipids are well divided on the basis of retention time in the instrumental conditions described in Section 2.5, while there is good separation of fatty acids on the basis of chain length and unsaturation. The sterols are well clustered in the central part of chromatogram, except for uvaol and oleanolic acid, which are C30 sterols with a different steric distribution and interaction with the column. Class separation using liquid chromatography (LC) followed by MS detection for species identification is very important in the analysis of glycerophospholipids extracted from a complex biological matrix [41-43]. Glycerophospholipids are distributed in the central part of the chromatogram, except for 1-palmitoyl-sn-glycero-3-phosphocholine, as



Fig. 1. Division of the different classes of grape lipids on the basis of retention time.

the retention time indeed also depends on the alkyl chain. At the end of chromatogram we find triacylglycerols in the glycerolipids category, which are less polar because of the long alkyl chain and the absence of a polar group; monoacylglycerols are instead found in the first 10 min of the chromatogram. Finally, we can observe that ceramide has a retention time of 19.2 min because of the d18/1/18/0 chain. A typical chromatogram of separation is shown in Fig. 2.

3.2. Fragmentation study and optimization of MS/MS conditions

The classification of long-chain lipid substances into different families is useful because mass spectrometric methods developed for lipid analysis have been centred around these divisions [29].



Fig. 2. UHPLC-ESI-MS/MS chromatograms of the quantifier MRM transition for the 33 lipids. (A) Carnitines, (B) sterols, (C) fatty acids, (E) glycerolipids, (F) glycerophospholipids, (G) sphingolipids, and (H) prenols.

In our method, structural information to confirm lipid identity was obtained by means of a preliminary fragmentation study, showing a characteristic fragmentation pattern for each class of compounds.

Q1 scan spectra of each lipid showed different ion species, due to the presence of ammonium formate in the mobile phase buffer and the possible consequent formation of adduct ions.

Fatty acids (FA) were typically analysed as negative ions using ESI. For almost all of them, the adduct ion $[M+HCOO]^-$ was the base peak, showing the $[M-H]^-$ ion as the most abundant fragment, except for *cis*-11-eicosenoic acid, which was detected as $[M-H]^-$ anion with a neutral loss of water to yield an $[M-H-18]^-$ ion.

A subclass of FA, acylcarnitines (CAR) was analysed using $[M+H]^+$ as precursor ion, showing their unique and informative MS/MS fragmentation pattern. Acylcarnitines dissociated to produce neutral losses of 59 and 161, as shown in Table 3.

Glycerolipids (GL), triacylglycerols (TAG) and diacylglycerols (DAG), could logically be placed in the same group as phospholipids (phosphatidylcholine, phosphatidylethanolamine, etc.) since both family members share a common glycerol backbone in their structure. However, these two types of lipids have entirely different chemical properties: neutral versus polar respectively [29]. The analysis of glycerolipids such as glyceryl trioleate and glyceryl tripalmitoleate, which have no electrostatic charge in solution, was carried out on the base of the molecular ion $[M+NH_4]^+$.

The most abundant MS/MS fragment ions correspond to the loss of one esterified fatty acyl group from the molecular ion as the free carboxylic acid, as well as ammonia, providing information about the fatty acyl groups of the TAG molecular species, as proposed by Murfy and Axersen in the TAG fragmentation scheme [29].

Glycerophospholipids (GP):phosphatidylcholines (PC) and phosphatidylglycerols (PG) were analysed, according to the polar head group of these molecules, with a preference for $[M+H]^+$ ions for PC species, as shown in Table 3.

The fragmentation pattern for glycerophospholipids showed the characteristic fragment ions for each different class: m/z 184 for the phosphocholine family.

Ceramide (C18) belongs to the class of sphingolipids (SP), which includes structurally very different compounds. Table 3 shows molecular ions $[M+H]^+$ used as precursors and the typical losses of H₂O to form $[M-H_2O+H]^+$ ions (*m*/*z* 548 used as quantifier ion), with subsequent amide bond cleavage and neutral loss of the fatty acid to yield an ion with *m*/*z* 264 (qualifier).

Finally, for the sterol lipid family (ST) we considered the adduct with ammonium $[M+NH_4]^+$. In our method we found the transition $[M+NH_4]^+ \rightarrow [M+NH_4-35]^+$, considered to be the characteristic fragment for the quantification of this class of compounds, as shown in Table 3.

3.3. Validation

The method was validated for 33 lipids. The linearity range was evaluated using the R^2 value, which was from 0.95 to 1.00. LOQ values were different for each compound and were in the range of 0.003–14.88 ng mL⁻¹. The linearity data were used to assess the percentage of matrix effect (%ME), which was calculated as (1–slope in solvent/slope in matrix), expressed as a percentage [38,44]. The % ME values shown in Table 2 were in the range \pm 20, except for oleoyl-L-carnitine hydrochloride (-26%), palmitoyl-L-carnitine hydrochloride (-33.9%), lanosterol (-25.8%), *cis*-11-eicosenoic acid (-21.5%) and 1-monopalmitoleoyl-rac-glycerol (-20.8%). %ME in the range between -20% and 20% can be considered as insignificant because such variability is close to RSD repeatability values [44].

Intra-day and inter-day repeatability were evaluated for each lipid. CV% did not exceed a value of 15% for intra-day assay and 20% for inter-day assay (Table 4). The relative recovery ranges were

over 90% for 12 compounds, between 80% and 90% for 8 compounds, between 70% and 80% for 5 compounds, between 60% and 70% for 6 compounds and below 60% for 1 compound. All these method validation results are summarized in Table 4.

None of the blank samples gave any signal that interfered with the peaks of the analytes after injection of the highest point in the calibration curve, showing no memory effect in the chromatographic run.

3.4. Quantification in real samples

One of the goals of this work was to demonstrate the feasibility of the analytical method developed for direct and rapid definition of lipid content in grape samples.

Table S2 summarises all the quantitative data for the 33 lipids quantified, expressed in terms of absolute amount ($\mu g g^{-1}$ of fresh weight), measured in 18 healthy grape varieties (10 red and 8 white), harvested at technological maturity, in the same year (2010) and belonging to 4 different genetic groups: V. vinifera, V. non-vinifera, Muscat and hybrid [39]. We injected each extracted samples two times: non diluted and diluted hundred times, in order to evaluate the most abundant lipids. The amounts are reported as $\mu g g^{-1}$ of fresh sample after normalization, on the basis of the internal standard, docosahexaenoic acid. The most abundant compounds found were oleanolic acid, 1,2-dilinoleoylsn-glycero-3-phosphocholine and glyceryl trioleate. In general, the amount of lipids was similar for all varieties, while we observed an increased amount of linoleic acid and oleic acid+cis-vaccenic acid in Vitis champini and Pinot noir. In P. noir we also found an increased amount of three fatty acids: linolenic acid, palmitic acid and cis-11-eicosanoic acid.

These preliminary observations suggest the existence of a certain diversity in the composition of grape lipids according to the cultivar, which requires more systematic confirmation. With regard to differences in the lipid profile or concentration linked to grape colour, we did not notice any particular trend for these samples. The wide genetic diversity of the grape samples chosen for this preliminary survey could have prevented the observation of minute differences inside red and white grapes belonging to a similar genetic group.

As regards the amount of lipids reported in the literature, it is difficult to make any comparison, because of the different varieties selected by other authors and because many papers have reported on the amount of lipids separately in tissue, skin, seed or pulp.

4. Conclusion

Due to their function, lipids are very important in biological systems. In plants in particular they have different roles and their relative abundance is subsequently influenced by genotype and phenotype. As knowledge of grape lipids is poor, a targeted LC–MS method for quantitative analysis of different classes of grape lipids was set up and optimized for the extraction, identification and quantification of different classes of grape lipids: fatty acids, sterols, glycerolipids, glycerophospholipids and sphingolipids.

Specifically, the chromatographic method allowed us to obtain good separation of the different classes of lipids and the MRM method allowed us to be very specific, avoiding interference and analysing lipids after minimal sample preparation. The method described was used to produce a lipid profile for different grape varieties. It was successfully applied to the analysis of 18 grape samples (10 red grape and 8 white grape varieties) from 4 different genetic groups, with 33 lipids being identified and quantified, 23 of these being confirmed by the q/Q value. This protocol could be easily adapted to analyse other simpler grape products, such as grape must in particular. In conclusion, our method could be applied to the study of lipids both in whole grape samples and grape tissues. Moreover, the analysis of lipids in must during winemaking could be useful in order to better understand and exploit yeast metabolism, given the crucial importance of these compounds in the fermentation process [25,45,46].

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.talanta.2015.03.003.

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Table S1: Classification of grape samples

Genetic Group Name	Genetic Group Code	Sample Name	Sample Color					
VisionantiniCom	1	Vitis Anderson	Red					
vitis non-vinijera	1	Vitis Champini	Red					
		Alicante Henri Bouschet	Red					
Hybrid	3	Sauvignon Blanc	White					
		Aleatico	Red					
		Millardet et Grasset	Red					
Muscat	4	Vermentino	White					
		White						
		Moscato Ottonel X Malvasia bianca di Candia-clone2	White					
		Merlot Noir	Red					
		Pinot Noir	Red					
		Syrah	Red					
		Cabernet Franc	Red					
Vitis vinifera	5	Sangiovese	Red					
		White						
		White						
		Peverella						
		Biancaccia	White					

Table S2: Quantification in real samples

		n	Ŀ	ri Bouschet	anc		Grasset		onel X Malvasia ndia clone1	onel X Malvasia ndia clone2				anc					
Class	Compound	Vitis Anderso	<i>Vitis</i> Champi	Alicante Hen	Sauvignon Bl	Aleatico	Millardet et (Vermentino	Moscato Otto bianca di Caı	Moscato Otto bianca di Caı	Merlot Noir	Pinot Noir	Syrah	Cabernet Fra	Sangiovese	Viogner	Garganega	Peverella	Biancaccia
Carniti	nes																		
	Oleoyl-L-carnitine hydrochloride	<loq< td=""><td><loq< td=""><td>0.0010</td><td><loq< td=""><td>0.0012</td><td><loq< td=""><td><loq< td=""><td><loq< td=""><td><loq< td=""><td><loq< td=""><td>0.0009</td><td><loq< td=""><td><loq< td=""><td><loq< td=""><td><loq< td=""><td><loq< td=""><td><loq< td=""><td>0.0012</td></loq<></td></loq<></td></loq<></td></loq<></td></loq<></td></loq<></td></loq<></td></loq<></td></loq<></td></loq<></td></loq<></td></loq<></td></loq<></td></loq<>	<loq< td=""><td>0.0010</td><td><loq< td=""><td>0.0012</td><td><loq< td=""><td><loq< td=""><td><loq< td=""><td><loq< td=""><td><loq< td=""><td>0.0009</td><td><loq< td=""><td><loq< td=""><td><loq< td=""><td><loq< td=""><td><loq< td=""><td><loq< td=""><td>0.0012</td></loq<></td></loq<></td></loq<></td></loq<></td></loq<></td></loq<></td></loq<></td></loq<></td></loq<></td></loq<></td></loq<></td></loq<></td></loq<>	0.0010	<loq< td=""><td>0.0012</td><td><loq< td=""><td><loq< td=""><td><loq< td=""><td><loq< td=""><td><loq< td=""><td>0.0009</td><td><loq< td=""><td><loq< td=""><td><loq< td=""><td><loq< td=""><td><loq< td=""><td><loq< td=""><td>0.0012</td></loq<></td></loq<></td></loq<></td></loq<></td></loq<></td></loq<></td></loq<></td></loq<></td></loq<></td></loq<></td></loq<></td></loq<>	0.0012	<loq< td=""><td><loq< td=""><td><loq< td=""><td><loq< td=""><td><loq< td=""><td>0.0009</td><td><loq< td=""><td><loq< td=""><td><loq< td=""><td><loq< td=""><td><loq< td=""><td><loq< td=""><td>0.0012</td></loq<></td></loq<></td></loq<></td></loq<></td></loq<></td></loq<></td></loq<></td></loq<></td></loq<></td></loq<></td></loq<>	<loq< td=""><td><loq< td=""><td><loq< td=""><td><loq< td=""><td>0.0009</td><td><loq< td=""><td><loq< td=""><td><loq< td=""><td><loq< td=""><td><loq< td=""><td><loq< td=""><td>0.0012</td></loq<></td></loq<></td></loq<></td></loq<></td></loq<></td></loq<></td></loq<></td></loq<></td></loq<></td></loq<>	<loq< td=""><td><loq< td=""><td><loq< td=""><td>0.0009</td><td><loq< td=""><td><loq< td=""><td><loq< td=""><td><loq< td=""><td><loq< td=""><td><loq< td=""><td>0.0012</td></loq<></td></loq<></td></loq<></td></loq<></td></loq<></td></loq<></td></loq<></td></loq<></td></loq<>	<loq< td=""><td><loq< td=""><td>0.0009</td><td><loq< td=""><td><loq< td=""><td><loq< td=""><td><loq< td=""><td><loq< td=""><td><loq< td=""><td>0.0012</td></loq<></td></loq<></td></loq<></td></loq<></td></loq<></td></loq<></td></loq<></td></loq<>	<loq< td=""><td>0.0009</td><td><loq< td=""><td><loq< td=""><td><loq< td=""><td><loq< td=""><td><loq< td=""><td><loq< td=""><td>0.0012</td></loq<></td></loq<></td></loq<></td></loq<></td></loq<></td></loq<></td></loq<>	0.0009	<loq< td=""><td><loq< td=""><td><loq< td=""><td><loq< td=""><td><loq< td=""><td><loq< td=""><td>0.0012</td></loq<></td></loq<></td></loq<></td></loq<></td></loq<></td></loq<>	<loq< td=""><td><loq< td=""><td><loq< td=""><td><loq< td=""><td><loq< td=""><td>0.0012</td></loq<></td></loq<></td></loq<></td></loq<></td></loq<>	<loq< td=""><td><loq< td=""><td><loq< td=""><td><loq< td=""><td>0.0012</td></loq<></td></loq<></td></loq<></td></loq<>	<loq< td=""><td><loq< td=""><td><loq< td=""><td>0.0012</td></loq<></td></loq<></td></loq<>	<loq< td=""><td><loq< td=""><td>0.0012</td></loq<></td></loq<>	<loq< td=""><td>0.0012</td></loq<>	0.0012
	Palmitoyl-L-carnitine hydrochloride	0.0006	0.0003	0.0011	0.0006	0.0008	0.0007	0.0009	0.0004	0.0009	0.0008	0.0007	0.0009	0.0028	0.0014	0.0007	0.0011	0.0006	0.0008
Sterols																			
	Desmosterol	0.44	0.16	0.22	0.25	0.24	1.07	0.35	0.24	0.34	0.38	1.61	0.26	1.28	<loq< td=""><td>0.26</td><td>N.D.</td><td>0.51</td><td><loq< td=""></loq<></td></loq<>	0.26	N.D.	0.51	<loq< td=""></loq<>
	Ergosterol	1.22	1.00	0.93	N.D.	1.17	0.78	1.70	1.00	N.D.	1.10	3.31	3.77	2.92	<loq< td=""><td>1.88</td><td>4.19</td><td><loq< td=""><td>0.65</td></loq<></td></loq<>	1.88	4.19	<loq< td=""><td>0.65</td></loq<>	0.65
	Lanosterol	7.94	16.10	7.54	8.74	9.74	23.43	5.20	10.93	10.67	8.71	8.65	10.88	15.88	<loq< td=""><td>16.24</td><td>12.09</td><td>12.17</td><td>4.41</td></loq<>	16.24	12.09	12.17	4.41
	Uvaol	31.61	51.81	16.41	14.25	7.11	39.00	4.32	28.43	29.83	10.13	9.86	16.30	8.03	14.42	21.87	11.91	13.60	28.27
Fatty a	cids																		
	Arachidic acid	2.04	4.21	3.39	4.64	9.00	8.85	7.57	15.23	3.92	4.62	10.57	5.55	6.51	5.10	4.43	9.28	4.92	9.87
	Behenic acid	1.16	2.07	3.19	1.98	3.39	5.50	3.65	6.72	1.68	2.43	6.22	1.68	4.86	5.01	1.80	9.98	3.70	4.68
	cis-11-eicosenoic acid	0.65	0.85	1.54	0.41	0.28	0.35	0.42	1.16	<loq< td=""><td>0.49</td><td>2.00</td><td>0.65</td><td><loq< td=""><td><loq< td=""><td><loq< td=""><td><loq< td=""><td><loq< td=""><td><loq< td=""></loq<></td></loq<></td></loq<></td></loq<></td></loq<></td></loq<></td></loq<>	0.49	2.00	0.65	<loq< td=""><td><loq< td=""><td><loq< td=""><td><loq< td=""><td><loq< td=""><td><loq< td=""></loq<></td></loq<></td></loq<></td></loq<></td></loq<></td></loq<>	<loq< td=""><td><loq< td=""><td><loq< td=""><td><loq< td=""><td><loq< td=""></loq<></td></loq<></td></loq<></td></loq<></td></loq<>	<loq< td=""><td><loq< td=""><td><loq< td=""><td><loq< td=""></loq<></td></loq<></td></loq<></td></loq<>	<loq< td=""><td><loq< td=""><td><loq< td=""></loq<></td></loq<></td></loq<>	<loq< td=""><td><loq< td=""></loq<></td></loq<>	<loq< td=""></loq<>
	Erucic acid	0.17	0.15	0.13	0.12	0.11	0.13	0.15	0.12	0.14	0.14	0.38	0.19	0.62	0.39	0.17	0.43	0.45	0.13
	Heptadecanoic acid	0.21	0.24	0.18	0.35	0.18	0.93	0.59	0.39	0.40	0.19	0.76	0.39	N.D.	N.D.	0.14	N.D.	N.D.	0.22
	Lignoceric acid	4.89	3.63	5.09	4.48	5.91	13.67	8.54	7.99	6.84	6.70	9.46	3.86	64.24	12.23	6.39	20.04	5.17	4.60
	Linoleic acid	6.06	14.91	1.22	8.33	0.71	3.02	1.07	3.17	2.17	2.30	21.27	6.77	4.17	1.48	1.35	1.16	2.96	1.08
	Linolenic acid	1.78	1.39	0.83	0.99	0.91	1.48	0.79	0.61	0.57	0.54	5.11	6.30	0.74	0.74	0.97	0.66	0.47	0.80
	Myristic acid	0.63	1.03	101.04	0.87	0.66	1.19	0.62	0.93	0.98	0.65	1.00	1.40	<loq< td=""><td>6.79</td><td>0.44</td><td>0.85</td><td>0.69</td><td>0.77</td></loq<>	6.79	0.44	0.85	0.69	0.77
	Myristoleic acid	0.13	0.13	3.13	0.09	0.13	0.16	0.12	0.16	0.14	<loq< td=""><td>0.34</td><td>0.19</td><td><loq< td=""><td>0.78</td><td>0.16</td><td>0.48</td><td>0.31</td><td>0.08</td></loq<></td></loq<>	0.34	0.19	<loq< td=""><td>0.78</td><td>0.16</td><td>0.48</td><td>0.31</td><td>0.08</td></loq<>	0.78	0.16	0.48	0.31	0.08
	Oleic acid + cis-Vaccenic acid	3.90	10.19	1.21	3.05	0.62	2.34	1.43	2.65	1.31	2.18	22.99	7.02	2.10	1.11	1.47	1.04	2.70	1.08
	Palmitic acid	13.59	16.31	11.04	19.88	8.09	30.43	29.22	27.25	11.35	11.79	40.47	28.03	8.27	N.D.	7.98	5.85	6.62	13.73
	Palmitoleic acid	0.49	0.19	4.87	N.D.	<loq< td=""><td>0.28</td><td>0.16</td><td>0.39</td><td>0.26</td><td>0.22</td><td>1.17</td><td>0.96</td><td>N.D.</td><td>N.D.</td><td>0.25</td><td>N.D.</td><td>N.D.</td><td>0.20</td></loq<>	0.28	0.16	0.39	0.26	0.22	1.17	0.96	N.D.	N.D.	0.25	N.D.	N.D.	0.20
	Stearic acid	14.41	17.19	17.85	31.77	15.76	49.70	47.87	35.81	15.38	17.36	37.44	28.03	24.92	0.80	14.31	19.45	14.00	18.70
Glycero	olipids																		

	1.2.3-tripentadecanoylglycerol	0.22	0.30	0.70	0.43	0.83	0.33	0.42	0.44	0.68	0.26	0.26	0.42	0.46	0.76	0.53	N.D.	0.61	0.48
	1-linoleoyl-rac-glycerol	0.35	0.65	7.23	0.32	<loq< td=""><td>0.97</td><td>0.31</td><td>0.15</td><td>0.34</td><td>0.24</td><td>1.75</td><td>1.66</td><td>N.D.</td><td>N.D.</td><td>0.20</td><td>0.75</td><td>N.D.</td><td>0.21</td></loq<>	0.97	0.31	0.15	0.34	0.24	1.75	1.66	N.D.	N.D.	0.20	0.75	N.D.	0.21
	1-monopalmitoleoyl-rac- glycerol	0.34	0.28	42.98	0.42	0.35	0.33	0.39	0.37	0.34	0.39	0.36	0.45	0.67	0.71	0.30	0.63	0.50	0.35
	1-oleoyl-rac-glycerol	0.13	0.13	0.11	0.15	0.12	0.16	0.14	0.10	0.09	0.12	0.27	0.56	0.17	0.16	0.06	0.15	0.06	0.11
	Glyceryl trioleate	109.44	95.86	346.68	283.58	75.42	226.80	238.48	82.85	109.51	318.33	204.57	268.08	255.20	154.90	218.97	332.98	195.51	174.65
	Glyceryl tripalmitoleate	<loq< td=""><td><loq< td=""><td>2.10</td><td><loq< td=""><td>2.21</td><td><loq< td=""><td><loq< td=""><td><loq< td=""><td>2.09</td><td><loq< td=""><td>2.29</td><td>1.91</td><td><loq< td=""><td>7.21</td><td>1.43</td><td><loq< td=""><td><loq< td=""><td><loq< td=""></loq<></td></loq<></td></loq<></td></loq<></td></loq<></td></loq<></td></loq<></td></loq<></td></loq<></td></loq<></td></loq<>	<loq< td=""><td>2.10</td><td><loq< td=""><td>2.21</td><td><loq< td=""><td><loq< td=""><td><loq< td=""><td>2.09</td><td><loq< td=""><td>2.29</td><td>1.91</td><td><loq< td=""><td>7.21</td><td>1.43</td><td><loq< td=""><td><loq< td=""><td><loq< td=""></loq<></td></loq<></td></loq<></td></loq<></td></loq<></td></loq<></td></loq<></td></loq<></td></loq<></td></loq<>	2.10	<loq< td=""><td>2.21</td><td><loq< td=""><td><loq< td=""><td><loq< td=""><td>2.09</td><td><loq< td=""><td>2.29</td><td>1.91</td><td><loq< td=""><td>7.21</td><td>1.43</td><td><loq< td=""><td><loq< td=""><td><loq< td=""></loq<></td></loq<></td></loq<></td></loq<></td></loq<></td></loq<></td></loq<></td></loq<></td></loq<>	2.21	<loq< td=""><td><loq< td=""><td><loq< td=""><td>2.09</td><td><loq< td=""><td>2.29</td><td>1.91</td><td><loq< td=""><td>7.21</td><td>1.43</td><td><loq< td=""><td><loq< td=""><td><loq< td=""></loq<></td></loq<></td></loq<></td></loq<></td></loq<></td></loq<></td></loq<></td></loq<>	<loq< td=""><td><loq< td=""><td>2.09</td><td><loq< td=""><td>2.29</td><td>1.91</td><td><loq< td=""><td>7.21</td><td>1.43</td><td><loq< td=""><td><loq< td=""><td><loq< td=""></loq<></td></loq<></td></loq<></td></loq<></td></loq<></td></loq<></td></loq<>	<loq< td=""><td>2.09</td><td><loq< td=""><td>2.29</td><td>1.91</td><td><loq< td=""><td>7.21</td><td>1.43</td><td><loq< td=""><td><loq< td=""><td><loq< td=""></loq<></td></loq<></td></loq<></td></loq<></td></loq<></td></loq<>	2.09	<loq< td=""><td>2.29</td><td>1.91</td><td><loq< td=""><td>7.21</td><td>1.43</td><td><loq< td=""><td><loq< td=""><td><loq< td=""></loq<></td></loq<></td></loq<></td></loq<></td></loq<>	2.29	1.91	<loq< td=""><td>7.21</td><td>1.43</td><td><loq< td=""><td><loq< td=""><td><loq< td=""></loq<></td></loq<></td></loq<></td></loq<>	7.21	1.43	<loq< td=""><td><loq< td=""><td><loq< td=""></loq<></td></loq<></td></loq<>	<loq< td=""><td><loq< td=""></loq<></td></loq<>	<loq< td=""></loq<>
Glycero	ophospholipids																		
94 - 10 - 9, 64 (1961)	1.2-dilinoleoyl-sn-glycero-3- phosphocholine	189.66	166.29	130.76	167.93	196.09	328.39	77.36	89.35	191.15	80.06	222.77	155.84	145.69	134.04	80.51	213.93	194.81	146.57
	1.2-dioleoyl-sn-glycero-3- phosphocholine	36.08	59.57	32.21	35.87	29.03	61.23	18.30	26.45	45.13	42.68	93.19	69.34	45.46	25.48	34.88	59.37	31.84	18.05
	1.2-dioleoyl-sn-glycero-3- phospho -rac-(1-glycerol)sodium salt	1.34	0.89	0.79	0.84	1.22	1.76	0.92	1.18	1.41	0.94	1.80	1.63	3.86	2.03	0.77	3.32	1.70	0.64
	1-palmitoyl-sn-glycero-3- phosphocholine	0.58	1.44	1.43	1.10	1.09	1.52	0.49	0.66	1.42	0.77	1.02	1.27	1.07	0.93	2.68	0.92	0.72	0.82
Sphinge	olipids																		
	Ceramide	0.01	0.01	1.42	0.01	0.01	0.01	0.02	0.01	0.01	0.01	0.02	0.02	0.02	<loq< td=""><td>0.01</td><td>0.01</td><td>0.01</td><td>0.01</td></loq<>	0.01	0.01	0.01	0.01
Prenols	3																		
	Oleanolic acid	5397.61	8779.73	4094.63	5497.21	4424.58	8791.33	3532.99	3682.31	5077.43	4027.33	5712.77	5148.74	5774.34	4141.20	5192.31	5089.01	4887.84	3427.64

Plants are commonly exposed to a large number of stresses, which can be divided into two main categories: abiotic (environmental) and biotic (biological). One of these environmental stresses is represented by mechanical damage to leaf tissue due to rain, snow, wind, animals, pathogens, or the plant themselves (Benikhlef et al. 2013). Plants have many external structural defences; when this initial protection strategy is not enough to protect themselves, plants are able to modulate their metabolic pathways to produce chemical compounds having defence properties.

The use of leaf discs is widely adopted in various kinds of experiments for different vegetable species (e.g. cucumbers, sunflowers, lactuceae, chilli peppers, tomatoes, cacao, Chinese cabbage and kidney beans) and in particular in studies regarding the effect of different types of biotic stress on the biochemical response of the grapevine. This makes it necessary to understand metabolic perturbation after injury. Since there is little knowledge regarding mechanical wounding of grapevine leaves, the aim of this study was to analyse changes in phenolic, lipid and carotenoid content in Bianca grapevine leaves subjected to mechanical wounding (leaf discs) at 0, 6, 12, 24, 48, 96 and 120 hours after injury, testing two different sizes of leaf discs (1.1 cm and 2.8 cm in diameter) in order to determine the role of these compounds in response to mechanical stress. To our knowledge, this is the first work which has studied the effect of cutting stress in widely used leaf disc experiments. Comparing both leaf disc sizes, similar metabolism perturbation was found. In our work, bigger differences in stilbenes and stilbenoids were found in 1.1 cm diameter discs compared to those 2.8 cm in diameter, with an accumulation of some compounds, such as alpha-viniferin, pallidol and ampelopsin H + vaticanol C-like isomer. Our results indicate that mechanical wounding induced accumulation of compounds with less or as yet undescribed toxic activity against pathogens, especially in 1.1 cm diameter discs, assuming their implication in response to abiotic stress. The smallest differences were found in compounds with confirmed activity against pathogens, in relation to the size of the discs. In lipids and carotenoids the
differences were less visible and the trend was mostly the same after mechanical wounding in both sizes, with an increase in fatty acids such as linoleic acid, linolenic acid and oleic + *cis*-vaccenic acid during the first 12 hours after injury, followed by a return to basal level. These results allowed us to surmise their role in response to abiotic stress, in particular to mechanical wounding of grapevine leaves.

The metabolic results of this work can be used to better apply the best leaf disc technique to evaluate metabolic changes due to biotic stress, having previous knowledge about the perturbation caused by abiotic stress. These findings are significant for experiments studying the different behaviour of resistant varieties (totally or partially) and sensitive varieties, in terms of the biochemical mechanisms involved in resistance to the disease. A better understanding of resistance biochemistry may lead to improved selection of resistant plants in order to reduce fungicide treatments. The lack of information about the effect of mechanical wounding on the grapevine leaf metabolism led to the concept behind this experiment.

My personal involvement in this project started with the experimental design. I personally performed the experiments, analysis and data processing and I was responsible for writing the manuscript and managing the comments and improvements to the text by other authors.

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Chapter III

Lipid, phenol and carotenoid changes in 'Bianca' grapevine leaves after mechanical wounding: a case study ORIGINAL ARTICLE



Lipid, phenol and carotenoid changes in 'Bianca' grapevine leaves after mechanical wounding: a case study

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Abstract Metabolic changes can occur in plants in response to abiotic stress. Extensive use of leaf discs (mechanical wounding) in studies regarding the effect on the biochemical response of the grapevine to different types of biotic stress makes it necessary to understand metabolic perturbation after injury. In this study, we investigate how mechanical wounding can affect the metabolism of grapevine leaf tissue using Bianca variety as case study. Two sizes of leaf discs (1.1 and 2.8 cm in diameter) were excised from leaves, and phenol, lipid and carotenoid perturbation were investigated 0, 6, 12, 24, 48, 96 and 120 h post cutting. In our study, we found an accumulation of molecules belonging to stilbenoid and stilbene classes such as trans-resveratrol, trans-piceide, Zmiyabenol C, E-cis-miyabenol C and ampelopsin D + quadrangularin A after abiotic stress. The increase in fatty acids such as linoleic acid, linolenic acid and oleic + cisvaccenic acid during the first 12 h after injury, followed by a return to basal level, allowed us to surmise their role in response to abiotic stress, in particular to mechanical wounding in grapevine leaves. Different-sized discs caused a different response to the tissue, with a higher accumulation in 1.1-cm-

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diameter discs, especially of phenol compounds. The results of this work can be used to better understand metabolic changes due to biotic stress, having previous knowledge about the perturbation caused by abiotic stress.

Keywords Mechanical wounding · Grapevine · Abiotic stress · Metabolism

Introduction

Plants are exposed daily to a large number of environmental stresses; one of these is represented by mechanical damage to leaf tissue due to rain, snow, wind, animals, pathogens or the plants themselves (Benikhlef et al. 2013). Different physical barriers in plants are the first defence mechanism against mechanical damage. When physical barriers are not enough in the fight against insects and injury has occurred, plants are able to modulate their metabolic pathways to produce chemical factors having defensive properties. Indeed, wound activates local and systemic responses including metabolic changes and the induction of gene expression (Reymond et al. 2000; León et al. 2001) associated with induced defence reactions (Benikhlef et al. 2013). The study of mechanical damage provides an opportunity to study perturbation after abiotic stress, compared to that inflicted by herbivores, under controlled conditions (Brilli et al. 2011).

In the group of polyphenols, the flavonoid pathway is crucial for plant defence against biotic and abiotic stress, since it plays a vital role in the interaction of plants with their surrounding environment (Dai et al. 1994, 1995; Noel et al. 2005). Plant stilbenes, a small group of phenylpropanoids identified as phytoalexins, are known to be massively accumulated not only in the plant-pathogen interaction but also in response to abiotic stresses including wounding (Chiron et al.

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2000; Chung et al. 2003; Vannozzi et al. 2012; Höll et al. 2013). Other chemical classes of small molecules are also known to be modulated when abiotic stress is inflicted on the plant. Vu et al. (2015) found a significant change in 254 out of 264 lipids in response to mechanical wounding in Arabidopsis thaliana, due to activation of an array of enzymes. Their work demonstrated that despite individual plants shown small differences in lipid composition, there is a significant change in the levels of lipids as a response to wounding, with structural lipids showing a decrease, while the concentration of many other lipids such as galactolipids, phosphatidylglycerols, phosphatidic acids, trigalactosyldiacylglycerols and tetra-galactosyldiacylglycerols, tri-acylglycerols and some sterols increased (Vu et al. 2015). For the carotenoid class, activation of their de novo accumulation after oxidative stress due to abiotic stress has been reported (Tôyama 1972). Bouvier et al. (1998) showed that gene expression of geranylgeranyl pyrophosphate synthase (GGPS), responsible for the synthesis of the immediate precursor of carotenoids, and capsanthin-capsorubin synthase, which synthesizes the final carotenoid products, is induced in chilli pepper fruits by wounding (Bouvier et al. 1998).

Leaf damage triggers wound responses in plants, and it seems that a large number of the genes involved in the wounding response are comparable with those activated after pathogen infections (Savatin et al. 2014). This indicates that abiotic stress needs to be efficiently contrasted.

There is little knowledge regarding mechanical wounding of grapevine leaves, although the excision of leaf discs is widely used in studies regarding the effect on the biochemical responses of the grapevine due to different biotic stresses. In particular, several studies have focused on biochemical, metabolomic and genetic responses caused by destructive diseases such as downy mildew, caused by a biotrophic oomycete, Plasmopara viticola (Berk. and Curt.) Berl. & de Toni (Boso et al. 2006; Kennelly et al. 2007; Bellin et al. 2009; Kono et al. 2015), and powdery mildew caused by the ascomycetous fungus Erysiphe necator Schwein (Willocquet et al. 1996; Delye and Corio-Costet 1998; Jailloux et al. 1998; Schnee et al. 2013). The Organization Internationale de la Vigne et du Vin (OIV; www.oiv.int) reports a method for the assessment of the degree of disease infection based on a leaf disc test: descriptors N. 452-1 and 455-1 for downy and powdery mildew respectively. Moreover, the use of leaf discs is extensively adopted in various kinds of experiments for different vegetable species: cucumbers (Zhao et al. 2013; Sun et al. 2013), sunflowers (Lava et al. 2015), lactuceae (Lebeda et al. 2012), chilli peppers (Park et al. 2013), tomatoes (Suthaparan et al. 2016), cacao (Nyadanu et al. 2012), Chinese cabbage (Zhang et al. 2012) and kidney beans (Keshavarz and Taheri 2015). The principal aim of this study was to analyse changes in phenolic, lipid and carotenoid content in grapevine leaves subjected to mechanical wounding

(leaf discs) at various time points after injury, testing two different sizes of leaf discs in order to determine the role of these compounds in response to mechanical stress. The metabolic results of this work can be used to better evaluate metabolic changes due to biotic stress, having previous knowledge about the perturbation caused by abiotic stress. To start investigating mechanical stress in grapevine leaves, we focused on a case study using Bianca variety that shows good resistance both to downy and powdery mildew and a very good tolerance to the frost (Kozma and Dula 2003; Peressotti et al. 2010). The significance of these findings is important for the experiments studying different behaviour of resistant (totally or partially) varieties and sensitive varieties in terms of the biochemical mechanisms involved in resistance to the disease. Up to our knowledge, this is the most comprehensive study on the effect of mechanical wounding on several classes of metabolites: polyphenols, carotenoids and lipids in grape leaf discs.

Materials and methods

Plant material and experimental design

Evaluation of mechanical wounding was performed using grapevine plants from the 'Bianca' cultivar. The Bianca plants were from the 'Fondazione Edmund Mach' grape collection, located in San Michele all'Adige (Trento), Italy (46° 12' 0" N, 11° 8' 0" E), grown in pots in greenhouse conditions. Bianca is a grapevine resistant to the pathogen P. viticola, obtained by hybridization of Vitis vinifera 'Bouvier' and 'Villard Blanc' (Csizmazia and Bereznai 1968). The plants were divided into three homogenous groups; each group represented one biological replicate. For each plant, the third, fourth and fifth leaves beneath the shoot apex were detached, then rinsed with ultrapure water to eliminate surface residues. Leaf discs 1.1 and 2.8 cm in diameter were excised from each leaf and placed randomly onto wet paper in petri dishes. Petri dishes were incubated in a growth chamber at 21 °C until sampling. Tissues were sampled 0, 6, 12, 24, 48, 96 and 120 h post cutting (hpc) then ground under liquid nitrogen to obtain a frozen powder.

Targeted phenolic compound analysis

Phenolic compounds were determined according to Vrhovsek et al. (2012b), with some modifications (Vrhovsek et al. 2012b). Briefly, 0.4 mL of chloroform and 0.6 mL of methanol/water (2:1) were added to 0.1 g of fresh leaf powder. A 20 μ L aliquot of gentisic acid (50 mg/L) and rosmarinic acid (50 mg/L) was added as internal standard. The extraction mixture was shaken for 15 min in an orbital shaker, then centrifuged for 5 min at 15,000×g at 4 °C. The upper

aqueous-methanolic phase was collected. The extraction was repeated by adding 0.6 mL of methanol and water (2:1 v/v) and 0.2 mL of chloroform; the samples were centrifuged for 5 min at $15,000 \times g$ at 4 °C. The aqueous-methanolic phase was collected and combined with the previous one. Both fractions were united and evaporated to dryness under N₂. Samples were reconstituted in 500 uL of methanol and water (1:1 v/v), centrifuged and transferred carefully into a HPLC vial with glass insert before injection. Chromatographic analysis was carried out using a Waters Acquity UPLC system (Milford) coupled to a Waters Xevo triple-quadrupole mass spectrometer detector (Milford). Compounds were identified based on their reference standard, retention time and qualifier and quantifier ion and were quantified using their calibration curves and expressed as milligram per kilogram of fresh leaves (Vrhovsek et al. 2012b).

Targeted lipid compound analysis

Lipid analysis was carried out according to Della Corte et al. (2015) using Folch's extraction method (Folch et al. 1957; Della Corte et al. 2015) with some modifications. Briefly, 0.1 g of fresh leaf powder was weighed, and 0.3 mL of methanol was added and vortexed for 30 s; 0.6 mL of chloroform containing butylated hydroxyl toluene (BHT 500 mg/L) was added, followed by the addition of 10 µL of internal standard (docosahexaenoic acid 100 µg/mL). Samples were placed in an orbital shaker for 60 min. Afterwards, 0.25 mL of water was added; after 10 min, the samples were centrifuged at 3600 rpm for 10 min. The total lower lipid-rich layer was collected. The extraction was repeated by adding 0.4 mL of chloroform/methanol/water 86:14:1 v/v/v. The samples were centrifuged at 3600 rpm for 10 min; the total lower lipid-rich layer was collected. Both fractions were united and evaporated to dryness under N2. Samples were reconstituted in 300 µL of acetonitrile/2-propanol/water (65:30:5 v/v/v) containing the internal standard cholesterol at a concentration of 1 µg/mL and transferred into a HPLC vial with insert before LC-MS/ MS analysis. Separation was performed using a UHPLC Dionex 3000 (Thermo Fischer Scientific Germany) coupled to an API 5500 triple-quadrupole mass spectrometer (Applied Biosystems/MDS Sciex). Compounds were identified based on their reference standard, retention time and qualifier and quantifier ion and were quantified using their calibration curves and expressed as milligram per kilogram of fresh leaves (Della Corte et al. 2015).

Targeted carotenoid and tocopherol analysis

Carotenoids and tocopherols were analysed according to Wehrens et al. (2013). Briefly, 0.1 g of fresh leaf powder was extracted with 2 mL of a mix of methanol/chloroform/ water in a ratio of 2:2:1. Twenty microlitres of trans- β -apo-8'-

carotenal (100 µg/mL) was used as internal standard. After vortexing and shaking for 15 min, samples were centrifuged to help the phase separation; the chloroform phase of the extraction was collected. Ten microlitres of a 0.1% triethylamine solution was added to prevent rearrangement of carotenoids. Samples were dried with N₂ and stored at -80 °C until analysis. Dried samples were suspended in 50 µL of ethyl acetate and transferred to dark vials. Chromatographic analysis was performed in a 1290 Infinity Binary UHPLC (Agilent) equipped with an RP C30 column (250 × 2.1 mm, 3 µm), coupled to a 20 × 4.6 mm C30 guard column. Spectra components and elution profiles were determined as in Wehrens et al. (2013). Compounds were quantified from linear calibration curves built with standard solutions and expressed as milligram per kilogram of fresh leaves.

Statistical analysis

One-way ANOVA followed by Newman-Keuls test was applied to determine the differences over the time course for the two different sets (1.1 and 2.8 cm in diameter) using STATISTICA software (version 9.1). Student's *t* test was applied to investigate the differences between the different diameter discs at the same time points using STATISTICA software (version 9.1).

Results

Two different sizes of leaf discs (1.1 and 2.8 cm in diameter) were excised from leaves of the Bianca grape variety. The impact of mechanical wounding on the phenolic, lipid and carotenoid metabolism was tested. The zero time point of each size was identified as the control and samples were collected 6, 12, 24, 48, 96 and 120 h post injury. A total of 48 phenolics, 27 lipids and 16 compounds in the tocopherol, carotenoid, xanthophyll and chlorophyll classes were identified and quantified, and their concentration is expressed as milligram per kilogram of fresh leaves (Table S1). At first, we evaluated the metabolic perturbation over the time course in the two different sizes of leaf discs; afterwards, we compared the perturbation between the two sizes.

Metabolic perturbation over time

Heatmaps (Figs. 1, 2 and 3) representing a \log_2 fold change between time points (6, 12, 24, 48, 96 and 120 h post injury) and the zero time point (control) show an overview of metabolic perturbation caused by mechanical wounding over the time course considering 1.1 and 2.8 cm in diameter leaf discs separately. Blue and red boxes represent lower and higher concentration of specific compounds in relative time points compared to the control (zero time point). As shown in Fig. 1 Effect of mechanical wounding on phenolic metabolites during the first 120 h post cutting. Heatmaps represent log2 fold change (with zero time point) of phenolic compounds in 1.1 and 2.8-cm-diameter leaf discs 6, 12, 24, 48, 96 and 120 h post cutting. Blue and red boxes represent lower and higher concentrations compared to the control (0 h post cutting). Asterisks indicate significant differences (p < 0.05) between time points and the control (0 h post cutting)



Fig. 1, in both leaf disc sizes, we found a significant accumulation of stilbene and stilbenoid compounds. In Fig. 4, the sum of quantified stilbene and stilbenoid compounds is reported, showing an increase in their content after 48 h with a maximum at 96 h post damage. Different trends were reported for stilbenes and stilbenoids, based on whether they were monomers, dimers, trimers or tetramers. Monomers such as *trans*resveratrol, *trans*-piceide and isorhapontin increased 6–12 h post injury. Dimer compounds such as *trans*-epsilon-viniferin and pallidol showed an accumulation starting at 24 h post mechanical damage, but we found the most important variations in trimers and tetramers, with a consistent accumulation of these metabolites starting 24 h after injury. Few significant differences were reported within compounds belonging to

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other classes of phenolic compounds; in particular, flavonols such as kaempferol-3-O-rutinoside, quercetin-3-glucoside + quercetin-3-galactoside, quercetin-3-O-glucuronide and rutin were modulated in 1.1 cm in diameter leaf discs (Fig. 1). Among the 27 lipids quantified (Fig. 2), after injury, we obtained significant accumulations of fatty acid-specific compounds such as linolenic acid, linoleic acid, oleic + *cis*vaccenic acid and palmitic acid mostly with a similar trend in the two different sizes of discs during the time course. Accumulation of some of these has previously been reported in other plants after wounding (Conconi et al. 1996; Lee et al. 1997; Ryu and Wang 1998). Linoleic acid, linolenic acid oleic + *cis*-vaccenic acid showed similar trends after mechanical damage, with early accumulation starting in the first 12 h post Metabolic changes in grapevine leaves after mechanical wounding

Fig. 2 Effect of mechanical wounding on lipid compounds during the first 120 h post cutting. Heatmaps represent \log_2 fold change (with zero time point) of lipid compounds in 1.1 and 2.8-cm-diameter leaf discs 6, 12, 24, 48, 96 and 120 h post cutting. Blue and red boxes represent lower and higher concentrations compared to the control (0 h post cutting). Asterisks indicate significant differences (p < 0.05) between time points and the control (0 h post cutting)



damage and then a progressive return to basal levels at 48– 96 h. Uvaol and oleanolic acid (C30 compounds) showed slower accumulation starting after 12–24 h, with a progressive trend (Fig. 5). Heptadecanoic acid and palmitic acid concentration was perturbed by mechanical damage only in 1.1-cmdiameter discs, with a return to the basal level 96 h after mechanical wounding (Table S1). No perturbation was found among carotenoid and tochopherol compounds after injury in the damaged tissue (Fig. 3). The quantity of this class of compounds was less affected after mechanical damage. Mechanical wounding caused an early decrease in chlorophyll and xanthophyll concentration compared to zero time point in both size discs, with statistical relevance only for 2.8 cm in diameter leaf discs (Fig. 6). Looking at the entire time course using the ANOVA test, the differences found were really low for both leaf disc sizes (Table S1).

Metabolic perturbation between leaf disc sizes

To evaluate differences between the two sizes, a Student's t test was applied for each time point; in Table S1 and in Figs. 4, 5 and 6, asterisks indicate significant differences between 1.1 and 2.8-cm discs. Comparing the two sizes of discs, we saw a greater accumulation of stilbene + stilbenoid

Fig. 3 Effect of mechanical wounding on carotenoid, chlorophyll, xanthophyll and tocopherol compounds during the first 120 h post cutting. Heatmaps represent log₂ fold change (with zero time point) of lipid compounds in 1.1 and 2.8-cmdiameter leaf discs 6, 12, 24, 48, 96 and 120 h post cutting. Blue and red boxes represent lower and higher concentrations compared to the control (0 h post cutting). Asterisks indicate significant differences (p < 0.05) between time points and the control (0 h post cutting)



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Fig. 4 Effect of mechanical wounding on stilbene + stilbenoid metabolites during the first 120 h post cutting. *Bars* represent \pm STDEV n = 3. One-way ANOVA followed by Newman-Keuls test was able to determine the differences (*letters in the graph*) over the time points for

each of the two different sets (1.1 and 2.8 cm in diameter); *asterisks* indicate significant differences between different diameter discs: *p value <0.05, **p value <0.01, **p value <0.001 evaluated using the *t* test

compounds in 1.1-cm-diameter leaf discs compared to 2.8cm ones, except for monomers, where we found a similar trend for both types of discs (Fig. 4). The most perturbed compounds we found are alpha-viniferin and ampelopsin H

+ vaticanol C-like isomer, with a maximum increase at 96 h post injury and a concentration of 189.0 and 127.5 mg/kg in 1.1-cm-diameter discs and of 47.5 and 51.3 mg/kg respectively (Table S1). Differences in lipid compounds are

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Fig. 5 Effect of mechanical wounding on lipid compounds during the first 120 h post cutting. *Bars* represent \pm STDEV *n* = 3. One-way ANOVA followed by Newman-Keuls test was able to determine the differences (*letters in the graph*) over the time points for each of the two different sets

reported mostly for fatty acids, glycerolipids and glycerophospholipids. As reported for stilbene compounds, in lipids, we also found greater content increases in 1.1-cmdiameter leaf discs than 2.8-cm-diameter leaf discs, but for

(1.1 and 2.8 cm in diameter); *asterisks* indicate significant differences between different diameter discs: *p value <0.05, **p value <0.01, ***p value <0.001 evaluated using the t test

some of them such as arachidic acid, behenic acid, myristoleic acid, 1-oleoyl-rac-glycerol, glyceryl tripalmitoleate, 1,2-dilinoleoyl-sn-glycero-3-phosphocholine and 1,2-dioleoyl-sn-glycero-3-phospho-rac-(1-glycerol), we



Fig. 6 Effect of mechanical wounding on carotenoid, chlorophyll, xanthophyll and tocopherol compounds during the first 120 h post cutting. *Bars* represent \pm STDEV *n* = 3. One-way ANOVA followed by Newman-Keuls test was able to determine the differences (*letters in the*

found a significant difference in control (zero time point) with a higher basal content in 2.8 cm in diameter discs.

The difference we found in the tocopherol, carotenoid, xanthophyll and chlorophyll classes is mostly due to the higher concentration in 2.8 cm in zero time point diameter discs compared to 1.1 cm; we found no specific differences for the other time points.

Discussion

Plants are usually exposed to several types of stress; in this study, we investigated how mechanical wounding can affect the metabolism of grapevine leaf tissue. To our knowledge, this is the first work which has studied the effect of mechanical wounding in widely used leaf disc experiments on several classes of metabolites.

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graph) over the time points for each of the two different sets (1.1 and 2.8 cm in diameter); *asterisks* indicate significant differences between different diameter discs: *p value <0.05, **p value <0.01, ***p value <0.001 evaluated using the t test

It was confirmed that stilbene and stilbenoid metabolism is affected in response to mechanical wounding in grapevine leaves of the Bianca variety, depending on the dimension of the leaf discs. Stilbenes and stilbenoids in Vitis are constitutively product in different organs, but their most important role is their involvement as phytoalexins against pathogens; indeed, in the presence of biotic stress, their production is induced (Jeandet et al. 2002; Chong et al. 2009). Different derivates of resveratrol have different levels of toxicity for pathogens (Langcake and Pryce 1977; Adrian et al. 1997; Pezet et al. 2004). In our study, we found an accumulation of less toxic molecules belonging to stilbenoid and stilbene classes after abiotic stress. Trans-resveratrol started to accumulate soon after mechanical damage, and after 24 h, its level started to decrease; this compound was then glycosylated into transpiceide, a non-toxic compound, and polymerized into dimer, trimer and tetramer compounds (Fig. 1). Twenty-four hours after injury, an accumulation of Z-miyabenol C, E-cismiyabenol C and ampelopsin D + quadrangularin A occurred. These compounds were identified for the first time in grapevine leaves infected with P. viticola by Mattivi et al. (2011). Their accumulation can be caused by plant-pathogen interaction, but their toxic activity against pathogens has not yet been verified, and likewise after abiotic/mechanical stress, as shown in this study. In other studies, not only stilbene and stilbenoid compounds were shown to be modulated by biotic stress; Dai et al. (1994, 1995) found an accumulation of resveratrol and flavonoids after P. viticola infection that may play an important role in the resistant and intermediate resistant Vitis against the pathogen probably restricting the pathogen development (Dai et al. 1994, 1995). As regards lipids, it has been demonstrated that composition changes in response to wounding in Arabidopsis plants (Vu et al. 2015). Oxylipins, a group of compounds with biological activities such as an antimicrobial effect, regulation of plant cell death and signalling activity (Prost et al. 2005), are formed by polyunsaturated fatty acids; they were de novo synthesized in response to environmental stress including mechanical wounding (Howe and Schilmiller 2002). Production of oxylipins seems to be initiated by phospholipase A2-mediated release of unsaturated fatty acids from membrane lipids (Farmer and Ryan 1992; Dhondt et al. 2000), and it has been demonstrated that phospholipase A2 increases rapidly in response to wounding in tomato leaves (Narváez-Vásquez et al. 1999). Several previous works have focused on the study of jasmonic acid, a fatty acid-derived phytohormone (derived from C18 FA 18:3) known for its role in response to several stresses, in particular to wounding in Arabidopsis (McConn et al. 1997; Creelman

Fig. 7 Randomization scheme for 1.1-cm-diameter leaf discs. One biological replicate was made up of 12 homogeneous plants; the third, fourth and fifth leaves from each plant were detached, and around 20 discs from each leaf were excised. To have sufficient material for metabolic analysis, a minimum of two petri dishes are necessary for each sample. Using 1.1-cmdiameter discs, we were able to have at least one disc from each leaf (for example, red leaf and red discs) in each petri dish

and Mullet 1997). It is also known that precursors of jasmonic acid are able to modulate plant response after biotic and abiotic stress (Kachroo and Kachroo 2009). In our study, we focused on grapevine leaf perturbation due to mechanical wounding investigating different classes of lipids, such as carnitines, sterols, fatty acids, glycerolipids, glycerophospholipids, sphingolipids and prenols. It was shown that besides stilbenoids, also some molecules belonging to the fatty acid class are affected by mechanical stress (Figs. 1, 2 and 3). The increase in specific unsaturated fatty acid compounds such as linoleic acid, linolenic acid and oleic + *cis*-vaccenic acid after mechanical wounding in the first hours post injury allows us to surmise for the first time their role in Bianca grapevine leaves in response to abiotic stress, in particular to mechanical wounding (Fig. 2).

As regards carotenoid and tocopherol metabolites, no significant variations were detected over the time course after mechanical wounding. As regards classes of chlorophylls and their degradation compounds (xanthophylls), we found a decrease during the first 6 h post damage compared to the control (zero time point), especially in 2.8-cm-diameter leaf discs, followed by stabilization (Fig. 6).

Comparing both leaf disc sizes, similar metabolism perturbation was found over the time. Bigger differences in stilbenes and stilbenoids were found in 1.1-cm-diameter discs compared to those 2.8 cm in diameter, with an accumulation of especially some compounds, such as alpha-viniferin, pallidol and ampelopsin H + vaticanol C-like isomer. The differences in the concentrations of these compounds were about three times higher in 1.1-cm leaf discs (Table S1). If we take in consideration circumference/area ratio of leaf discs, we found



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that the value is 2.5 times higher in 1.1-cm disc (3.7) compared to 2.8-cm disc (1.4), and therefore, the damage surface results relatively higher in small discs; these can probably help us to understand the higher accumulation we found for some compounds in 1.1-cm discs. Our results indicate that mechanical wounding induced accumulation over the time of some compounds assuming their implication in response to abiotic stress.

In order to exclude that metabolic modifications observed are also due to the sampling timing, it would be interesting to compare the obtained result on leaf discs with the results obtained on intact leaf in the same experimental conditions. This is unfortunately not feasible since the leaves of the same plant and even more from different plants are very different in the content of metabolites (Vrhovsek et al. 2012a), and it would be impossible to find a proper control sample (intact leaf) which could be compared to the experiment on the randomized leaf disc experiment.

There is no recommendation regarding a size for the disc technique; anyway, despite for some metabolites the cut stress causes higher variability in smaller size discs, they are in any case more commonly used in practice for different reasons. One of them is sampling itself since using 1.1-cm discs, the sampling can be more representative in order to properly perform randomization (Fig. 7). In our experience, 12 plants for each biological replicate were necessary, and from each plant, the third, fourth and fifth leaves were used for the experiment as reported in the previous studies to be most susceptible for the infection (Vrhovsek et al. 2012a). Metabolite composition can be influenced by the leaf age, leaf development stage and leaf position on the shoot; for this reason, we think that it is necessary to perform a good randomization to reduce the undesirable variability. As shown in Fig. 7, using 1.1-cm-diameter disc excision, it was possible to obtain a sufficient number of discs to have at least one disc from a single leaf in each petri dish. In this way, better randomization in terms of the biological replicate over the time course was obtained. For the same experiment applying the excision of bigger discs, it is not possible to obtain a sufficient number of discs from one leaf to cover all the time points. It is also recommended to avoid taking discs from the major veins of the leaf, which cannot be avoided working with 2.8-cm-diameter discs, and this also works in a favour using smaller discs. On the other hand, working with 2.8 cm in diameter leaf discs, we can obtain the necessary amount of samples for the analysis with a faster excision; furthermore, as we have shown in our work, the effect of mechanical wounding on bigger discs is diminished due to the lower ratio between circumference and area, with a smallest damage surface as result.

Many plant-pathogen studies have been performed using leaf disc technique, but for the first time, we focused the attention on metabolic perturbation due to mechanical wounding in grapevine. It was shown that compound accumulation needs to be taken into account when performing metabolic investigation. The results of this study may help in the future application of leaf disc techniques, in order to better understand the effect of abiotic combined with biotic stress, since in this kind of experiment, both types of stress are always involved. These results also show that when carrying out experiments on the disc, it is always necessary to have identical control samples for each time point, in order not to underestimate external factors and other stresses not involved in the research. In this way, the result for each time point and each metabolite is the difference between the absolute value of the treated sample (biotic + abiotic stress) subtracted by the value of the control sample (abiotic stress). Moreover, during metabolic analysis focused on biotic stress, we need to take in consideration the significant differences between infected and control samples for each time point using a t test and not only the changes over the time course to be sure to evaluate only the perturbation we are interested to. Anyway, depending on the purpose of the study, the disc dimension should be chosen, taking into account that each choice is a compromise of pro and cont.

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Supplementary Table 1: Quantification of phenolic compounds in 1.1 cm and 2.8 cm diameter leaf discs; the concentration is represented as mg/Kg of fresh leaves. One way ANOVA followed by Newman-Keuls test was able to determine the differences during the time point for 1.1 cm discs (lowercase letters) and 2.8 cm discs (uppercase letters); asterisks indicate significant differences between different diameter discs p-value<0.05 (*),p-value<0.01 (**), p-value<0.001 (**) evaluated by t-test.

	-																				
	1.1 cm	2.8 cm	t-test	1.1 cm	2.8 cm	t-test	1.1 cm	2.8 cm	t-test	1.1 cm	2.8 cm	t-test	1.1 cm	2.8 cm	t-test	1.1 cm	2.8 cm	t-test	1.1 cm	2.8 cm	t-test
Class Compound	10	рс	_	61	прс		12	hpc		24	l hpc		48	hpc		96	hpc		120	/ hpc	
Benzoic Acid Derivatives																					
gallic acid	2.126 (a)	1.713 (A)	NS	2.077 (a)	1.55 (A)	NS	1.604 (a)	1.722 (A)	NS	1.633 (a)	1.712 (A)	NS	1.644 (a)	1.435 (A)	NS	1.788 (a)	1.228 (A)	NS	1.36 (a)	1.401 (A)	NS
vanillin	0.032 (a)	0.105 (A)	*	0.065 (a)	0.065 (A)	NS	0.039 (a)	0.068 (A)	NS	0.032 (a)	0.054 (A)	NS	0.045 (a)	0.042 (A)	NS	0.021 (a)	0.059 (A)	NS	0.049 (a)	0.028 (A)	NS
vanillic acid	0.009 (a)	0.039 (B)	**	0.013 (a)	0.023 (AB)	NS	0.011 (a)	0.01 (A)	NS	0.021 (a)	0.01 (A)	NS	0.014 (a)	0.014 (A)	NS	0.008 (a)	0.015 (A)	NS	0.008 (a)	0.014 (A)	NS
Coumarins																					
esculin	1.35 (a)	1.406 (A)	NS	1.462 (a)	1.341 (A)	NS	1.417 (a)	1.08 (A)	NS	1.604 (a)	1.161 (A)	NS	1.552 (a)	1.198 (A)	NS	1.464 (a)	1.248 (A)	NS	1.391 (a)	1.198 (A)	NS
Phenylpropanoids																					
		178.371								173.089			172.385				159.529				
trans-coutaric acid	178.297 (a)	(A)	NS	187.603 (a)	178.201 (A)	NS	180.062 (a)	158.013 (A)	NS	(a)	169.767 (A)	NS	(a)	162.847 (A)	NS	159.825 (a)	(A)	NS	168.444 (a)	162.896 (A)	NS
caffeic acid	0.849 (a)	0.96 (A)	NS	0.969 (a)	0.864 (A)	NS	0.707 (a)	0.753 (A)	NS	0.784 (a)	0.844 (A)	NS	0.601 (a)	0.777 (A)	NS	0.614 (a)	0.699 (A)	NS	0.618 (a)	0.612 (A)	NS
caftaric acid	1206.945 (a)	(A)	NS	1251.322 (a)	1228.531 (A)	NS	(a)	11/3.402 (A)	NS	11/4.41/ (a)	1220.218 (A)	NS	(a)	1219.536 (A)	NS	1207.755 (a)	(A)	NS	(a)	(A)	NS
fertaric acid	62 578 (a)	58 46 (A)	NS	(3) 85 485 (a)	60 531 (A)	NS	(°) 76 685 (a)	56 12 (A)	NS	(e) 74 796 (a)	61 338 (A)	NS	79 76 (a)	58 11 (A)	NS	76 896 (a)	57 238 (A)	NS	(3) 77 985 (a)	56 675 (A)	NS
ferulicacid	0.043 (a)	0.034 (A)	NS	0.03 (a)	0.012 (4)	NS	0.043 (a)	0.027 (A)	NS	0.016 (a)	0.018 (A)	NS	0.027 (a)	0.021 (A)	NS	0.013 (a)	0.012 (A)	NS	0.025 (a)	0.015 (A)	NS
sinanic acid	0.043 (a)	0.004 (A)	NS	0.65 (a)	0.012 (A)	NS	0.558 (b)	0.027 (A)	NS	0.010 (a)	0.010(A)	NS	0.027 (a)	0.021 (A)	NS	0.015 (a)	0.374 (A)	NS	0.511 (ab)	0.019 (A)	NS
Dihydrochalcones	0.401 (u)	0.401 (A)	115	0.400 (00)	0.450 (A)	113	0.550 (6)	0.425 (A)	113	0.404 (00)	0.401 (A)	115	0.41 (u)	0.434 (74)	115	0.411 (u)	0.574 (A)	113	0.511(05)	0.405 (A)	113
phlorizin	2 455 (a)	2 423 (A)	NS	2 735 (a)	2 523 (A)	NS	2 726 (a)	2 257 (A)	NS	2 934 (a)	2 66 (A)	NS	2 875 (a)	2 509 (A)	NS	2 418 (a)	2 76 (A)	NS	2 95 (a)	2 529 (A)	NS
Elavonas	2.455 (d)	2.425 (A)	115	2.755 (0)	2.525 (A)	113	2.720 (0)	2.257 (A)	113	2.554 (0)	2.00 (A)	115	2.075 (0)	2.505 (A)	115	2.410 (u)	2.70 (A)	113	2.55 (u)	2.525 (A)	113
luteolin 7.0 glucocide	0.11 (5)	0.100 (A)	MC	0.105 (5)	0.110 (A)	NC	0.105 (5)	0.094 (A)	NC	0.007 (a)	0.105 (A)	NC	0.002 (5)	0.105 (A)	NC	0 102 (5)	0.112 (A)	NC	0.106 (5)	0.095 (A)	NC
Flavan-3-ols	0.11 (a)	0.105 (A)	145	0.105 (a)	0.115 (A)	145	0.105 (a)	0.034 (A)	145	0.0 <i>37</i> (a)	0.105 (A)	145	0.055 (a)	0.105 (A)	145	0.102 (8)	0.112 (A)	145	0.100 (a)	0.035 (A)	145
catechin	2 642 (2)	2 604 (A)	MC	2 255 (2)	2 256 (A)	NC	2 028 (5)	2 402 (A)	NC	2 /01 (5)	4.019 (A)	NC	2 468 (5)	5 10 (A)	NC	2 274 (2)	2 /21 //)	NC	2 722 (2)	2 247 (A)	NC
catechin	2.042 (d)	1.257 (A)	NC	5.255 (a)	5.230 (A)	NS	5.056 (d)	5.495 (A)	NS	5.491 (d)	4.019 (A)	NS	5.400 (d)	5.19 (A)	NC	5.274 (d)	5.451 (A)	NS	5.722 (d)	5.547 (A)	NS
epicatechin gallate	1.1 (d)	1.557 (A)	NC	0.973 (a)	1.115 (A)	NS	1.411 (d)	1.214 (A)	NS	0.07 (d)	1.461 (A)	NS	1.541 (d)	1.096 (A)	NC	1.570 (d)	1.009 (A)	NS	1.550 (a)	1.514 (A)	NS
epicatechin galate	1.724 (d)	1.951 (A)	NC	1.654 (d)	1.957 (A)	NS	2.409 (d)	2.341 (A)	NS	2.307 (d)	2.075 (A)	NS	1.900 (d)	1.995 (A)	NC	2.592 (a)	2.107 (A)	NS	2.097 (a)	2.202 (A)	NS
epiganocatechin	2.917 (a)	3.338 (A)	NS NC	3.112 (a)	2.92 (A)	NS	3.009 (a)	3.145 (A)	NS	Z.722 (d)	3.267 (A)	NS	2.921 (a)	3.282 (A)	NS	2.608 (a)	2.99 (A)	NS	2.041 (a)	2.7 (A)	/NS *
epiganocatecrini ganate	5.292 (a)	4.000 (A)	NS NC	5.057 (a)	5.198 (A)	NS	5.995 (d)	5.441 (A)	NS	5.063 (a)	5.147 (A)	NS	5.594 (a)	5.440 (A)	NS	5.479 (a)	5.325 (A)	NS	5.242 (a)	4.976 (A)	
galocatechin	10.909 (a)	11.831 (A)	NS NC	13.771 (a)	11.061 (A)	NS	12.///(d)	11.322 (A)	NS	11.298 (a)	10.941 (A)	NS	10.797 (a)	10.227 (A)	NS	10.049 (a)	10.314 (A)	NS	11.333 (a)	9.347 (A)	NS NC
	15.901 (a)	22.829 (A)	NS	19.041 (a)	21.383 (A)	NS	19.879 (a)	19.756 (A)	NS	20.063 (a)	24.008 (A)	NS	20.785 (a)	20.933 (A)	NS	17.249 (a)	25.562 (A)	NS	21.288 (a)	23.303 (A)	NS NG
procyanidin B2 + B4 (as B2)	4.286 (a)	4.941 (A)	NS	4.259 (a)	4.64 (A)	NS	4.824 (a)	4.805 (A)	NS	5.380 (d)	4.945 (A)	NS	5.259 (a)	5.343 (A)	NS	3.009 (a)	5.692 (A)	NS *	4.622 (a)	5.572 (A)	NS NG
procyanidin B3 (as B1)	1.294 (a)	1.395 (A)	NS	1.123 (a)	1.547 (A)	NS	1.48 (a)	1.567 (A)	NS	1.344 (a)	1.852 (A)	NS	1.36 (a)	1.972 (A)	NS	1.143 (a)	2.417 (A)	•	1.726 (a)	1.303 (A)	NS
Flavonois	0.000	0.004 (4)		0.554 (-1-)	0.040(4)		0.040(.1.)	0.500 (4)	**	0.046 (.1.)	0.005 (4)		0.004 (.1.)	0 704 (4)		4 000 (11)	0.705 (4)		4 400 (1)	0.04 (4)	
isornamnetin-3-O-glucoside	0.644 (a)	0.621 (A)	NS	0.551 (ab)	0.616 (A)	NS	0.848 (ab)	0.598 (A)		0.946 (ab)	0.665 (A)	NS	0.981 (ab)	0.731 (A)	NS	1.033 (ab)	0.705 (A)	NS	1.488 (D)	0.91 (A)	NS
Isornamnetin-3-rutinoside	0.814 (a)	1.033 (A)	NS	0.937 (a)	1.062 (A)	NS	0.899 (a)	0.922 (A)	NS *	1.126 (a)	0.905 (A)	NS	0.935 (a)	1.011 (A)	NS	1.029 (a)	0.881 (A)	NS	0.951 (a)	0.971 (A)	NS
kaempferol-3-O-glucoside	1.874 (a)	2.209 (A)	NS	2.166 (a)	1.992 (A)	NS	2.336 (a)	1.704 (A)	*	2.693 (a)	2.118 (A)	NS	2.238 (a)	1.956 (A)	NS	2.084 (a)	2.046 (A)	NS	2.246 (a)	2.094 (A)	NS
kaempferol-3-O-glucuronide	37.254 (a)	39.716 (A)	NS	46.288 (a)	37.229 (A)	NS	45.26 (a)	31.562 (A)	**	47.926 (a)	38.194 (A)	NS	46.401 (a)	34.435 (A)	NS	41.701 (a)	34.28 (A)	NS	43.957 (a)	37.375 (A)	NS
kaempferol-3-O-rutinoside	0.543 (b)	0.79 (A)	NS	0.723 (ab)	0.688 (A)	NS	0.892 (a)	0.627 (A)	NS	0.94 (a)	0.731 (A)	•	0.792 (ab)	0.749 (A)	NS	0.73 (ab)	0.652 (A)	NS	0.836 (ab)	0.714 (A)	NS
myricetin quarcatin-2-alucosida+quarcatin-2-aalactosida (as qua-2-	10.027 (a)	9.887 (A)	NS	10.834 (a)	10.712 (A)	NS	10.999 (a)	11.054 (A)	NS	10.3 (a)	10.825 (A)	NS	10.642 (a)	11.395 (A)	NS	6.961 (a)	10.975 (A)	NS	10.537 (a)	10.391 (A)	NS
glc)	21.762 (b)	25.102 (A)	NS	28.072 (a)	25.15 (A)	NS	30.382 (a)	21.649 (A)	**	32,273 (a)	25.099 (A)	*	31,424 (a)	23.808 (A)	NS	28.634 (a)	24.674 (A)	NS	32.764 (a)	25.651 (A)	*
guercetin-3-O-glucoside-arabinoside	0.153 (a)	0.246 (A)	NS	0.247 (a)	0.19 (A)	NS	0.231 (a)	0.132 (A)	NS	0.307 (a)	0.253 (A)	NS	0.275 (a)	0.139 (A)	NS	0.185 (a)	0.204 (A)	NS	0.231 (a)	0.24 (A)	NS
	0.200 (0)			825.844						868.634	0.200 ()		851.071	01200 (11)		0.200 (0)	742.961		(-)		
quercetin-3-O-glucuronide	734.293 (b)	729.58 (A)	NS	(ab)	739.492 (A)	NS	848.788 (a)	729.888 (A)	NS	(a)	774.825 (A)	NS	(a)	715.364 (A)	NS	788.2 (ab)	(A)	*	819.84 (ab)	748.526 (A)	NS
rutin	4.523 (b)	5.428 (A)	NS	6.139 (a)	5.316 (A)	NS	6.183 (a)	5.111 (A)	NS	6.526 (a)	5.522 (A)	NS	6.253 (a)	5.082 (A)	NS	5.882 (a)	5.117 (A)	NS	6.204 (a)	5.365 (A)	NS
taxifolin	0.113 (a)	0.214 (A)	NS	0.307 (a)	0.294 (A)	NS	0.218 (a)	0.377 (A)	NS	0.119 (a)	0.239 (A)	*	0.248 (a)	0.277 (A)	NS	0.132 (a)	0.187 (A)	NS	0.154 (a)	0.19 (A)	NS
Stilbenes + Stilbenoids																					
α-viniferin	ND	ND	NS	ND	ND	NS	ND	ND	NS	ND	0.93 (A)	NS	20.216 (a)	6.322 (A)	**	189.065 (c)	47.484 (B)	**	136.476 (b)	60.448 (B)	**
<i>cis</i> -piceide	3.803 (ab)	5.987 (A)	NS	4.588 (b)	6.784 (A)	NS	3.437 (ab)	4.035 (A)	NS	2.89 (ab)	5.25 (A)	NS	2.983 (ab)	5.017 (A)	NS	2.796 (ab)	4.506 (A)	NS	2.436 (a)	4.204 (A)	NS
<i>cis</i> + <i>trans</i> -ω-viniferin	ND	ND	NS	ND	ND	NS	ND	ND	NS	0.031 (a)	0.038 (A)	NS	0.182 (a)	0.236 (A)	NS	1.004 (a)	0.337 (A)	NS	0.945 (a)	0.174 (A)	NS

	<i>trans</i> -ε-viniferin	ND	ND	NS	ND	0.069 (A)	NS	ND	0.036 (A)	NS	1.035 (a)	2.127 (AB)	NS	5.289 (ab)	6.516 (B)	NS	10.12 (b)	4.261 (AB)	NS	10.521 (b)	3.503 (AB)	NS
	trans-piceide	0.744 (a)	1.72 (A)	**	1.197 (a)	2.023 (A)	NS	1.68 (a)	1.991 (A)	NS	1.679 (a)	3.358 (AB)	**	2.818 (c)	4.045 (B)	NS	3.991 (b)	4.315 (B)	NS	4.692 (b)	4.576 (B)	NS
	trans-resveratrol	0.11 (b)	0.163 (C)	NS	0.552 (b)	1.291 (AB)	*	1.036 (a)	1.822 (A)	*	1.278 (a)	1.868 (A)	NS	1.651 (a)	1.653 (A)	NS	1.265 (a)	1.094 (AB)	NS	1.636 (a)	0.569 (BC)	**
	ampelopsin D + quadrangularin A	ND	ND	NS	ND	ND	NS	ND	ND	NS	0.157 (a)	0.262 (AB)	NS	0.453 (a)	0.597 (B)	NS	1.213 (b)	0.621 (B)	NS	1.486 (b)	0.493 (AB)	*
	ampelopsin H + vaticanol C-like isomer	ND	ND	NS	ND	ND	NS	ND	ND	NS	2.862 (a)	5.945 (A)	***	32.484 (a)	27.399 (AB)	NS	127.518 (b)	51.353 (B)	*	119.18 (b)	54.683 (B)	*
	astringin	0.042 (a)	0.079 (A)	NS	0.31 (ab)	0.332 (A)	NS	0.342 (b)	0.312(A)	NS	0.12 (ab)	0.254 (A)	**	0.14 (ab)	0.245 (A)	NS	0.25 (ab)	0.274 (A)	NS	0.203 (ab)	0.252 (A)	NS
	E-cis-miyabenol	ND	ND	NS	ND	ND	NS	ND	ND	NS	0.095 (a)	0.041 (A)	NS	1.745 (a)	0.846 (A)	NS	6.816 (b)	2.064 (A)	NS	4.992 (b)	1.698 (A)	NS
	isohopeaphenol	ND	ND	NS	ND	ND	NS	ND	ND	NS	0.572 (a)	1.448 (A)	NS	6.735 (a)	7.765 (AB)	NS	28.315 (b)	11.637 (B)	NS	28.486 (b)	12.195 (B)	NS
	isorhapontin	0.079 (a)	0.241 (B)	**	0.166 (a)	0.294 (B)	NS	0.27 (a)	0.327(B)	NS	0.211 (a)	0.544 (A)	**	0.421 (ab)	0.613 (A)	NS	0.444 (ab)	0.57 (A)	NS	0.674 (b)	0.616 (A)	NS
	pallidol	ND	ND	NS	ND	ND	NS	ND	0.523 (A)	NS	3.572 (a)	2.629 (AC)	NS	10.014 (b)	7.206 (B)	NS	14.782 (b)	6.219 (B)	*	15.69 (b)	4.497 (BC)	*
	Z-miyabenol C	ND	ND	NS	ND	ND	NS	ND	ND	NS	ND	ND	NS	1.335 (a)	0.689 (AB)	NS	6.838 (c)	1.637 (B)	NS	4.225 (b)	1.555 (B)	NS
Other.	5																					
	arbutin	1.026 (a)	1.488 (AB)	**	1.026 (a)	1.278 (A)	NS	1.046 (a)	1.332 (A)	NS	1.196 (a)	1.501 (AB)	**	1.445 (c)	1.645 (AB)	NS	2.389 (b)	1.946 (B)	**	2.354 (b)	1.902 (B)	NS
	.										110.043			101.426								
	caffeic acid+catechin condensation product	88.996 (a)	97.433 (A)	NS	100.367 (a)	97.257 (A)	NS	105.49 (a)	78.991 (A)	NS	(a)	99.623 (A)	NS	(a)	85.377 (A)	NS	97.111 (a)	80.008 (A)	*	97.563 (a)	87.376 (A)	NS

Supplementary Table 1: Quantification of lipids compounds in 1.1 cm and 2.8 cm diameter leaf discs; the concentration is represented as mg/Kg of fresh leaves. One way ANOVA followed by Newman-Keuls test was able to determine the differences during the time point for 1.1 cm discs (lowercase letters) and 2.8 cm discs (uppercase letters); asterisks indicate significant differences between different diameter discs p-value<0.05 (*), p-value<0.01 (**), p-value<0.001 (**) evaluated by t-test.

	1.1 cm	2.8 cm	t-test	1.1 cm	2.8 cm	t-test	1.1 cm	2.8 cm	t-test	1.1 cm	2.8 cm	t-test	1.1 cm	2.8 cm	t-test	1.1 cm	2.8 cm	t-test	1.1 cm	2.8 cm	t-test
Class Compound	0	hpc	-	6	6 hpc		12 hpc		-	24	hpc		48	прс		96	hpc		120	hpc	
Carnitines																					
Palmitoyl-L-carnitine hydrochloride	0.02 (a)	0.020 (A)	NS	0.018 (a)	0.018 (A)	NS	0.019 (a)	0.021 (A)	NS	0.02 (a)	0.023 (A)	NS	0.021 (a)	0.018 (A)	NS	0.02 (a)	0.019 (A)	NS	0.020 (a)	0.020 (A)	NS
Sterols																					
Desmosterol	0.292 (a)	0.427 (A)	NS	0.298 (ab)	0.332 (A)	NS	0.325 (ab)	0.389 (A)	NS	0.348 (ab)	0.392 (A)	NS	0.368 (b)	0.449 (A)	NS	0.34 (ab)	0.413 (A)	NS	0.315 (ab)	0.362 (A)	NS
Ergosterolo	1.181 (a)	1.177 (A)	NS	0.955 (a)	0.278 (A)	NS	0.327 (a)	0.366 (A)	NS	0.377 (a)	0.381 (A)	NS	0.454 (a)	0.338 (A)	**	0.699 (a)	0.581 (A)	NS	1.158 (a)	0.582 (A)	NS
Uvaol	0.524 (a)	1.575 (A)	NS	0.507 (a)	1.354 (A)	NS	0.711 (a)	1.634 (A)	NS	1.064 (ab)	2.11 (A)	NS	1.699 (bc)	2.38 (A)	NS	2.004 (c)	2.052 (A)	NS	1.745 (bc)	2.542 (A)	NS
Fatty acids																					
Arachidic acid	0.952 (b)	1.554 (A)	*	1.187 (ab)	1.619 (A)	NS	1.626 (a)	1.418 (A)	NS	1.356 (ab)	1.45 (A)	NS	1.491 (ab)	1.067 (A)	NS	1.257 (ab)	1.128 (A)	NS	1.614 (a)	1.202 (A)	NS
Behenic acid	1.846 (a)	2.961 (A)	*	3.034 (a)	3.502 (A)	NS	3.563 (a)	3.414 (A)	NS	2.654 (a)	3.204 (A)	NS	3.165 (a)	2.951 (A)	NS	3.258 (a)	2.744 (A)	NS	3.804 (a)	3.119 (A)	NS
Erucic acid	0.055 (a)	0.052 (A)	NS	0.049 (a)	0.047 (A)	NS	0.053 (a)	0.047 (A)	NS	0.047 (a)	0.051 (A)	NS	0.059 (a)	0.032 (A)	***	0.061 (a)	0.029 (A)	*	0.075 (a)	0.033 (A)	***
Heptadecanoic acid	0.185 (ab)	0.175 (A)	NS	0.18 (ab)	0.165 (A)	NS	0.34 (c)	0.108 (A)	*	0.028 (abc)	0.132 (A)	**	0.286 (bc)	0.127 (A)	**	0.154 (a)	0.065 (A)	NS	0.22 (ab)	0.082 (A)	**
Lignoceric acid	3.936 (a)	5.305 (A)	NS	4.654 (a)	6.392 (A)	NS	6.378 (a)	6.213 (A)	NS	5.259 (a)	6.032 (A)	NS	5.538 (a)	6.207 (A)	NS	5.952 (a)	4.931 (A)	NS	6.155 (a)	5.971 (A)	NS
Linoleic acid	0.18 (c)	0.228 (AB)	NS	0.65 (ac)	0.532 (AB)	NS	1.048 (ab)	0.535 (AB)	**	1.124 (ab)	0.754 (B)	NS	1.301 (b)	0.578 (AB)	**	0.634 (ac)	0.295 (AB)	NS	0.893 (ab)	0.177 (A)	*
Linolenic acid	1.456 (a)	1.219 (A)	NS	4.284 (abc)	3.309 (ABC)	NS	6.034 (c)	3.739 (C)	NS	5.611 (bc)	3.571 (BC)	NS	4.28 (abc)	2.86 (ABC)	NS	1.867 (a)	1.878 (ABC)	NS	2.872 (ab)	1.41 (AB)	NS
Myristic acid	0.874 (c)	0.646 (A)	NS	0.506 (ab)	0.713 (A)	NS	0.829 (bc)	0.867 (A)	NS	0.724 (abc)	0.71 (A)	NS	0.538 (abc)	0.32 (A)	NS	0.378 (a)	0.295 (A)	NS	0.45 (bc)	0.449 (A)	NS
Myristoleic acid	0.065 (a)	0.047 (C)	*	0.059 (a)	0.029 (ABC)	NS	0.039 (a)	0.026 (ABC)	NS	0.028 (a)	0.035 (BC)	NS	0.065 (a)	0.01 (AB)	NS	0.01 (a)	0.003 (A)	NS	0.018 (a)	0.003 (A)	NS
Oleic acid+cis-Vaccenic acid	0.928 (a)	0.705 (A)	NS	2.159 (ab)	2.196 (A)	NS	3.672 (b)	1.664 (A)	NS	2.928 (ab)	1.852 (A)	NS	3.935 (b)	1.535 (A)	***	1.193 (a)	0.762 (A)	NS	1.971 (ab)	1.766 (A)	NS
Palmitic acid	9.253 (a)	12.049 (A)	NS	13.941 (a)	9.768 (A)	NS	32.532 (b)	10.466 (A)	NS	19.111 (ab)	11.54 (A)	*	24.379 (ab)	11.096 (A)	*	9.034 (a)	6.962 (A)	NS	15.49 (a)	10.174 (A)	NS
Palmitoleic acid	0.136 (a)	0.078 (A)	NS	0.208 (a)	0.218 (A)	NS	0.232 (a)	0.164 (A)	NS	0.155 (a)	0.152 (A)	NS	0.173 (a)	0.1 (A)	NS	0.087 (a)	0.077 (A)	NS	0.09 (a)	0.057 (A)	NS
Stearic acid	6.916 (a)	8.657 (B)	NS	7.507 (a)	2.676 (A)	*	25.84 (b)	3.389 (A)	NS	9.409 (a)	3.171 (A)	NS	12.768 (a)	3.663 (A)	*	2.439 (a)	0.705 (A)	*	7.483 (a)	2.985 (A)	**
Glycerolipids																		NS			
1,2,3-Tripentadecanoylglycerol	0.077 (a)	0.025 (A)	NS	0.055 (a)	0.018 (A)	*	0.065 (a)	0.014 (A)	*	0.064 (a)	0.026 (A)	NS	0.057 (a)	0.013 (A)	*	0.025 (a)	0.007 (A)	NS	0.033 (a)	0.01 (A)	NS
1-Oleoyl-rac-glycerol	0.097 (ab)	0.232 (A)	*	0.165 (ab)	0.192 (A)	NS	0.113 (ab)	0.249 (A)	*	0.221 (b)	0.225 (A)	NS	0.085 (ab)	0.088 (B)	NS	< LOQ	< LOQ	NS	< LOQ	< LOQ	NS
Glyceryl trioleate	0.939 (ab)	0.764 (A)	NS	0.81 (b)	1.085 (A)	*	1.061 (ab)	1.039 (A)	NS	1.244 (a)	1.074 (A)	NS	1.021 (ab)	0.857 (A)	NS	1.141 (ab)	0.902 (A)	NS	1.226 (a)	0.962 (A)	NS
Glyceryl tripalmitoleate	0.098 (a)	0.033 (A)	*	0.054 (a)	0.025 (A)	NS	0.085 (a)	0.028 (A)	NS	0.076 (a)	0.037 (A)	NS	0.089 (a)	0.017 (A)	*	0.053 (a)	0.02 (A)	*	0.046 (a)	0.014 (A)	NS
Glycerophospholipids																					
1,2-Dilinoleoyl-sn-glycero-3-phosphocholine	31.713 (a)	35.373 (A)	*	33.594 (ab)	35.325 (A)	NS	40.775 (ab)	38.175 (A)	NS	40.907 (ab)	43.677 (A)	NS	41.457 (ab)	36.666 (A)	NS	46.902 (b)	42.241 (A)	NS	43.074 (ab)	48.957 (A)	NS
1,2-Dioleoyl-sn-glycero-3-phosphocholine 1,2-Dioleoyl-sn-glycero-3-phospho-rac-(1-glycerol)sodium	37.401 (a)	41.656 (A)	NS	36.856 (a)	36.023 (A)	NS	35.684 (a)	40.076 (A)	NS	36.294 (a)	38.861 (A)	NS	34.623 (a)	34.244 (A)	NS	34.455 (a)	31.26 (A)	NS	29.757 (a)	30.783 (A)	NS
salt	0.15 (a)	0.253 (A)	*	0.141 (a)	0.223 (A)	NS	0.143 (a)	0.235 (A)	*	0.152 (a)	0.24 (A)	NS	0.156 (a)	0.178 (A)	NS	0.227 (a)	0.224 (A)	NS	0.193 (a)	0.198 (A)	NS
1-Palmitoyl-sn-glycero-3-phosphocholine	1.604 (ab)	1.815 (B)	NS	1.16 (a)	1.255 (A)	NS	1.755 (b)	1.462 (AB)	**	1.477 (ab)	1.221 (A)	NS	1.540 (ab)	1.262 (A)	NS	1.235 (a)	1.237 (A)	NS	1.16 (a)	1.473 (AB)	NS
Sphingolipids																					
Ceramide	0.023 (a)	0.019 (A)	NS	0.023 (a)	0.023 (A)	NS	0.019 (a)	0.016 (A)	NS	0.043 (a)	0.018 (A)	NS	0.026 (a)	0.017 (A)	*	0.03 (a)	0.024 (A)	NS	0.026 (a)	0.02 (A)	NS
Prenols																					
Oleanolic acid	1.267 (a)	2.784 (A)	NS	2.571 (a)	3.112 (AB)	NS	1.919 (a)	4.258 (AB)	*	2.175 (a)	3.865 (AB)	*	4.802 (c)	4.795 (B)	NS	13.088 (b)	7.641 (C)	**	11.431 (b)	10.26 (D)	NS

Supplementary Table 1: Quantification of carotenoid, chlorophyll, xantophyll and tocopherol compounds in 1.1 cm and 2.8 cm diameter leaf discs; the concentration is represented as mg/Kg of fresh leaves. One way ANOVA test was able to determine the differences during the time point for 1.1 cm discs (lowercase letters) and 2.8 cm discs (uppercase letters); asterisks indicate significant differences between different diameter discs p-value<0.05 (*),p-value<0.001 (***) evaluated by t-test.

	1.1 cm	2.8 cm	t-test	1.1 cm	2.8 cm	t-test	1.1 cm	2.8 cm	t-test	1.1 cm	2.8 cm	t-test	1.1 cm	2.8 cm	t-test	1.1 cm	2.8 cm	t-test	1.1 cm	2.8 cm	t-test
	01	hpc		6	hpc		12	hpc	-	24	hpc	-	48	hpc		96	hpc	_	120	hpc	_
Class Compound			-						-			-			-			-			-
Tocopherols																					
α Tocopherol	272.318 (a)	239.583 (A)	NS	248.702 (a)	171.547 (A)	NS	245.416 (a)	152.861 (A)	NS	336.019 (a)	213.074 (A)	NS	270.345 (a)	169.844 (A)	NS	304.063 (a)	181.499 (A)	NS	310.777 (a)	199.773 (A)	NS
γ Tocopherol	31.645 (a)	47.755 (A)	NS	37.672 (a)	39.753 (A)	NS	33.313 (a)	41.786 (A)	*	35.607 (a)	44.664 (A)	NS	30 (a)	35.281 (A)	NS	31.66 (a)	35.113 (A)	NS	36.962 (a)	36.714 (A)	NS
Carotenoids																					
α Carotene	14.488 (ab)	23.019 (A)	**	13.617 (ab)	18.711 (A)	NS	13.657 (ab)	15.439 (A)	NS	15.6 (a)	19.726 (A)	NS	10.708 (b)	15.146 (A)	NS	15.032 (a)	15.559 (A)	NS	13.546 (ab)	14.208 (A)	NS
β Carotene	127.152 (a)	127.199 (A)	NS	109.309 (a)	100.198 (A)	NS	109.646 (a)	84.604 (A)	NS	146.073 (a)	114.887 (A)	NS	99.853 (a)	91.629 (A)	NS	137.981 (a)	96.846 (A)	NS	122.531 (a)	92.011 (A)	NS
Phytoene	114.073 (a)	110.538 (A)	NS	100.66 (a)	54.782 (A)	NS	90.584 (a)	49.584 (A)	NS	111.47 (a)	75 (A)	NS	72.431 (a)	64.044 (A)	NS	89.466 (a)	61.136 (A)	NS	78.26 (a)	63.961 (A)	NS
Xanthophyls																					
Lutein	215.293 (a)	251.836 (B)	NS	182.678 (a)	181.828 (B)	NS	188.184 (a)	161.907 (A)	NS	207.505 (a)	205.806 (AB)	NS	163.08 (a)	181.324 (A)	NS	199.979 (a)	164.358 (A)	NS	188.871 (a)	160.515 (A)	NS
Neoxanthin	105.265 (a)	139.309 (B)	*	91.27 (a)	95.547 (A)	NS	96.185 (a)	82.637 (A)	NS	97.115 (a)	94.334 (A)	NS	80.491 (a)	92.734 (A)	NS	94.791 (a)	91.801 (A)	NS	101.241 (a)	80.889 (A)	*
Chlorophylls																					
	1128.057	1119.233														1188.498					
Chloropyll a	(a)	(A)	NS	956.242 (a)	861.745 (A)	NS	934.498 (a)	756.427 (A)	NS	1188.056 (a)	915.999 (A)	NS	860.122 (a)	822.042 (A)	NS	(a)	839.078 (A)	NS	1036.986 (a)	734.455 (A)	NS
Chloropyll b Chloropyll a epimer as chlorophyll	524.579 (a)	736.503 (B)	**	423.728 (a)	535.201 (A)	NS	450.932 (a)	459.008 (A)	NS	517.864 (a)	550.419 (A)	NS	379.171 (a)	484.709 (A)	NS	483.035 (a)	450.632 (A)	NS	426.945 (a)	419.309 (A)	NS
а	119.049 (a)	123.237 (A)	NS	103.640 (a)	123.261 (A)	NS	110.036 (a)	126.123 (A)	NS	124.291 (a)	145.240 (A)	NS	102.192 (a)	141.820 (A)	NS	118.946 (a)	108.027 (A)	NS	94.954 (a)	116.838 (A)	NS
Chloropyll b epimer as chlorophyll																					
b	121.049 (a)	163.667 (A) 1112.644	•	96.199 (a)	140.217 (A)	NS	108.516 (a)	125.466 (A)	NS	117.195 (a)	153.629 (A)	NS	89.262 (a)	138.861 (A)	•	112.743 (a)	124.129 (A)	NS	94.298 (a)	114.181 (A)	NS
Pheophytin a as chlorophyll a	709.173 (a)	(B)	*	504.04 (a)	717.745 (A)	NS	585.399 (a)	645.095 (A)	NS	575.317 (a)	747.862 (A)	NS	414.312 (a)	674.549 (A)	**	496.96 (a)	586.256 (A)	NS	435.295 (a)	556.052 (A)	NS
Pheophytin a' as chlorophyll a	126.972 (b)	190.202 (B)	NS	87.361 (ab)	128.432 (A)	NS	102.247 (ab)	114.636 (A)	NS	104.074 (ab)	144.216 (AB)	NS	75.586 (a)	125.807 (A)	**	92.176 (ab)	106.061 (A)	NS	78.692 (ab)	99.995 (A)	NS
Pheophytin b as chlorophyll b	22.118 (a)	40.351 (B)	**	15.384 (a)	29.203 (A)	*	18.721 (a)	22.499 (A)	NS	19.488 (a)	29.949 (A)	NS	14.337 (a)	25.429 (A)	**	16.785 (a)	21.872 (A)	NS	15.025 (a)	20.603 (A)	*
Pheophytin b' as chlorophyll b	2.916 (a)	5.362 (B)	*	1.906 (a)	4.007 (AB)	NS	2.572 (a)	3.192 (A)	NS	2.229 (a)	4.349 (AB)	*	1.801 (a)	3.672 (AB)	**	2.078 (a)	2.988 (A)	NS	1.676 (a)	2.876 (A)	*

Preface to Chapter IV



The 'Bianca' grapevine cultivar is a Hungarian hybrid, obtained by crossing Bouvier and the resistant grapevine Villard Blanc in 1963 in the Eger wine region of north-east Hungary. It is cultivated principally in Hungary, but some vineyards with this variety can be found in the Russian wine region of Krasnodar Krai and in Moldavia. The grape was officially registered for use in wine production in 1982 and is used today to make a wide assortment of wines, from dry to sweet dessert wines. It shows good resistance both to downy and powdery mildew and has very good tolerance to frost. A major QTL, named *Rpv3*, has been found to account for Bianca's partial resistance to downy mildew; for this reason Bianca is popular among organic vine growers. The grape clusters of Bianca tend to be medium-sized to very large, but the berries always tend to be small, with a noticeable waxy coating.

In this work we monitored metabolite changes in leaf discs of the resistant variety Bianca after inoculation with a suspension of *Plasmopara viticola*, with the aim of discovering biomarkers for specific stages of the host defence. In particular we evaluated primary and secondary metabolism at 12, 24, 48 and 96 hours post inoculation. We used existing protocols of LC-MS/MS for identification and quantification of lipids and phenols, and GC-MS for identification and semi-quantification of volatile organic compounds (VOCs). Moreover, we validated a new GC-MS protocol for the identification and quantification of primary compounds.

The method was successfully applied to Bianca leaf discs dataset with the identification and quantification of 48 metabolites. This work highlighted some important

aspects of the host response to *P. viticola* in a commercial variety under controlled conditions, providing biomarkers for a better understanding of the mechanism of plant defense and a potential application in field studies of resistant varieties. Our results gave a picture of plant metabolome perturbation with the finding of 53 molecules perturbed in Bianca leaf discs after *P. viticola* inoculation compared to the control. These compounds could be a potential biomarkers in Bianca variety after its infection with *P. viticola*. In general we found a primary metabolism perturbation during the first 24-48 hours post infection and a later modification on metabolites belonging to secondary metabolism at 48-96 hours post infection.

My personal contribution to this work started with the experimental design; I personally performed the experiment, the extraction and analysis of compounds and the data processing. I was also involved in the method validation of primary metabolites. I was responsible for writing the manuscript and managing the comments and improvements to the text by other authors.

Information and images of Bianca grape adaxial and abaxial leaf surfaces were retrieved from the *Vitis International Variety Catalogue – VIVC.*

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Chapter IV

Identification of biomarkers for defense response to *Plasmopara viticola* in a resistant grape variety





Identification of Biomarkers for Defense Response to *Plasmopara viticola* in a Resistant Grape Variety

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Chitarrini G, Soini E, Riccadonna S, Franceschi P, Zulini L, Masuero D, Vecchione A, Stefanini M, Di Gaspero G, Mattivi F and Vrhovsek U (2017) Identification of Biomarkers for Defense Response to Plasmopara viticola in a Resistant Grape Variety. Front. Plant Sci. 8:1524. doi: 10.3389/fpls.2017.01524 Downy mildew (Plasmopara viticola) is one of the most destructive diseases of the cultivated species Vitis vinifera. The use of resistant varieties, originally derived from backcrosses of North American Vitis spp., is a promising solution to reduce disease damage in the vineyards. To shed light on the type and the timing of pathogen-triggered resistance, this work aimed at discovering biomarkers for the defense response in the resistant variety Bianca, using leaf discs after infection with a suspension of P. viticola. We investigated primary and secondary metabolism at 12, 24, 48, and 96 h post-inoculation (hpi). We used methods of identification and quantification for lipids (LC-MS/MS), phenols (LC-MS/MS), primary compounds (GC-MS), and semi-quantification for volatile compounds (GC-MS). We were able to identify and quantify or semiquantify 176 metabolites among which 53 were modulated in response to pathogen infection. The earliest changes occurred in primary metabolism at 24-48 hpi and involved lipid compounds, specifically unsaturated fatty acid and ceramide; amino acids, in particular proline; and some acids and sugars. At 48 hpi, we also found changes in volatile compounds and accumulation of benzaldehyde, a promoter of salicylic acid-mediated defense. Secondary metabolism was strongly induced only at later stages. The classes of compounds that increased at 96 hpi included phenylpropanoids, flavonols, stilbenes, and stilbenoids. Among stilbenoids we found an accumulation of ampelopsin H + vaticanol C, pallidol, ampelopsin D + quadrangularin A, Z-miyabenol C, and a-viniferin in inoculated samples. Some of these compounds are known as phytoalexins, while others are novel biomarkers for the defense response in Bianca. This work highlighted some important aspects of the host response to P. viticola in a commercial variety under controlled conditions, providing biomarkers for a better understanding of the mechanism of plant defense and a potential application in field studies of resistant varieties.

Keywords: biomarkers, Plasmopara viticola, hybrid, plant pathogen, Bianca, grapevine, resistance, metabolomics

INTRODUCTION

Downy mildew is one of the most destructive diseases of the grapevine caused by the biotrophic oomycete *Plasmopara viticola* (Berk. and Curt.) Berl. & de Toni. This pathogen was introduced from North America into Europe in the second half of the nineteenth century. The cultivated species *Vitis vinifera* is susceptible to *P. viticola*. Disease management strategies rely on the use of fungicides potentially harmful to humans and the environment (Negatu et al., 2016; Christen et al., 2017; Rortais et al., 2017). In some situations, chemical protection is also economically challenging, due to the costs of synthetic fungicides and the labor involved in spraying.

The pathogen is able to infect green tissues and establish biotrophism widely across the *Vitis* genus. Unlike the European *V. vinifera*, some accessions in North American wild species have evolved host resistance. Resistant accessions are able to activate defense responses upon pathogen infection, which culminate in localized necrosis, resulting into lower rates of sporangia release compared to susceptible accessions (Bellin et al., 2009; Polesani et al., 2010).

Resistant accessions of wild species have been crossed with cultivated varieties to introgress resistance. The use of resistant varieties is a promising strategy for viticulture to cope with downy mildew (Bisson et al., 2002). Among these, the variety Bianca is widely cultivated in Hungary, Moldova, and Russia and is one of the few resistant accessions in which the genetic basis of resistance has been elucidated (Bellin et al., 2009). Moreover, Bianca, which is an hybrid between Bouvier and the resistant grapevine Villard Blanc obtained in 1963 (Csizmazia and Bereznai, 1968), is officially registered for use in wine production in 1982 (Kozma and Dula, 2003). A large part of the resistance phenotype of Bianca is explained by the Rpv3 locus, located in chromosome 18. In Bianca and in all known resistant descendants of the "Villard Blanc," the Rpv3 locus controls the ability to trigger a localized hypersensitive response (HR) soon after the initiation of the infection (Bellin et al., 2009; Di Gaspero et al., 2012). HR in the proximity of infection sites confines biotrophic pathogens, restricting their endophytic growth (Jones and Dangl, 2006). Early inducible responses include cell wall deposition, release of reactive oxygen species and hypersensitive cell death (HR) at the infection site, controlled by interactions between avirulence gene products and plant receptors, and it can be the result of multiple signaling pathways (Heath, 2000).

Plant defense responses require energy and activation of signaling molecules, primarily supplied by primary metabolism of carbohydrates, organic acids, amines, amino acids, and lipids (Bolton, 2009; Rojas et al., 2014). HR also stimulate the expression of defense responses near the infected area and the systemic acquired resistance of plant (Greenberg and Yao, 2004). Several studies have shown the importance of secondary metabolites for expressing plant defense, often related to specific functions such as toxicity against pathogens, or acting as signal molecules after stress (Bennett and Wallsgrove, 1994; Gershenzon and Dudareva, 2007). The induction of stress-related metabolites known as phytoalexins contributed to

the inhibition of biotrophic pathogens in resistant grapevines (Dercks and Creasy, 1989; Derckel et al., 1999; Slaughter et al., 2008; Godard et al., 2009; Ferri et al., 2011; Gessler et al., 2011). Stilbenes is a class of phytoalexins that provided active compounds with antifungal activity against various pathogens, including *P. viticola* (Dercks and Creasy, 1989). The pattern of stilbene accumulation upon *P. viticola* infection differs among *Vitis* species. Stilbene concentration showed earlier and higher increase in resistant varieties as compared to susceptible ones. In other cases, downy mildew resistance was observed in the absence of stilbene accumulation (Keller, 2015). This suggests the necessity to investigate which secondary metabolites play a key role in resistance and which of them are reliable biomarkers of the defense response in resistant varieties.

We expect that several classes of primary and secondary metabolites are modulated in Bianca during the defense response to *P. viticola*. In this scenario, metabolomics is the most suitable approach for monitoring a wide range of molecules. Indeed, several metabolomics studies have been already reported in grapevine. Some of them aimed at highlighting intervarietal variation in berry composition (Mulas et al., 2011; Gika et al., 2012; Degu et al., 2014; Teixeira et al., 2014; Bavaresco et al., 2016). Other studies aimed at identifying metabolite changes in infected leaves (Batovska et al., 2009; Ali et al., 2012; Becker et al., 2013; Algarra Alarcon et al., 2015). However, the metabolite changes that are brought about by the resistance mechanism have not yet been fully described.

In this work, we monitored metabolite changes in leaf discs of the resistant variety Bianca after infection with a suspension of *P. viticola*, with the aim of discovering biomarkers for specific stages of the host defense. In particular, we evaluated both primary and secondary metabolism at 12, 24, 48, and 96 h post-inoculation (hpi). We used existing protocols of LC-MS/MS for identification and quantification of lipids and phenols, and GC-MS for identification and semi-quantification of volatile organic compounds (VOCs). Moreover, we validated a new GC-MS protocol for the identification and quantification of primary compounds, including organic acids, amino acids, amines, sugars, and lipids, which yielded 48 metabolites in Bianca leaf discs.

MATERIALS AND METHODS

Plant Material

Metabolite analyses were performed using leaves from twonode cuttings of the cultivar "Bianca." The mother plants were held at Fondazione Edmund Mach grape collection, San Michele all'Adige, Italy ($46^{\circ}12'0''$ N, $11^{\circ}8'0''$ E). Own-rooted vines (n = 45) were grown in potted soil in controlled greenhouse conditions. Water was supplied by drip irrigation in order to avoid premature infections of downy mildew on leaves. At the stage of 12-leaf shoots, the plants were sorted into three homogenous groups; each group represented a biological replicate. At the time of the experiment plants were healthy, with no evidence of foliar diseases.

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Artificial Inoculation of Leaf Discs and Incubation under Controlled Conditions

The third, fourth, and fifth fully expanded leaves beneath the apex were detached from each plant, rinsed with ultrapure water. From each leaf, 1.1 cm diameter discs were excised with a cork borer and placed randomly onto wet paper in Petri dishes with the abaxial side up. Around 100 discs per condition per time point (es. 12 hpi, inoculated, biological replicate 1 = 100 discs) were used. Leaf discs were left to equilibrate at 21°C for 12 h after punching and prior to inoculation. P. viticola spores were collected from natural infected leaves in an untreated vineyard on 2014 and immediately frozen at -20° C. Before the experiment, they were propagated by infecting a susceptible variety and collecting fresh sporulation. After sporulation, the fresh spores were immediately used to prepare the experiment suspension. Discs were sprayed with *P. viticola* inoculum suspension at 1×10^6 sporangia/mL. Sealed Petri dishes were incubated in a growth chamber at 21°C until sampling. Mock inoculations (not inoculated) were done with ultrapure water as control. Leaf discs were sampled at 12, 24, 48, and 96 hpi/mock, then ground under liquid nitrogen to obtain a frozen powder. Three biological replicates were sampled at each time point.

Targeted Primary Compound Analysis and Method Validation Sample Preparation

The extraction of primary metabolites was carried out according to Fiehn et al. (2008) with some modifications. Briefly, 0.1 g of fresh leaf powder was subjected to extraction by adding 1 mL of cool (-20°C) extraction solvent, composed of isopropanol/acetonitrile/water (3:3:2 v/v/v). A 20 µL aliquot of a solution containing palmitic-D3, nicotinic-D4, and glucose-D7 (1000 mg/L) was added as an internal standard. The extraction mixture was vortexed for 10 s, shaken at 4°C for 5 min and centrifuged at 12,000 g for 2 min at 5°C. A second round of extraction was carried out following the same procedure. The two supernatants were merged and re-suspended in a final volume of 5 mL using the extraction solvent. A total of 250 µL of supernatant was placed in a 1.5 mL Eppendorf tube and evaporated to dryness under N2. The residue was re-suspended in 500 µL of acetonitrile/water (50:50 v/v), vortexed for 10 s, sonicated and centrifuged at 12,000 g for 2 min. The supernatant was then transferred into a 1.5 mL Eppendorf tube and dried out under N2. The dried extract was subject to derivatization, first by adding 20 µL of methoxamine hydrochloride in pyridine (20 mg/mL) to inhibit cyclization of reducing sugars and shaken at 30°C for 1 h; then by adding 80 µL of N-methyl-Ntrimethylsilyl-trifluoroacetamide with 1% trimethylchlorosilane for trimethylsilylation of acidic protons and shaken at 37°C for 30 min. Finally, 5 µL of a solution containing decane and heptadecane (1000 mg/L) were added in order to monitor the chromatographic analysis and the instrumental conditions. The derivatized extract was then transferred into vials for analysis. One microliter of derivatized extract was injected for GC/MS analysis.

Instrumental Conditions

Analyses were performed using a Trace GC Ultra combined with a mass spectrometer TSQ Quantum GC and an autosampler Triplus (Thermo Electron Corporation, Waltham, MA, United States). A RXI-5-Sil MS w/Integra-Guard®(fused silica) (30 m \times 0.25 mm \times 0.25 μ m) column was used for compound separation. Helium was used as the carrier gas at 1.2 mL/min and the injector split ratio was set to 1:10. The injector, transfer line and source temperature were set to 250°C. The initial oven temperature was kept at 65°C for 2 min, increased by 5.2°C/min to 270°C and held at 270°C for 4 min. These conditions were shown to represent a good compromise in order to obtain a not excessively long chromatographic run, a high number of compounds and good peak separation. The mass spectrometer was operated in electron ionization mode. Data acquisition was performed in full scan mode from 50 to 700 m/z. Data processing was performed using XCALIBURTM 2.2 SOFTWARE.

Method Validation

The method for primary metabolites was validated according to the currently accepted US Food and Drug Administration (FDA) bio-analytical method validation guide (US Department of Health and Human Services, 2001). Validation assays were established on calibration standards and quality control (QC) samples prepared as a pool of grape samples, extracted and derivatized according to the procedure described above. QC samples were used to evaluate the recovery of each compound and the stability of sample, intra- and inter-day variability, and to evaluate the efficiency of the extraction procedure. The standard mix was used to determine the limit of detection (LOD), limit of quantification (LOQ), and linearity range for each compound. Matrix calibration curves built using QC samples were compared with solvent calibration curves. Matrix effect (ME) values were determined using the slope ratios: $ME\% = 100 \times (1 - slope)$ solvent calibration curve/slope matrix calibration curve) (Kwon et al., 2012). LOQ and LOD were evaluated at the concentration in which the quantifier transition presented a signal-to-noise (S/N) ratio of >10 and >3, respectively. Intra- and inter-day variability were evaluated using the coefficient of variation (CV%) of QC samples injected 10 times on 1 day and then for 5 consecutive days. The recovery test was estimated on 10 spiked grape samples and calculated as the average of the "measured value/expected value" ratio (%). Each compound was identified and quantified against the standard, using one, or in the case of a few compounds, two specific m/z characteristics for the individual metabolite (extracted ion monitoring) and excluding saturated fragments. The fragments used for quantitation and the linear retention index (RI) are reported in Supplementary Table S1. Compounds were expressed as mg/kg of fresh leaves.

Targeted Lipid Compound Analysis

Lipid analysis was carried out according to Della Corte et al. (2015), using Folch's extraction method (Folch et al., 1957; Della Corte et al., 2015) with some modifications. Briefly, 0.3 mL of methanol were added to 0.1 g of fresh leaf powder and vortexed for 30 s, then 0.6 mL of chloroform containing butylated hydroxyl

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toluene (500 mg/L) were added, followed by the addition of 10 μ L of internal standard (docosahexaenoic acid 100 µg/mL). Samples were placed in an orbital shaker for 60 min. After the addition of 0.25 mL of water, samples were centrifuged at 3600 rpm for 10 min. The total lower lipid-rich layer was collected and re-extracted by adding 0.4 mL of chloroform/methanol/water 86:14:1 v/v/v. The samples were centrifuged at 3600 rpm for 10 min, the total lower lipid-rich layer was collected. Both chloroform fractions were merged and evaporated to dryness under N2. Samples were re-suspended in 300 µL of acetonitrile/2propanol/water (65:30:5 v/v/v) containing the internal standard cholesterol at a concentration of 1 µg/mL and transferred into a HPLC vial. Separation was performed using a UHPLC Dionex 3000 (Thermo Fisher Scientific, Germany), with a RP Ascentis Express column (15 cm × 2.1 mm; 2.7 µm C18) purchased from Sigma, following a 30 min multistep linear gradient following Della Corte et al. (2015). The UHPLC system was coupled with an API 5500 triple-quadrupole mass spectrometer (Applied Biosystems/MDS Sciex) equipped with an electrospray (ESI)source. Compounds were identified using Analyst Software based on their true reference standard, retention time and qualifier and quantifier ion, and were quantified using their calibration curves and expressed as mg/kg of fresh leaves.

Targeted Phenolic Compound Analysis

Phenolic compounds were determined according to Vrhovsek et al. (2012), with some modifications. Briefly, 0.4 mL of chloroform and 0.6 mL of methanol:water (2:1) were added to 0.1 g of fresh leaf powder. A 20 µL aliquot of gentisic acid (50 mg/L) and rosmarinic acid (50 mg/L) were added as internal standards. The extraction mixture was shaken for 15 min in an orbital shaker, then centrifuged for 5 min at 15,000 g at 4°C. The upper aqueous-methanolic phase was collected. The extraction was repeated by adding 0.6 mL of methanol and water (2:1 v/v) and 0.2 mL of chloroform; the samples were centrifuged for 5 min at 15,000 g at 4°C. The aqueous-methanolic phase was collected and combined with the previous one. Both fractions were merged and evaporated to dryness under N2. Samples were re-suspended in 500 µL of methanol and water (1:1 v/v), centrifuged and transferred carefully into an HPLC vial. Chromatographic analysis was performed using a Waters Acquity UPLC system (Milford) with a Waters Acquity HSS T3 column (100 mm \times 2.1 mm; 1.8 μ m) following Vrhovsek et al. (2012). Mass spectrometry detection was performed on a Waters Xevo triple-quadrupole mass spectrometer detector (Milford) with an electrospray (ESI) source (Vrhovsek et al., 2012). Compounds were identified based on their reference standard, retention time and qualifier and quantifier ion, and were quantified using their calibration curves and expressed as mg/kg of fresh leaves. Data processing was performed using Waters MassLynx V4.1 software.

Volatile Compound Analysis

Volatile compounds were extracted with solid phase microextraction, using a method adapted from Matarese et al. (2014) and Salvagnin et al. (2016). The extraction was carried out with some modifications; briefly, 0.1 g of fresh leaves were placed in 10 mL glass vials with 2 mL of buffer

(0.1 m Na₂HPO₄ and 50 mM citric acid; pH 5), 0.2 g of NaCl, and 5 µL of 1-heptanol (25 mg/L) as internal standard. Samples were kept at 60°C for 20 min and compounds in the headspace were captured for 35 min at 60°C. A Trace GC Ultra gas chromatograph coupled to a Quantum XLS mass spectrometer (Thermo Electron Corporation, Waltham, MA, United States) was used to separate the compounds with a fused silica Stabilwax[®]-DA column (30 m \times 0.25 mm i.d. \times 0.25 μ m) (Restek Corporation, Bellefonte, United States). The headspace was sampled using 2-cm DVB/CAR/PDMS 50/30 µm fiber from Supelco (Bellefonte, PA, United States). The compounds were desorbed in the GC inlet at 250°C for 4 min. The GC oven parameters were set following Salvagnin et al. (2016). The MS detector was operated in scan mode (mass range 40-450 m/z) with a 0.2 s scan time and the transfer line to the MS system was maintained at 250°C. Data processing was performed using XCALIBURTM 2.2 SOFTWARE. For the identification of volatile compounds we used letter "A" for compounds with comparable mass spectra and retention time to those of the pure standard, "B" for those with a RI match on a similar phase column with the database NIST MS Search 2.0, and "C" for those identified in the mass spectral database NIST MS Search 2.0 (Sumner et al., 2007). The experimental linear temperature RI of each compound was calculated using a series of n-alkanes (C10-C30) in the same experimental conditions as the samples. The results were expressed in a semi-quantitative manner and expressed in µg/kg using 1-heptanol as the internal standard.

Data Analysis

Statistical analysis and data visualization were performed with custom R scripts (R Core Team, 2017). Missing values were imputed with a random value including between zero and LOQ. The concentrations were transformed using the base 10 logarithm, in order to make data distribution more normal-like (van den Berg et al., 2006). Principal component analysis (PCA) was performed on the obtained multidimensional dataset, after mean centering and unit scaling, using the FactoMineR and Factoextra R packages (Lê et al., 2008; Kassambara and Mundt, 2017). The *t*-statistic was computed using the Stats package (R Core Team, 2017), while network visualization exploited the ggraph package (Pedersen, 2017).

RESULTS

In leaf discs inoculated with *P. viticola* and in mock-inoculated controls, we identified 176 compounds (Supplementary Table S2) belonging to acids (18), amino acids (13), amines and others (3), sugars (14), carnitines (1), sterols (3), fatty acids (14), glycerolipids (4), glycerophospholipids (4), sphingolipids (1) prenols (1), benzoic acid derivates (4), coumarins (2), phenylpropanoids (6), dihydrochalcones (1), flavones (1), flavan-3-ols (9), flavonols (11), stilbenes and stilbenoids (14), and other phenolics (2). All these metabolites were annotated with identification level 1 (with standards) and their concentration was expressed as mg/kg of fresh leaves. The volatile acids (3), alcohols (7), aldehydes (9), benzenoids (4), ketones (4),

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terpenoids (14), other VOCs (5), and unknown VOCs (5) were semi-quantified as the equivalent of the internal standard (1-heptanol) and their concentration was expressed as $\mu g/kg$ of fresh leaves (Supplementary Table S2). The concentration reported represents the average value of three biological replicates \pm standard error. For the identification of VOCs, we reported the confidence levels for metabolite identification defined by the Metabolomics Standards Initiative (Sumner et al., 2007): level A is assigned to compound for which the mass spectrum and the retention time match with the one of the pure standard; level B indicates that the RI of the compound and of the reference standard matches on a similar phase column; level C is assigned when the compounds mass spectrum is available into mass spectra databases (Supplementary Table S2).

Validation Results of the Primary Compound Method

Unlike for lipid, phenolic, and volatile compounds, a validated protocol for identification and quantification of primary metabolites in grapevine leaves was missing at the beginning of this study. We thus adopted a method established by Fiehn et al. (2008) on grape berries and performed a validation step to confirm the identity of each compound in a leaf matrix. All the standards were injected to obtain their fragmentation patterns and to calculate their retention indices. The calculated retention indices and mass spectra were compared with the NIST MS Search 2.0 database. The method was validated with the injection of relative standards for 96 compounds: 29 acids, 17 amino acids, 12 amines and others, 24 sugars and 14 fatty acids (Supplementary Figure S1). All the validation results are summarized in Supplementary Table S1. The ME values evaluated by comparing the calibration curves (matrix and solvent) were in the range between -20 and 20%, except for salicylic acid, citric acid, glycine, beta-alanine, tyrosine, fructose, and myoinositol, which slightly exceeded the limit of $\pm 20\%$ established by the validation method guide; this value can be considered as insignificant, because it is close to the relative standard deviation values of repeatability (European Commission, 2011). Intra- and inter-day repeatability were evaluated for each compound and expressed as CV%. The value should not exceed 15% for intra-day and 20% for inter-day; again in this case we had very good results, except for oxalic acid (intra-day 18.2%; inter-day 26.4) and malonic acid (intra-day 15.2%; inter-day 44.6%). The recovery ranges were over 90% for 74 compounds, between 80 and 90% for 13 compounds, between 70 and 80% for four compounds, and between 50 and 70% for five compounds. Using solvent calibration curves we evaluated the linearity ranges and the LOD and LOQ limits for each compound reported in Supplementary Table S1. In general, we obtained good validation results for the method, which make us confident about the possibility of applying the method for accurate quantification of primary compounds in different matrices.

The fatty acid derivatization step can modify the profile, with the formation of oxidation or isomerization products (Rigano et al., 2016) and as previously reported, the best option is to use trimethylsilyl diazomethane, with the production of methyl esters (FAMEs), avoiding the poor separation of fatty acid compounds and substantial interference (Topolewska et al., 2015). In our method, we validated all the compounds following the derivatization used by Fiehn et al. (2008), but we found a consistent residue in blank injections of some compounds, such as palmitic acid, stearic acid, and arachidic acid in particular during the sample runs. Due to this interference, we were not able to correctly quantify fatty acid compounds in our matrix using the GC-MS method therefore their quantification was performed using LC-MS/MS.

Metabolite Changes during the Defense Response

Global metabolite changes in the resistant host upon pathogen inoculation were first visualized by using PCA (**Figure 1**). In the plot, the position of the three biological replicates and their average is reported for each time point (12, 24, 48, and 96 h) for inoculated and not inoculated leaves. PCA of all compounds revealed good separation between the factors of the study and the temporal evolution is clearly captured by the first PCA component (Dim 1), accounting for 35.7% of total variance. The second component (Dim 2), accounting for 13.5% of total variance, discriminates leaf discs undergoing a defense response to *P. viticola* inoculation from mock inoculated controls (**Figure 1**).

In order to identify which class of metabolites was responsible for this separation, we performed PCA (Figure 2) separately for primary metabolites (Figure 2A), lipids (Figure 2B), phenolic compounds (Figure 2C), and VOCs (Figure 2D). Again the time trend is clearly distinguishable (almost captured by the first component), and also a good separation between the two conditions can be noticed for specific time points for the different classes of compounds. Indeed, we observe for primary compounds a clear separation between the two conditions along the second dimension (which capture 24.1% of the total variance) at 48 hpi and, looking at the two components (explaining a total of 55.3% of the variance), possibly at 24 hpi (Figure 2A). Lipids show the greatest differences at 24 hpi, where the inoculated and control samples are separated mainly along the second component, which explains 16.4% of the total variance (Figure 2B). Phenols are involved in the plant response only later, at 96 hpi, with the first component capturing 52.3% of the variance and possibly explaining both the time course and the differences the two conditions (Figure 2C). Finally, VOCs PCA separates inoculated and not inoculated samples at 48 hpi, and possibly at 96 hpi, mainly along the first component, which explains 51.5% of the variance due to both the time trend and the differences between the two classes in the last two time points (Figure 2D).

In order to select biomarkers for each specific stage of the defense response, we computed the t-statistic for all the metabolites for each time point, since it takes into account both the difference between the means and the estimate of the biological variability. To concentrate on the compounds most different between inoculated and control samples, we focused on the subset of metabolites (176) and time points (4) having

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an arbitrary absolute value for the *t*-statistic greater than 3 (|t|> 3): 64 values of the *t*-statistic satisfied our constraint. In terms of compounds we are dealing with 53 metabolites, which are different between inoculated and control samples in at least one time point (Supplementary Figure S2 and Table S3). The results of this analysis are represented in network form in Figure 3. The network contains 53 nodes, each one representing one metabolite. A link is drawn between two metabolites only if both are among those most different (|t| > 3) at the same time point. In the same visualization the compound classes is highlighted by the color of the nodes and the time course information by color of the link (Figure 3). Time point specific cliques are characterizing the structure of the network: the metabolites shown in one of these cliques show differences between the two conditions at that specific time point. The smaller number of nodes in the network at 12 hpi indicates that metabolic changes were minimal at this time point, only involving a small group of volatile compounds, glycine, ampelopsin D + quadrangularin A, trans-resveratrol, kaempferol-3-O-rutinoside, and pallidol. Several lipids and primary metabolites are highly modulated at 24 hpi, as already noticed looking at Figure 2. No lipids are highly modulated at 48 hpi, and many polyphenols are mainly involved at 96 hpi. Moreover, it is immediately apparent that ceramide and trans-piceid shows a central position in the network, meaning that these compounds were highly modulated at all the time points. Some interesting results are represented by metabolites connected with two different colored links; these were different at two time points, as exemplified by transresveratrol (Figure 3).

To further investigate this interesting subset of compounds and as further check for both the selection criterion and the visualization proposed, we explored the trends of log10 concentration over time for some key metabolites. These plots show results consistent with the network representation (Supplementary Figure S3). Ceramide concentration in inoculated leaves was already higher at 12 hpi compared with the controls and reached the highest concentration at 96 hpi (0.32 mg/kg; Supplementary Table S2). Trans-piceid concentration was already high at 12 hpi and reached the highest concentration at 48 hpi (5.29 mg/kg) (Supplementary Table S2). Among the polyphenols, trans-e-viniferin was the compound that modulated earliest at 48 hpi, while other trimeric and tetrameric stilbenoids, such as ampelopsin H + vaticanol C, pallidol, ampelopsin D + quadrangularin A, Z-miyabenol C and a-viniferin for example, were modulated at 96 hpi (Supplementary Figure S3). Accumulation of trans-resveratrol occurred early after infection (12 hpi), and was followed by a decrease in concentration at 24 and 48 hpi, and a resumption of accumulation at 96 hpi (Supplementary Figure S3).

A considerable number of compounds belonging to each class increased in concentration over time in both inoculated and not inoculated samples (Supplementary Table S2); this would explain the high variance in the first dimension of PCA, which is associated with the time course (Figure 1). The progressive accumulation of stress-related compounds in leaf discs, regardless pathogen inoculation, can be explained by other stresses affecting the tissues as a consequence of leaf removal, punching of the leaf lamina, and artificial conditions of leaf disc

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incubation. We found accumulation of some lipid compounds, such as arachidic acid, oleanolic acid, and uvaol, in inoculated and control samples (Supplementary Table S2). In polyphenols we also observed an accumulation of flavonols and some trimers and tetramers belonging to the stilbene and stilbenoid class during the first 48 h, irrespective of pathogen infection, and then differentiation at 96 hpi (Supplementary Table S2). These results are consistent with previous reports of metabolite changes caused by mechanical wounding (Chitarrini et al., 2017).

DISCUSSION

Grapevine and *P. viticola* interaction is still poorly understood in terms of metabolites: there is the need to improve the knowledge about how the plant system is perturbed after stress. In this study, a metabolomic approach has revealed major changes in primary and secondary metabolism of a resistant grape variety during the defense response to *P. viticola*. The identification of biomarkers, specific of four stages of the defense response, from 12 to 96 hpi, reflected a progression of physiological events that bring about resistance.

In addition to the importance of secondary metabolites in the fight against pathogens, the role of primary metabolism needs to be taken into account, since it is not only an energy provider but also regulates defense responses in plants in the presence of potential pathogens or pathogen-derived elicitors (Rojas et al., 2014). We expected that a defense response against an endophytic biotroph could be triggered only after the establishment of intimate contact between pathogen haustoria and host plasma membranes. In fact, we observed minimal metabolite changes in the host within 12 hpi, compatibly with a scenario in which *P. viticola* oospores/zoospores take several hours to germinate on the leaf lamina, target the stomata, form appressoria, break through the cell wall of mesophyll cells and develop functional haustoria. We identified a few biomarkers for this very early stage of host–pathogen interaction. Most of them were volatile compounds, which may interfere with the pathogen endophytic invasion of mesophyll air spaces.

The classes of biomarkers specific to 24 and 48 hpi suggested that early host responses to *P. viticola* were being set in place during those stages. We detected a sharp shift in primary metabolism. Leaf discs undergoing the defense response showed a cumulative amount of sugars, organic acids, and amino acids 6.7% higher than controls at 12 hpi, 9.4% higher at 24 hpi, 14.1% higher at 48 dpi, and 11% lower at 96 dpi. These data suggest that most of the metabolic effort for containing pathogen infection was carried out by 48 hpi and the metabolic cost for this effort was paid at 96 hpi. In leaf discs undergoing the defense response, organic acids were 13.3% higher than controls at 12 hpi

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and 24.6% higher at 24 hpi. Vice versa, sugars and amino acids were consistently lower at the same time points in leaf discs undergoing the defense response (-2.3 and -12.1% sugars at 12 and 24 hpi, respectively; -8.9 and 19.9% amino acids at 12 and 24 hpi, respectively). At 48 hpi sugars and amino acids were 33.9 and 42.3% higher in leaf discs undergoing the defense response compared to controls. The cost of expressing defense has been shown in barley as a peak of respiration rate during the expression of host resistance to *Blumeria graminis* (Brown and Rant, 2013).

Primary metabolism is important for energy supply but it also has a role providing precursors of secondary metabolites, building block of PR proteins, and components of the defense signaling cascade (Rojas et al., 2014). Less et al. (2011) found different regulation of specific genes in *Arabidopsis* related to primary metabolism, due to abiotic and biotic stress response; in particular, up-regulation of genes involved in energy production processes and down-regulation of genes associated with assimilatory processes was found (Less et al., 2011). We found changes in primary compounds at 24 and 48 hpi, in particular we observed an interesting modulation for proline. In *Arabidopsis*, both supply and catabolism of proline are components of salicylic acid-mediated resistance, contributing to cell death in response to *Pseudomonas* (Deuschle et al., 2004; Cecchini et al., 2011). The role of proline in the Bianca grapevine variety after *P. viticola* infection should be elucidated with further experiments, however, for the moment we can identify this molecule as a putative biomarker.

Lipids represent a class of compounds with structural diversity and complexity. They are critical components of plant cell membranes and provide energy for metabolic activities. We found changes at 24 hpi in particular with a faster decrease in some unsaturated fatty acids after *P. viticola* infection. Ceramide started accumulating very early in infected samples compared to the control, and continued to accumulate after biotic stress up to 96 hpi; it was previously reported that ceramides can be essential as signaling molecules in the activation of defense-related plant programmed cell death (Kachroo and Kachroo, 2009; Berkey et al., 2012).

Subsequently, secondary metabolism was affected more strongly by the pathogen, with changes in the volatile compounds at 48–96 hpi and at the latest at 96 hpi in phenolic compounds. Some phenolic compounds, such as phenylpropanoids and flavonoids, have previously been identified and considered responsible for distinguishing the resistant cultivar Regent from the susceptible Trincadeira (Ali et al., 2012). We found higher concentrations of these compounds in our infected samples compared with the control at 96 hpi (Supplementary Table S2); this result suggests their involvement as biomarkers of

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resistance to the pathogen in the Bianca grapevine. Transresveratrol production in grapevine leaves after pathogen infection was identified by Langcake and Pryce (1977). It has been demonstrated that trans-resveratrol is a precursor of fungal toxicity compounds identified as phytoalexins; these compounds can be produced by grapevine leaves after abiotic and biotic stress and can be used in the grapevine as a marker of resistance against pathogens (Jeandet et al., 2002). The accumulation of trans-resveratrol at 12 hpi in our infected samples can reflect the role of this molecule as a precursor of other toxic molecules, and the very early trans-resveratrol accumulation in Bianca is probably due to a rapid response to the pathogen. In our study we found a major increase in some molecules deriving from resveratrol, such as trans-E-viniferin at 48 hpi and subsequently trans- and cis-piceid, isoraphontin, ampelopsin H + vaticanol C-like isomer, a-viniferin and pallidol at 96 hpi. During the first hpi, a low accumulation of viniferins (grapevine specific stress related metabolites) was found after pathogen infection, probably due to their accumulation at a later stage (4-7 days after inoculation), as previously described (Pezet et al., 2004; Jean-Denis et al., 2006; Slaughter et al., 2008). The time course for the appearance of these viniferins is in full agreement with the sequential mechanism extensively discussed by Bavaresco et al. (2012), progressing from initial synthesis of resveratrol toward the formation of dimers and then the higher oligomers. Such a path requires growth through the subsequent addition of one resveratrol unit to an existing dimer, leaving one part of the initial structure unchanged. The biosynthesis of dimers and higher oligomers appears to be of key importance in resistance, in agreement with the observations of Malacarne et al. (2011) regarding a population of Merzling × Teroldego. This paper indeed highlights that there is a negative correlation between the content of different oligomers and the percentage of sporulation observed following infection, while this was not the case for the monomers trans-resveratrol and trans-piceid, which were also found in non-tolerant genotypes with high sporulation. Moreover, the importance of viniferin oligomers is further confirmed by the concentration values required to induce inhibition of mildew development recently reported by Gabaston et al. (2017).

The peak of accumulation of phenolic compounds at 96 hpi was anticipated at 48 h by the accumulation of phenylalanine in inoculated samples (**Figure 3** and Supplementary Table S2). Phenylalanine is the precursor of the phenylpropanoid pathway, leading to the synthesis of flavonoids and stilbenes by stilbenes, two classes of compounds that increased at 96 (Sparvoli et al., 1994; Flamini et al., 2013).

Among the volatiles compounds, we found an increase in benzaldehyde production at 48 and 96 hpi in infected samples (Supplementary Figure S2) Benzaldehyde is considered as a growth suppressor and spore inhibitor, with activity against *Botrytis cinerea*, also at a low concentration (Martínez, 2012). Benzaldehyde also promotes salicylic acid accumulation, induces expression of PR proteins and increases TMV resistance in tobacco (Ribnicky et al., 1998). The higher concentration we found in infected Bianca samples at 48 and 96 hpi (around 1.5 times higher compared to the control) seems to suggest its involvement as a putative biomarker against *P. viticola* growth or diffusion.

Based on our results, we can argue that all the compounds significantly differentiated in infected samples have a role in Bianca-*P. viticola* interaction. In particular, 53 metabolites have been identified as putative biomarkers in hybrid Bianca grapevine leaves after *P. viticola* infection. Some of them are known biomarkers of resistance (viniferins). Among the others, some are likely to be putative biomarkers of resistance in Bianca leaf discs after *P. viticola* infection, such as benzaldehyde and proline, while we cannot exclude that the presence of metabolites induced by the mechanical wounding resulting from the leaf disks preparation.

To the best of our knowledge, this is the first time that an extensive metabolomic study has been undertaken using a hybrid grape variety to better understand metabolic perturbation after *P. viticola* infection, finding early stage biomarkers for different chemical classes of metabolites. These results can represent a starting point for better understanding grapevine resistance and can lead to discoveries regarding new mechanisms for plant–pathogen interaction between the grapevine and *P. viticola*.

We also obtained good results for method validation in relation to the identification and quantification of 97 primary compounds belonging to different chemical classes: acids, amino acids, amines, sugars, and fatty acids, using a GC-MS method for separation and identification. The method can easily be applied to further analysis for the identification and quantification of primary compounds in different matrices.

AUTHOR CONTRIBUTIONS

GC, LZ, AV, MS, GD, FM, and UV designed the experiment. GC, GD, LZ, and AV performed the experiment. GC, ES, and DM did the extractions, analytical analysis, and data treatment. GC, ES, and DM developed and validated the method. SR and PF conducted all the statistical analyses. All authors discussed the results and implications and commented on the manuscript at all stages.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fpls.2017.01524/ full#supplementary-material

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Supplementary Figure 1: Different classes of primary compounds taken into account for the GC-MS/MS validation method.



Supplementary Figure 2: t-statistic values of our metabolites. For network analysis we took into account only metabolites with |t| > 3.



Supplementary Figure 3: Graphs for specific metabolites. The Log10-transformed metabolite concentration of the three biological replicates over time is represented for each of the metabolites (I= inoculated samples; NI= not inoculated samples). The line represents the mean of the three biological replicates. Where missing values were present they were imputed with a random value between zero and LOQ.

Class	Compound	RT (min)	RI	m/z	Intra- day CV (%)	Inter-day CV (%)	Recovery (%)	Matrix effect (%)	Linearity range (mg/L)	LOD (mg/L)	LOQ (mg/L)
Acids											
	oxalic acid	9.36	1131	175+190	18.2	26.4	118.9	0	0.5-100	0.167	0.5
	malonic acid	11.17	1203	233	15.2	44.6	97.2	17	0.05-100	0.017	0.05
	benzoic acid	12.20	1242	179	1.8	8.4	50.4	-21	0.005-100	0.002	0.005
	nicotinic acid	13.37	1288	180	1.3	3.1	131.7	-6.1	0.005-50	0.002	0.005
	maleic acid	13.67	1300	245	1.4	5.1	107.3	0	0.005-200	0.002	0.005
	succinic acid	13.98	1312	247	1.3	3.8	106.7	1.5	0.005-150	0.002	0.005
	glyceric acid	14.45	1330	189	0.7	6.8	80.5	-11.4	0.005-150	0.002	0.005
	fumaric acid	14.91	1350	245	3.1	6.4	92.7	-6	0.01-20	0.003	0.01
	glutaric acid	16.25	1404	261	1.8	4.2	105.9	-8.8	0.005-150	0.002	0.005
	citramalic acid	17.81	1469	247	1.5	4.5	100.1	-8.1	0.005-40	0.002	0.005
	malic acid	18.31	1489	335	1.6	4.4	82.4	-7.1	0.005-200	0.002	0.005
	salicilic acid	18.63	1505	209	1.7	8.8	102.6	-25.3	0.5-150	0.167	0.5
	pyroglutamic acid	18.83	1514	156	1.6	7.1	126.2	-9.6	0.1-10	0.033	0.1
	cinnamic acid	19.51	1542	205	1.4	5.1	63.9	-12.4	5-100	1.667	5
	mevalonic acid	19.86	1558	233+247	1.8	16.5	62.6	-17.6	0.5-200	0.167	0.5
	threonic acid	19.96	1563	292	3.8	6.3	122.4	-19.7	0.005-40	0.002	0.005
	α -ketoglutaric acid	20.25	1575	198	2.8	4.4	90.2	-5.9	1-100	0.333	1
	3-hydroxy-3- methylglutaric acid	20.81	1601	247	1.5	4.8	100.8	-19.5	0.05-40	0.017	0.05
	tartaric acid	21.68	1639	423	4.8	10.3	79.4	4.4	0.005-200	0.002	0.005
	shikimic acid	25.26	1814	189	1.3	3.6	87.7	0	0.5-100	0.167	0.5
	citric acid	25.36	1818	257+273	2.2	5.1	87.4	-24.2	0.005-10	0.002	0.005
	isocitric acid	25.43	1823	245+273	1.5	3.3	93.4	-9	5-150	1.667	5
	hippuric acid	25.61	1832	206	7.9	17.4	83.7	-2.4	20-100	6.667	20
	5-keto gluconic acid	27.67	1938	364	2.7	4.3	91.3	-2.4	0.005-150	0.002	0.005
	ascorbic+dehydroascorbic acid	25.80	1841	173	2.3	7.4	112.7	-10.7	0.01-20	0.003	0.01
	quinic acid	26.16	1860	419	3.3	5.8	103.2	-5.7	0.005-200	0.002	0.005
	glucuronic	27.85	1947	333	2.7	4.6	106.8	5.3	0.01-150	0.003	0.01
	galacturonic acid	28.06	1960	333	2.7	4.5	100.4	5.3	0.05-100	0.017	0.05
	abscisic acid	32.42	2206	183	2.4	2.2	94.2	-4.5	2-150	0.667	2
Amino	acids										
	valine	8.23	1087	72	3.7	10.2	85.3	1.6	0.2-50	0.067	0.2
	alanine	8.61	1102	116	5.8	13.6	82.2	12.7	0.005-40	0.002	0.005
	norvaline	8.77	1107	72	3.8	7.3	133.5	14	1-100	0.333	1
	leucine	9.93	1153	86	3	6.3	134.4	14.9	0.2-100	0.067	0.2
	isoleucine	10.46	1174	86	2.8	7.4	153.8	16.8	0.2-50	0.067	0.2
	serine	12.50	1254	116	2.5	4.9	133.1	4.4	0.2-20	0.067	0.2
	threonine	13.46	1291	117	1.6	5.7	133	-17.3	0.5-10	0.167	0.5
	proline	13.49	1293	142	5.6	27.6	82.2	-22	0.1-100	0.033	0.1
	glycine	13.74	1303	248	2.9	6.9	123.8	45.7	0.005-10	0.002	0.005

Supplementary Table 1: GC-MS method validation results for the identification and quantification of primary compounds. RT Retention Time; RI Retention Index; LOD Limit of Detection; LOQ Limit of Quantification.

	aspartic acid	16.65	1421	160	2.5	19.3	96.5	11.7	5-150	1.667	5
	β-alanine	16.76	1426	248	2.6	6.7	116.4	-39.8	0.005-10	0.002	0.005
	y-aminobutyric acid	19.14	1527	304	1.3	6.4	103.9	-15.4	0.05-40	0.017	0.05
	asparagine	20.63	1582	159	5.1	21.2	137.4	21.1	10-150	3.333	10
	phenylalanine	21.21	1620	218	3.2	16.5	58.1	17.3	0.5-40	0.167	0.5
	ornithine+arginine	23.89	1746	174	11.4	21.6	96.6	-2.6	0.5-150	0.167	0.5
	lysine	25.92	1848	174	9.5	23.8	81.5	-12.5	0.2-100	0.067	0.2
	tyrosine	26.51	1877	179	10.2	24.6	87.2	-28	5-100	1.667	5
Amines	/ Others										
	2-pyrrolidinone	9.60	1138	142	2.4	3.6	108.1	-9.4	0.05-100	0.017	0.05
	isopentylamine	9.88	1152	174	2	6.2	118.1	-2	0.2-200	0.067	0.2
	uracil	14.50	1333	241	10.7	20.3	136.3	-4.5	0.005-20	0.002	0.005
	nicotinamide	17.93	1470	179	2.4	3.5	101.7	0.5	10-100	3.333	10
	cadaverine	25.61	1833	174	3.1	7.1	88.5	-17.3	0.02-100	0.007	0.02
	pyridoxiamine	26.20	1863	280	3	10.3	79.3	-13.6	1-150	0.333	1
	pyridoxal	26.35	1869	293	2.8	4.6	58.6	-0.6	0.02-150	0.007	0.02
	tryptamine	32.49	2213	174	3.1	15.3	90.2	-4.4	0.01-20	0.003	0.01
	spermidine	33.19	2251	144	9.5	17.3	102.5	3.8	1.0-40	0.333	1
	uridine	36.17	2436	217	5.7	10.8	133.4	4.6	10-200	3.333	10
	serotonine	36.35	2450	174	1.3	21.2	102.3	9.8	0.05-50	0.017	0.05
	adenosine	38.96	2630	230	1.5	3.1	91.4	-19.3	0.1-50	0.033	0.1
Sugars											
	maltol	13.22	1278	183	1.3	13.2	110.1	-15.8	1.0-50	0.333	1
	threitol	18.61	1504	217	2.1	7.7	125.4	14.9	0.005-40	0.002	0.005
	meso-erythriol	18.79	1512	217	2	7.3	120.8	7.9	0.01-40	0.003	0.01
	apiose	21.56	1636	217	0.8	6.6	131.7	22.3	0.2-150	0.067	0.2
	xylose	22.05	1659	217	1.5	4.5	113.3	10.4	0.005-40	0.002	0.005
	lyxose	22.18	1664	217	1.7	5.5	117.8	13.5	0.005-40	0.002	0.005
	ribose	22.53	1681	217	1.5	5.3	120.4	13.9	0.05-40	0.017	0.05
	xylitol	23.12	1709	217	2.6	7.3	106.8	-7.8	0.005-10	0.002	0.005
	α-rhamnose	23.32	1719	277	1	3.9	114.8	-8.3	0.005-100	0.002	0.005
	arabitol	23.43	1724	189	1.2	6.3	111.4	-17.4	0.01-100	0.003	0.01
	adonitol	23.52	1729	319	1.8	7	105.9	-15.9	0.005-50	0.002	0.005
	fucose	23.71	1738	117	2.2	2.3	112.7	3.1	0.01-50	0.003	0.01
	pinitol	25.59	1831	260	1.6	6.8	115	-12.5	0.005-40	0.002	0.005
	fructose	26.64	1886	307	1.9	4.4	75.2	-28.8	0.005-200	0.002	0.005
	mannose	26.71	1888	160	1.4	4	111.9	-2.6	0.005-100	0.002	0.005
	galactose	26.77	1892	319	1.5	5.2	102.4	-2.8	0.005-40	0.002	0.005
	glucose	27.26	1918	319	2	2.1	72.6	-13	0.005-200	0.002	0.005
	sorbitol	27.66	1938	319	2.6	7.1	104.1	0	0.005-20	0.002	0.005
	<i>myo</i> -inositol	30.41	2088	432	2.5	3.9	86.6	-36.8	0.005-150	0.002	0.005
	sucrose	39.10	2642	451	4.4	19.4	109.3	-18	0.005-150	0.002	0.005
	lactose	40.06	2708	204	3.2	7.9	91.2	-1.6	0.1-150	0.033	0.1
	trehalose	40.63	2745	204	4.3	8.8	118.8	-1.9	0.1-150	0.033	0.1

	maltose	40.85	2766	361	2.4	8	95.6	-1.7	0.1-150	0.033	0.1
	melibiose	42.80	2901	204	4.3	7.6	93.5	0.8	0.5-200	0.167	0.5
Fatty a	cids										
	myristoleic acid	25.66	1833	283	1.7	8.2	104.1	-17.4	5-100	1.667	5
	myristic acid	25.92	1848	285	1.6	5.7	100.2	2.3	0.5-100	0.167	0.5
	palmitoleic acid	29.19	2020	311	1.6	7	98.9	-2.3	2-200	0.667	2
	palmitic acid	29.60	2045	313	1.6	9.3	123.6	1.8	0.005-100	0.002	0.005
	margaric acid	31.33	2141	327	1	8.2	115.5	6.2	0.5-100	0.167	0.5
	linoleic acid	32.41	2205	337	1.4	8.5	90.6	3.1	2-200	0.667	2
	linolenic acid	32.49	2210	335	2	7.6	108.7	2.8	5-200	1.667	5
	oleic acid	32.54	2211	339	1.3	8.9	98.4	16.2	1-200	0.333	1
	cis-vaccenic acid	32.64	2218	339	0.9	9.7	103.5	8.2	2-200	0.667	2
	stearic acid	33.00	2240	341	1	8.2	88.3	-16.9	0.5-20	0.167	0.5
	cis-11-eicosenoic acid	35.73	2410	367	3.7	10.2	99	9.8	5-200	1.667	5
	arachidic acid	36.26	2438	369	4.1	9.9	107.4	13.3	0.005-40	0.002	0.005
	erucic acid	38.82	2609	395	7.9	13.7	108.5	5.2	10-200	3.333	10
	behenic acid	39.00	2639	397	11.5	11.7	131.1	-20	5.0-75	1.667	5
Supplementary Table 2: Quantification of primary compounds in Bianca grapevine samples in inoculated (I) and not inoculated (NI) at different time points, hours post infection (hpi). The concentrations reported represent the average value of 3 biological replicates ± error standard, expressed as mg/kg of fresh leaves.

	12 hpi		24	hpi	48	hpi	96 hpi				
Class Acids	Compound	N	Ι	r	Ι	NI	I	NI	I	NI	I
	malonic acid	4.568	± 0.128	4.104	± 0.418	4.343 ± 0.362	4.413 ± 0.190	4.153 ± 0.332	4.609 ± 0.328	4.031 ± 0.261	4.320 ± 0.177
	benzoic acid	2.751	± 0.539	2.713	± 0.507	3.063 ± 0.557	3.507 ± 0.808	3.155 ± 1.141	2.852 ± 0.531	3.936 ± 0.689	2.970 ± 1.033
	maleic acid	8.523	± 1.343	7.889	± 0.985	8.982 ± 1.877	4.802 ± 0.931	6.992 ± 0.610	10.777 ± 0.467	5.826 ± 0.753	9.087 ± 1.397
	succinic acid	23.505	± 1.625	20.167	± 1.149	18.189 ± 2.539	8.764 ± 0.865	13.098 ± 5.295	18.389 ± 0.247	12.011 ± 2.350	14.939 ± 0.875
	glyceric acid	149.175	± 39.088	173.101	± 21.091	163.783 ± 33.604	105.539 ± 6.644	104.695 ± 24.014	135.379 ± 6.654	111.246 ± 21.221	106.882 ± 20.738
	fumaric acid	2.668	± 0.116	2.527	± 0.211	2.625 ± 0.230	2.402 ± 0.222	2.293 ± 0.183	2.546 ± 0.064	2.030 ± 0.086	2.239 ± 0.109
	glutaric acid	1.754	± 0.094	1.632	± 0.177	1.551 ± 0.131	1.539 ± 0.133	1.468 ± 0.082	1.610 ± 0.025	1.510 ± 0.045	1.543 ± 0.062
	citramalic acid	2.028	± 0.301	2.365	± 0.097	2.246 ± 0.317	2.238 ± 0.280	1.944 ± 0.540	1.812 ± 0.217	1.200 ± 0.233	1.179 ± 0.309
	malic acid	1089.272	± 211.009	1143.834	± 95.929	1114.996 ± 211.083	992.011 ± 219.110	898.542 ± 197.604	1277.765 ± 56.426	745.360 ± 81.311	808.833 ± 209.315
	pyroglutamic acid	67.654	± 4.018	74.702	± 3.342	76.368 ± 4.918	72.467 ± 8.128	71.442 ± 8.776	104.290 ± 4.429	74.793 ± 4.420	105.435 ± 14.553
	threonic acid	9.817	± 1.354	8.942	± 1.246	7.946 ± 1.216	6.587 ± 2.005	5.709 ± 1.303	6.458 ± 0.323	5.376 ± 0.451	5.188 ± 0.553
	tartaric acid	7225.685	± 297.278	8284.564	\pm 731.983	7456.395 ± 675.729	10153.102 ± 694.855	7156.050 ± 902.174	6664.977 ± 196.984	6416.234 ± 453.763	5511.478 ± 599.635
	shikimic acid	125.679	± 4.598	165.898	\pm 36.497	160.320 ± 21.192	128.852 ± 12.498	121.657 ± 16.802	173.259 ± 43.980	110.571 ± 12.129	98.892 ± 8.791
	citric acid	93.761	± 9.736	103.546	\pm 3.017	138.375 ± 13.999	131.966 ± 14.023	143.766 ± 23.174	187.409 ± 8.809	171.119 ± 19.267	192.937 ± 39.122
	5-ketogluconic acid	40.609	± 5.632	50.331	± 8.298	45.659 ± 4.817	35.944 ± 5.787	32.202 ± 8.448	34.950 ± 4.974	27.253 ± 2.377	19.610 ± 3.251
	ascorbic acid	188.689	± 17.081	211.229	± 9.858	219.722 ± 21.167	127.248 ± 30.070	133.264 ± 40.608	223.241 ± 15.870	129.640 ± 6.750	111.920 ± 26.368
	quinic acid	25.901	± 3.348	31.059	± 2.973	26.279 ± 3.088	21.352 ± 2.611	20.018 ± 3.993	28.057 ± 3.848	19.543 ± 0.509	19.998 ± 1.411
4 .	abscisic acid	98.705	± 0.737	89.089	\pm 4.097	92.909 ± 8.535	92.959 ± 9.530	75.032 ± 6.892	83.630 ± 0.770	71.273 ± 1.485	79.323 ± 3.791
Amino d	acids	0.027	1 100	0.017	2.246	25.272 + 1.651	45.060 10.710	(2.00) 5.050	((220) 10(0	100 142 + 16 027	141.070 . 42.005
	valine	9.837	± 1.102	9.816	± 3.246	$25.3/2 \pm 1.651$	45.968 ± 12.718	63.996 ± 5.958	66.239 ± 4.968	$108.143 \pm 16.83/$	$141.9/8 \pm 42.695$
	launine	127.625	± 12.820	101.268	± 9.997	130.278 ± 18.012	88.493 ± 12.984	72.887 ± 24.993	124.058 ± 12.080	$124./14 \pm 13.440$	106.200 ± 36.350
	iaalawaina	8.337	± 1.002	9.233	± 1.431	10.110 ± 1.390	21.002 ± 5.037	25.570 ± 5.504	31.084 ± 2.113	33.029 ± 4.011	50.000 ± 15.189
	Isoleucine	25 284	± 1.142	27.621	± 1.382	19.393 ± 1.003	27.187 ± 3.517 25.877 ± 3.211	$32.1/1 \pm 2.90/$	30.922 ± 2.978 70.469 \pm 9.060	44.551 ± 5.471 76.014 \pm 0.025	04.249 ± 16.555 122.007 ± 26.089
	thraonina	30.646	± 1.309	27.021	± 3.780 ± 3.028	45.085 ± 7.902	16014 ± 1.471	43.201 ± 18.032 28.726 ± 0.582	70.408 ± 8.000 51.264 ± 4.803	70.914 ± 9.033 57.485 \pm 13.158	123.097 ± 20.088 66.878 ± 10.050
	nroline	32 835	+ 8747	29.490	± 9.541	33.130 ± 3.924 41.131 ± 4.591	10.914 ± 1.471 18.129 ± 0.661	26.720 ± 9.382 26.385 ± 5.479	51.504 ± 4.803 52.655 ± 6.143	75.863 ± 20.355	$45,830 \pm 10.039$
	glycine	2 266	+ 0.400	1 183	± 0.1341	1211 + 0159	0.941 + 0.124	0.957 ± 0.188	1519 ± 0.122	1142 + 0243	1243 ± 0.099
	aspartic acid	120.687	+ 10410	121 664	+ 3200	127224 + 5676	97007 + 12825	116674 + 19500	150.049 + 3.078	118449 + 9328	1.213 = 0.099 170223 + 11105
	<i>B</i> -alanine	1 183	± 0.108	1 180	± 0.138	1659 ± 0.079	1421 ± 0103	1264 ± 0206	1750 ± 0.085	2.646 ± 0.149	3173 ± 0177
	y-aminobutyric acid	76.250	± 7.600	56.301	± 3.658	69.438 ± 11.666	47.832 ± 2.464	46.862 ± 7.139	63.938 ± 4.650	46.947 ± 2.264	36.503 ± 4.466
	phenylalanine	82.333	± 4.844	88.209	± 1.913	114.704 ± 5.932	109.793 ± 8.497	138.812 ± 16.178	208.798 ± 7.241	234.205 ± 3.306	203.429 ± 38.391
	lysine	12.050	± 0.882	11.382	± 1.030	15.138 ± 1.389	13.710 ± 0.700	16.105 ± 2.081	16.579 ± 1.341	24.250 ± 1.603	18.196 ± 1.513
Amines	/Others										
	uracil	1.018	± 0.091	0.620	± 0.177	0.694 ± 0.156	0.570 ± 0.127	0.645 ± 0.037	0.811 ± 0.086	0.443 ± 0.089	0.863 ± 0.227
	pyridoxal	11.298	± 1.341	11.249	± 1.043	11.058 ± 1.315	9.958 ± 1.413	7.743 ± 1.312	10.161 ± 0.908	$7.738 \hspace{.1in} \pm \hspace{.1in} 0.807$	6.329 ± 1.091
	adenosine	25.803	± 1.069	22.211	± 1.121	22.631 ± 1.788	23.282 ± 0.564	23.230 ± 0.818	25.020 ± 0.699	21.960 ± 0.993	23.088 ± 0.611
Sugars	threitol	1.566	± 0.156	1.456	± 0.057	1.411 ± 0.100	1.218 ± 0.042	1.250 ± 0.070	1.416 ± 0.074	1.277 ± 0.166	1.340 ± 0.114

meso-erythriol	1.148	\pm	0.135	1.172	\pm	0.166	1.290	±	0.140	1.249	\pm	0.066	1.20)5 ±	0.286	1.369	±	0.091	1.238	\pm	0.139	1.434	±	0.070
xylose	7.035	\pm	0.368	6.598	\pm	0.306	8.129	±	1.098	7.847	\pm	0.482	10.07	'1 ±	0.578	10.869	±	0.513	9.878	\pm	0.554	12.904	±	1.338
ribose	6.020	\pm	0.464	5.286	±	0.382	6.220	±	1.019	4.667	\pm	0.160	4.60)1 ±	0.319	6.271	±	0.363	5.604	\pm	0.548	6.990	±	0.580
α -rhamnose	8.145	\pm	0.459	7.657	\pm	0.557	8.914	±	1.004	7.021	\pm	0.391	7.99	2 ±	0.405	9.575	±	0.329	9.167	\pm	0.585	9.874	±	0.421
arabitol	0.614	\pm	0.140	0.580	\pm	0.077	0.946	±	0.004	0.591	\pm	0.034	0.70)1 ±	0.077	0.937	±	0.113	0.910	\pm	0.100	0.961	±	0.044
adonitol	5.190	\pm	0.073	5.042	±	0.115	5.584	±	0.241	5.040	±	0.260	4.96	53 ±	0.451	6.799	±	0.614	5.231	±	0.373	5.956	±	0.593
fucose	18.641	\pm	1.094	19.196	±	0.948	22.257	±	2.409	20.307	±	0.246	20.57	'8 ±	0.963	24.685	±	0.922	20.297	±	0.966	23.918	±	0.769
fructose	566.374	\pm	93.893	602.380	±	60.235	627.578	±	90.834	527.922	±	67.471	414.56	52 ±	59.014	583.836	±	62.728	418.740	±	81.852	340.157	±	74.007
glucose	533.139	\pm	81.063	567.669	±	46.136	588.911	±	75.589	892.109	±	132.949	550.40)3 ±	115.948	527.171	±	49.398	414.350	±	83.898	324.749	±	71.233
sorbitol	1.408	±	0.175	1.214	±	0.135	1.480	±	0.169	1.794	±	0.270	1.68	39 ±	0.248	1.408	±	0.064	1.550	±	0.061	1.449	±	0.050
myo-inositol	2086.746	\pm	87.214	1973.240	±	118.251	2276.653	±	382.800	2268.049	±	235.955	1939.38	35 ±	90.294	2392.529	±	52.402	1772.351	±	83.732	1620.083	±	241.747
sucrose	2306.535	\pm	319.332	2230.674	±	332.372	2279.989	±	491.795	1372.737	±	415.686	1476.27	'3 ±	419.868	2394.537	±	176.044	1300.394	±	186.751	914.616	±	303.452
lactose	102.529	±	3.483	91.668	±	3.790	94.804	±	9.600	96.005	±	2.454	102.34	6 ±	1.673	113.069	±	5.166	91.019	±	1.044	97.372	±	13.899

	12	hpi	24	hpi	48	hpi	96	hpi
Class Compound	NI	Ι	NI	Ι	NI	I	NI	Ι
Carnitines								
palmitoyl-L-carnitine hydrochloride	0.0094 ± 0.0015	0.0136 ± 0.0018	0.0102 ± 0.0009	0.0098 ± 0.0002	0.0099 ± 0.0003	0.0100 ± 0.0005	0.0111 ± 0.0008	0.0123 ± 0.0011
Sterols								
desmosterol	0.8530 ± 0.2443	1.6528 ± 0.9359	0.5909 ± 0.2151	1.0677 ± 0.1911	2.6776 ± 0.9070	2.8860 ± 1.1835	0.7444 ± 0.3471	2.2783 ± 1.3839
lanosterol	3.6591 ± 0.7641	4.0217 ± 1.3897	3.5901 ± 2.8269	3.1185 ± 0.7350	2.0395 ± 1.1430	7.1136 ± 0.9571	0.8995 ± 0.8995	1.8462 ± 1.0829
uvaol	0.1933 ± 0.0415	0.2230 ± 0.0162	0.2219 ± 0.0201	0.2356 ± 0.0438	0.3906 ± 0.0645	0.4763 ± 0.0296	0.4581 ± 0.0222	0.6205 ± 0.1200
Fatty acids								
arachidic acid	1.8014 ± 0.2671	2.0517 ± 0.0981	2.2935 ± 0.0747	2.2685 ± 0.0869	2.3845 ± 0.1583	2.8529 ± 0.1282	2.5086 ± 0.2037	2.8705 ± 0.3379
behenic acid	3.6140 ± 0.4732	3.9973 ± 0.1815	3.8539 ± 0.2232	3.4973 ± 0.2308	3.4454 ± 0.2878	4.2055 ± 0.1408	3.8244 ± 0.2938	3.9746 ± 0.2191
erucic acid	0.5483 ± 0.0353	0.5301 ± 0.0370	0.6679 ± 0.0354	0.6169 ± 0.0677	0.6265 ± 0.0504	0.6139 ± 0.0396	0.7195 ± 0.0688	0.8018 ± 0.1143
heptadecanoic acid	0.3857 ± 0.0262	0.4234 ± 0.0658	0.4325 ± 0.0447	0.3798 ± 0.0306	0.4206 ± 0.0180	0.5266 ± 0.0905	0.5543 ± 0.0876	0.3581 ± 0.0129
lignoceric acid	7.0559 ± 0.6777	7.5801 ± 0.5759	8.0063 ± 0.6313	7.3177 ± 0.4939	7.6718 ± 0.3477	8.4514 ± 0.1053	$8,2334 \pm 0.6774$	7.3509 ± 1.4234
linoleic acid	0.8650 ± 0.1151	0.9158 ± 0.1869	0.9490 ± 0.1201	0.4695 ± 0.0171	0.4285 ± 0.0398	0.4917 ± 0.0101	0.4362 ± 0.0363	0.4083 ± 0.0193
linolenic acid	1.7469 ± 0.2308	32607 ± 11109	1.8597 ± 0.3735	0.9419 ± 0.0641	0.9883 ± 0.1103	1.0541 ± 0.1806	0.9878 ± 0.1073	0.9326 ± 0.1537
myristic acid	12406 ± 01223	13403 ± 0.0594	14877 ± 0.0980	12647 ± 01761	13383 ± 01287	14553 ± 0.0926	15876 ± 0.3102	15707 ± 01526
myristoleic acid	0.1816 ± 0.0157	0.1812 ± 0.0116	0.2144 ± 0.0012	0.1617 ± 0.0154	0.1995 ± 0.0220	0.1737 ± 0.0096	0.1705 ± 0.0135	0.1965 ± 0.0104
oleic acid+ <i>cis</i> -vaccenic acid	11266 + 01068	15006 + 04263	1.7163 + 0.4498	0.3776 + 0.0437	0.4639 + 0.4327	0.3956 + 0.2535	0.2602 + 0.2602	0.1809 + 0.1809
nalmitic acid	10.6169 + 1.7498	122662 + 12439	11.9569 + 2.8327	111579 + 21381	8.0167 + 0.8927	8.3329 + 2.7452	6.0506 ± 0.4424	10.0884 + 1.3027
palmitoleic acid	10415 ± 0.1806	10724 + 01818	0.9470 ± 0.1366	0.4981 + 0.0808	0.5795 ± 0.1207	0.5323 = 2.7732 0.5302 ± 0.0802	0.3289 ± 0.0822	0.4934 + 0.0881
stearic acid	0.0880 + 1.1203	0.6000 ± 0.0018	10.4877 ± 0.9624	10.7254 + 2.4001	0.5795 ± 0.1207 11.4102 + 0.6748	10.8848 ± 0.9477	13.1147 + 1.8376	15.7781 ± 2.0069
Glycarolinids	J.J000 ± 1.12/J	9.0090 ± 0.9400	10.4077 ± 0.9024	10.7234 ± 2.4001	11.4102 - 0.0740	10.0040 ± 0.9477	15.1147 ± 1.0570	15.7761 ± 2.0707
1 2 3-								
tripentadecanoylglycerol	0.0160 ± 0.0074	0.0143 ± 0.0030	0.0069 ± 0.0015	0.0138 ± 0.0042	<loq< td=""><td><loq< td=""><td><loq< td=""><td><loq< td=""></loq<></td></loq<></td></loq<></td></loq<>	<loq< td=""><td><loq< td=""><td><loq< td=""></loq<></td></loq<></td></loq<>	<loq< td=""><td><loq< td=""></loq<></td></loq<>	<loq< td=""></loq<>
1-oleoyl-rac-glycerol	0.1204 ± 0.0366	0.1088 ± 0.0277	0.0861 ± 0.0116	0.0587 ± 0.0027	0.0566 ± 0.0123	0.0525 ± 0.0032	0.0573 ± 0.0212	0.0326 ± 0.0058
glyceryl trioleate	ND	ND	ND	ND	ND	ND	0.1439 ± 0.0654	0.0611 ± 0.0601
glyceryl tripalmitoleate	0.0259 ± 0.0092	0.0298 ± 0.0025	0.0151 ± 0.0016	0.0203 ± 0.0050	0.0158 ± 0.0032	0.0126 ± 0.0040	0.0121 ± 0.0049	0.0081 ± 0.0010
Glycerophospholipids								
1,2-dilinoleoyl-sn-glycero- 3-phosphocholine	29.8792 ± 2.7491	29.5695 ± 1.8270	34.9049 ± 2.6737	30.1157 ± 1.9066	36.0143 ± 2.5093	28.9695 ± 2.8499	40.7558 ± 5.1545	41.3467 ± 2.9486
1,2-dioleoyl-sn-glycero-3- phosphocholine	23.9174 ± 3.9216	25.8122 ± 1.7495	25.6648 ± 2.4393	23.7158 ± 1.4728	26.2882 ± 1.5262	21.0150 ± 2.7616	24.1152 ± 0.7867	19.2591 ± 5.7697
1,2-dioleoyl-sn-glycero-3- phospho-rac-(1- glycerolycodium salt	0.3091 ± 0.0833	0.3008 ± 0.0608	0.2416 ± 0.0170	0.6754 ± 0.3663	0.3431 ± 0.0266	0.3061 ± 0.0321	0.3994 ± 0.0224	0.4742 ± 0.1090
1-palmitoyl-sn-glycero-3- phosphocholine	0.6521 ± 0.1163	0.7862 ± 0.0524	0.9670 ± 0.0301	0.7638 ± 0.0193	0.9632 ± 0.0857	0.8335 ± 0.0965	1.4127 ± 0.1839	0.8064 ± 0.2725
Sphingolipids								
ceramide	0.0203 ± 0.0031	0.1036 ± 0.0089	0.0285 ± 0.0026	0.1080 ± 0.0051	0.0260 ± 0.0016	0.1444 ± 0.0052	0.0288 ± 0.0043	0.3181 ± 0.0728
Prenols								
oleanolic acid	0.5667 ± 0.0608	0.7009 ± 0.1501	1.0615 ± 0.0831	1.1077 ± 0.1542	4.1770 ± 0.8777	5.9854 ± 1.2622	8.8932 ± 1.4374	7.9509 ± 0.2240

Supplementary Table 2: Quantification of lipids in Bianca grapevine samples in inoculated (I) and not inoculated (NI) samples at different time points, hours post infection (hpi). The concentrations reported represent the average value of 3 biological replicates ± error standard, expressed as mg/kg of fresh leaves.

Supplementary Table 2: Semi-quantification of volatile compounds in Bianca grapevine samples in inoculated (I) and not inoculated (NI) samples at different time points, hours post infection (hpi). The concentrations reported represent the average value of 3 biological replicates \pm error standard, expressed as $\mu g/kg$ of fresh leaves using 1-heptanol as internal standard. Retention time (RT), retention index (Ri) sperimental and theoretical is reported for each compound. The identification confidence is reported with the letter "A" to compare mass spectra and retention time with those of the pure standard, "B" for retention index match on a similar phase column, and "C" for identification with the mass spectral database.

Class Compound RT Ri ident. NI I NI II <							12 hpi		24	hpi	48]	hpi	96 hpi		
Acids Sper. Theor. 3-or-4-hexanoic-acid 35.84 1978 1977 B,C 30.483 \pm 3.008 40.443 \pm 5.555 43.517 \pm 8.175 42.583 \pm 2.888 37.189 \pm 3.453 73.061 \pm 13.254 35.123 \pm 4.866 69.702 \pm 6.499 nonancic-acid 39.83 2182 2180 A,B,C 15.664 \pm 4.800 15.500 \pm 4.226 13.182 \pm 3.571 11.490 \pm 1.065 9.990 \pm 3.532 16.024 \pm 7.958 8.426 \pm 3.189 6.929 \pm 0.478 actionic 37.80 1380 47.124 \pm 12.866 54.677 \mp 7.903 30.690 \pm 0.739 34.260 \pm 4.192 44.453 \pm 8.711 43.614 \pm 6.452 41.619 9.043 \pm 6.574 Alcohols	Class C	Compound	RT	Ri	Ri	ident.	NI	I	NI	Ī	NI	I	NI	I	
3-or-4-hexenoic-acid 35.84 1978 1977 B,C 30.483 \pm 3.008 40.443 \pm 5.555 43.517 \pm 8.175 42.583 \pm 2.888 37.189 \pm 3.453 73.061 \pm 13.254 35.123 \pm 4.866 69.702 \pm 6.409 nonanoic-acid 39.83 2182 2180 A,B,C 15.664 \pm 4.800 15.500 \pm 4.226 13.182 \pm 3.571 11.490 \pm 1.065 9.990 \pm 3.532 16.024 \pm 7.958 8.426 \pm 3.189 6.295 \pm 0.478 octanoic 37.82 2079 2073 A,B,C 47.124 \pm 12.866 54.677 \pm 7.903 30.690 \pm 0.739 34.260 \pm 4.192 44.453 \pm 8.711 43.614 \pm 6.452 41.619 9.049 37.805 \pm 0.674 Alcohols 1.920 1.141 17.049 A,E,C 8.718 \pm 1.312 40.542 \pm 5.769 48.383 \pm 11.426 42.847 \pm 8.743 44.560 \pm 3.979 36.102 \pm 6.739 43.401 \pm 6.824 1.1619 \pm 3.8	Acids	· · · ·		Sper.	Theor.										
nonanoic-acid 39.83 2182 2180 A,B,C 15.664 \pm 4.800 15.500 \pm 4.226 13.182 \pm 3.571 11.490 \pm 1.065 9.990 \pm 3.532 16.024 \pm 7.958 8.426 \pm 3.189 6.295 \pm 0.478 octanoic 37.82 2079 2073 A,B,C 47.124 \pm 12.866 54.677 \pm 7.903 30.690 \pm 0.739 34.260 \pm 4.192 44.453 \pm 8.711 43.614 \pm 6.452 41.619 \pm 9.049 37.805 \pm 0.674 Alcohols cyclobutanemethanol 19.29 1341 C 34.638 \pm 3.565 39.174 \pm 1.312 40.542 \pm 5.769 48.383 \pm 11.426 42.847 \pm 8.743 44.560 \pm 3.979 36.102 \pm 6.739 43.011 \pm 6.824 1-hexanol 2.048 1371 1380 A,B,C 78.68 \pm 13.701 82.788 \pm 15.928 106.862 \pm 5.120 113.007 \pm 5.724 144.793 \pm 15.411 173.290 \pm 10.063 167.780 \pm 26.285 20.359 \pm 32.423 30.	3-	-or-4-hexenoic-acid	35.84	1978	1977	B.C	30.483 ± 3.008	40.443 ± 5.555	43.517 ± 8.175	42.583 ± 2.888	37.189 ± 3.453	73.061 ± 13.254	35.123 ± 4.866	69.702 ± 6.409	
octanoic 37.82 2079 2073 A,B,C 47.124 12.866 54.677 7.903 30.690 ± 0.739 34.260 ± 4.453 ± 8.711 43.614 ± 6.452 41.619 ± 9.049 37.805 ± 0.674 Alcohols cyclobutanemthan 19.29 1341 C 34.660 ± 3.716 41.560 ± 3.707 36.605 39.174 ± 1.312 40.542 5.769 48.383 ± 1.1426 42.847 ± 8.743 44.560 ± 3.979 36.102 ± 6.739 43.401 ± 6.824 1-hexanol-2-ethyl 24.85 1504 1499 A,B,C 78.688 ± 13.701 82.788 ± 15.928 106.862 5.120 113.007 5.724 144.793 ± 15.411 17.3290 ± 10.633 167.780 26.285 220.359 ± 32.142 3-hexen-1-ol-cis/trans 21.52 1397 1391 B,C 304.912 ± 9.983 308.742 ± 47.158 397.190 ± 59	no	onanoic-acid	39.83	2182	2180	A.B.C	15.664 ± 4.800	15.500 ± 4.226	13.182 ± 3.571	11.490 ± 1.065	9.990 ± 3.532	16.024 ± 7.958	8.426 ± 3.189	6.295 ± 0.478	
Alcohols International	00	ctanoic	37.82	2079	2073	ABC	47.124 ± 12.866	54.677 ± 7.903	30.690 ± 0.739	34.260 ± 4.192	44.453 ± 8.711	43.614 ± 6.452	41.619 ± 9.049	37.805 ± 0.674	
cyclobutanemethanol 19.29 1341 C 34.638 \pm 3.565 39.174 \pm 1.312 40.542 \pm 5.769 48.383 \pm 11.426 42.847 \pm 8.743 44.560 \pm 3.979 36.102 \pm 6.739 43.401 \pm 6.824 1-hexanol 20.48 1371 1380 A,B,C 84.969 \pm 3.316 91.086 \pm 17.093 113.293 \pm 14.771 113.544 \pm 4.118 112.231 \pm 7.631 132.203 \pm 6.052 135.888 \pm 14.797 161.257 \pm 17.905 1-hexanol-2-ethyl 24.85 1504 1499 A,B,C 78.688 \pm 13.701 82.788 \pm 15.928 106.862 \pm 5.120 113.007 \pm 5.714 144.793 \pm 15.411 173.290 \pm 10.63 167.780 \pm 26.285 20.359 \pm 36.529 593.289 \pm 10.430 \pm 15.617 118.240 \pm 17.46	Alcohols					,,-									
I-hexanol 20.48 1371 1380 A,B,C 84.969 \pm 3.316 91.086 \pm 17.093 113.293 \pm 14.771 113.544 \pm 4.118 112.231 \pm 7.631 132.203 \pm 6.052 135.888 \pm 14.797 161.257 \pm 17.905 1-hexanol-2-ethyl 24.85 1504 1499 A,B,C 78.688 \pm 13.701 82.788 \pm 15.928 106.862 \pm 5.120 113.007 \pm 5.724 144.793 \pm 15.411 173.290 \pm 10.633 167.780 \pm 26.285 220.359 \pm 32.145 33.6742 \pm 47.158 397.190 \pm 59.570 388.059 \pm 32.760 420.740 \pm 46.102 408.996 \pm 21.048 445.728 \pm 36.140 192.293 \pm 30.648 3-nonen-1-ol 29.82 1698 1696 B,C 121.044 \pm 17.405 135.379 \pm 15.187 167.887 0.943 162.439 <td>CV</td> <td>vclobutanemethanol</td> <td>19.29</td> <td>1341</td> <td></td> <td>С</td> <td>34.638 ± 3.565</td> <td>39.174 ± 1.312</td> <td>40.542 ± 5.769</td> <td>48.383 ± 11.426</td> <td>42.847 ± 8.743</td> <td>44.560 ± 3.979</td> <td>36.102 ± 6.739</td> <td>43.401 ± 6.824</td>	CV	vclobutanemethanol	19.29	1341		С	34.638 ± 3.565	39.174 ± 1.312	40.542 ± 5.769	48.383 ± 11.426	42.847 ± 8.743	44.560 ± 3.979	36.102 ± 6.739	43.401 ± 6.824	
Inhexanol-2-ethyl 24.85 1504 1499 A,B,C 78.688 ± 13.701 82.788 ± 15.928 106.862 ± 5.120 113.007 ± 15.411 173.290 ± 10.063 167.780 ± 22.0359 ± 32.14 3-hexen-1-ol-cis/trans 21.52 1397 1391 B,C 304.912 ± 19.983 308.742 ± 47.158 397.190 ± 59.570 388.059 ± 32.760 420.740 ± 46.102 408.996 ± 21.048 445.728 ± 36.529 593.289 ± 10.742 1-nonanol 29.27 1675 1664 A,B,C 109.714 ± 12.994 103.03 ± 21.912 115.450 ± 15.866 121.771 ± 13.586 160.270 ± 14.746 181.987 ± 36.140 192.293 ± 30.645 3-nonen-1-ol 29.82 1698 1696 B,C 121.044 ± 17.375 ± 162.439 ± 15.562 195.647 ±	1-	-hexanol	20.48	1371	1380	ABC	84.969 ± 3.316	91.086 ± 17.093	113.293 ± 14.771	113.544 ± 4.118	112.231 ± 7.631	132.203 ± 6.052	135.888 ± 14.797	161.257 ± 17.905	
3-hexen-1-ol-cis/trans 21.52 1397 1391 B,C 304.912 19.983 308.742 447.158 397.190 59.570 388.059 ± 32.760 420.740 ± 46.102 408.996 ± 21.048 445.728 ± 36.529 593.289 ± 107.42 1-nonanol 29.27 1675 1664 A,B,C 109.714 ± 12.994 103.303 ± 21.912 115.450 ± 15.806 121.771 ± 13.586 160.270 ± 14.746 181.987 ± 36.529 593.289 ± 30.644 58.570 ± 46.102 408.996 ± 21.048 445.728 ± 36.529 593.289 ± 107.42 ± 30.642 ± 13.586 121.771 ± 13.586 155.885 ± 13.968 160.270 ± 14.746 181.987 ± 36.140 192.293 ± 30.644 $3-00en-1-ol$ 29.82 1698 1696 B,C 71.317 ± 5.752 75.789 ± 7.226 87.709 ± 12.876 90.057 ± 8.076 110.815 ± 3.581 118.240 ± 5.380 127.911 ± 13.718 128.730	1-	-hexanol-2-ethyl	24.85	1504	1499	ABC	78.688 ± 13.701	82.788 ± 15.928	106.862 ± 5.120	113.007 ± 5.724	144.793 ± 15.411	173.290 ± 10.063	167.780 ± 26.285	220.359 ± 32.143	
Innonanol 29.27 1675 1664 A,B,C 109,714 12,994 103,303 \pm 21,912 115,450 \pm 15,806 121,771 \pm 13,586 155,885 \pm 13,968 160,270 \pm 14,746 181,987 \pm 36,140 192,293 \pm 30,644 3-nonen-1-ol 29.82 1698 1696 B,C 121,044 \pm 17,405 135,379 \pm 15,187 167,887 \pm 0,943 162,439 \pm 15,562 195,647 \pm 13,321 224,391 \pm 5,378 203,201 \pm 25,064 246,893 \pm 15,278 1-octanol 26,71 1572 1563 A,B,C 71.317 \pm 5,752 75.789 \pm 7.226 87.709 \pm 12.876 90.057 \pm 8.076 110.815 \pm 3.581 118.240 \pm 5.380 127.911 \pm 13.718 128.730 \pm 8.720 Aldehydes 2,4-heptadienal 25.09 1513 1503 B,C 138.441 \pm 13.542 157.877 \pm 10.188 135.462 \pm 11.982 161.276 \pm 6.259 210.994 \pm 39.470 156.023 \pm 18.286 219.590 \pm 11.771 <td>3.</td> <td>-hexen-1-ol-cis/trans</td> <td>21.52</td> <td>1397</td> <td>1391</td> <td>B.C.</td> <td>304.912 ± 19.983</td> <td>308.742 ± 47.158</td> <td>397.190 ± 59.570</td> <td>388.059 ± 32.760</td> <td>420.740 ± 46.102</td> <td>408.996 ± 21.048</td> <td>445.728 ± 36.529</td> <td>593.289 ± 107.423</td>	3.	-hexen-1-ol-cis/trans	21.52	1397	1391	B.C.	304.912 ± 19.983	308.742 ± 47.158	397.190 ± 59.570	388.059 ± 32.760	420.740 ± 46.102	408.996 ± 21.048	445.728 ± 36.529	593.289 ± 107.423	
3-nonen-1-ol 29.82 1608 1606 B,C 121.044 \pm 17.405 167.887 \pm 0.933 162.439 \pm 13.321 224.391 \pm 5.378 203.01 \pm 25.064 246.893 \pm 15.27k 10.805 \pm 10.805 </td <td>1.</td> <td>-nonanol</td> <td>29.27</td> <td>1675</td> <td>1664</td> <td>ABC</td> <td>109.714 ± 12.994</td> <td>$103\ 303\ \pm\ 21\ 912$</td> <td>115450 ± 15806</td> <td>121.771 ± 13.586</td> <td>155885 ± 13968</td> <td>160.270 ± 14.746</td> <td>181.987 ± 36.140</td> <td>192.293 ± 30.649</td>	1.	-nonanol	29.27	1675	1664	ABC	109.714 ± 12.994	$103\ 303\ \pm\ 21\ 912$	115450 ± 15806	121.771 ± 13.586	155885 ± 13968	160.270 ± 14.746	181.987 ± 36.140	192.293 ± 30.649	
Index Index <t< td=""><td>3.</td><td>-nonen-1-ol</td><td>29.82</td><td>1698</td><td>1696</td><td>B C</td><td>121.044 ± 17.405</td><td>135379 ± 15187</td><td>167.887 ± 0.943</td><td>162.439 ± 15.562</td><td>195.647 ± 13.321</td><td>$224\ 391\ \pm\ 5\ 378$</td><td>$203\ 201\ \pm\ 25\ 064$</td><td>246893 ± 15278</td></t<>	3.	-nonen-1-ol	29.82	1698	1696	B C	121.044 ± 17.405	135379 ± 15187	167.887 ± 0.943	162.439 ± 15.562	195.647 ± 13.321	$224\ 391\ \pm\ 5\ 378$	$203\ 201\ \pm\ 25\ 064$	246893 ± 15278	
Aldehydes 1512 1502 1512 15102 15102 15102 15102 15102 15102 15102 15102 15102 15102 15102 15102 15102 15102 15102 15102 15102 15102 15102 15102 15102 15102 15102 15102 15102 15102 15102 15102 15102 15102 15102 15102 15102 15102 15102 15102 15102 15102 15102 15102 15102 15102 15102 15102 15102 15102 15102 15102 15102 15102 15102 15102 15102 15102 15102 15102 15102 15102 15102 15102 15102 15102 15102 15102 15102 15102 15102 15102 15102 15102 15102 15102 15102 15102 15102 15102 15102 15102 15102 15102 15102 15102 15102 15102 15102 15102 15102 15102 15102 15102 15102 15102 <	1.	-octanol	26.71	1572	1563	ABC	71317 ± 5752	75789 ± 7226	87709 ± 12876	90.057 ± 8.076	110.815 ± 3.581	118240 ± 5380	127.911 ± 13.718	128730 ± 8720	
Antiplets 2,4-heptadienal 25.09 1513 1503 B,C 138.441 \pm 13.542 153.274 \pm 10.861 157.877 \pm 10.188 135.462 \pm 11.982 161.276 \pm 6.259 210.994 \pm 39.470 156.023 \pm 18.286 219.590 \pm 11.771 2-heptanal 19.29 1341 1330 B,C 53.207 \pm 3.518 56.077 \pm 4.332 63.835 \pm 4.697 63.423 \pm 3.192 80.194 \pm 16.664 53.604 \pm 7.801 62.934 \pm 4.386 2,4-hexadienal 22.32 1422 1409 B,C 429.970 \pm 61.955 519.578 \pm 126.424 767.780 \pm 115.477 746.795 \pm 78.308 586.665 \pm 64.827 874.412 \pm 20.499 559.579 \pm 79.047 673.904 \pm 93.316	Aldehvdes	s securior	20.71	1072	1505	п,в,с	11.517 = 5.752	15.169 = 1.220	07.709 = 12.070	<i>y</i> 0.0 <i>01</i> = 0.0 <i>1</i> 0	110.015 = 5.501	110.210 = 5.500	127.911 = 15.710	120.750 = 0.720	
2.4-hexadienal 22.32 1422 1409 B,C 429.970 \pm 61.955 519.578 \pm 126.424 767.780 \pm 115.477 746.795 \pm 78.308 586.665 \pm 64.827 874.412 \pm 20.499 559.579 \pm 79.047 673.904 \pm 93.16	2	, 4-hentadienal	25.09	1513	1503	BC	$138\ 441\ +\ 13\ 542$	153274 + 10861	$157\ 877\ +\ 10\ 188$	135462 + 11982	161 276 + 6 259	210.994 + 39.470	156023 + 18286	219590 + 11771	
2,4-hexadienal 22.32 1422 1409 B,C 429.970 \pm 61.955 519.578 \pm 126.424 767.780 \pm 115.477 746.795 \pm 78.308 586.665 \pm 64.827 874.412 \pm 20.499 559.579 \pm 79.047 673.904 \pm 93.316	2,	-hentenal	19 29	1341	1330	B C	53.207 + 3.518	56.077 + 4.332	63.835 + 4.860	54.905 + 4.697	63423 + 3192	80.194 + 16.664	53.604 + 7.801	62.934 + 4.386	
2_{3} = 10.400 = 10.75 = 10.75 = 10.75 = 10.75 = 10.75 = 10.76 = 110.47 = 10.75 = 10.50 = 500.005 = 04.027 = 014.412 = 20.47 = 015.50 = 015.50 = 015.50 = 015.50 = 015.50 = 015.50 = 015.50 = 015.50 = 015.50 = 015.50 = 015.50 = 015.50 = 015.50 = 015.50 = 015.50 = 015.50 = 015.50 = 015.50 = 015.50 = 015.50 = 015.50 = 015.50 = 015.50 = 015.50 = 015.50 = 015.50 = 015.50 = 015.50 = 015.50 = 015.50 = 015.50 = 015.50 = 015.50 = 015.50 = 015.50 = 015.50 = 015.50 = 015.50 = 015.50 = 015.50 = 015.50 = 015.50 = 015.50 = 015.50 = 015.50 = 015.50 = 015.50 = 015.50 = 015.50 = 015.50 = 015.50 = 015.50 = 015.50 = 015.50 = 015.50 = 015.50 = 015.50 = 015.50 = 015.50 = 015.50 = 015.50 = 015.50 = 015.50 = 015.50 = 015.50 = 015.50 = 015.50 = 015.50 = 015.50 = 015.50 = 015.50 = 015.50 = 015.50 = 015.50 = 015.50 = 015.50 = 015.50 = 015.50 = 015.50 = 015.50 = 015.50 = 015.50 = 015.50 = 015.50 = 015.50 = 015.50 = 015.50 = 015.50 = 015.50 = 005.50 = 005.50 = 005.50 = 005.50 = 005.50 = 005.50 = 005.50 = 005.50 = 005.50 = 005.50 = 005.50 = 005.50 = 005.50 = 005.50 = 005.50 = 005.50 = 005.50 = 005.50 = 005.50 = 005.50 = 005.50 = 005.50 = 005.50 = 005.50 = 005.50 = 005.50 = 005.50 = 005.50 = 005.50 = 005.50 = 005.50 = 005.50 = 005.50 = 005.50 = 005.50 = 005.50 = 005.50 = 005.50 = 005.50 = 005.50 = 005.50 = 005.50 = 005.50 = 005.50 = 005.50 = 005.50 = 005.50 = 005.50 = 005.50 = 005.50 = 005.50 = 005.50 = 005.50 = 005.50 = 005.50 = 005.50 = 005.50 = 005.50 = 005.50 = 005.50 = 005.50 = 005.50 = 005.50 = 005.50 = 005.50 = 005.50 = 005.50 = 005.50 = 005.50 = 005.50 = 005.50 = 005.50 = 005.50 = 005.50 = 005.50 = 005.50 = 005.50 = 005.50 = 005.50 = 005.50 = 005.50 = 005.50 = 005.50 = 005.50 = 005.50 = 005.50 = 005.50 = 005.50 = 005.50 = 005.50 = 005.50 = 005.50 = 005.50 = 005.50 = 005.50 = 005.50 = 005.50 = 005.50 = 005.50 = 005.50 = 005.50 = 005.50 = 005.50 = 005.50 = 005.50 = 005.50 = 005.50 = 005.50 = 005.50 = 005.50 = 005.50 = 005.50 = 005.50 = 005.50 = 005.50 = 005.50 = 005.50 = 005.50 = 005.50 = 005.50 = 005.50 = 005.50 = 005.50 = 005.50 =	2	4-hevadienal	22 32	1422	1409	B C	429.970 + 61.955	519578 + 126424	767780 + 115477	746795 ± 78308	586665 + 64827	874.412 + 20.499	559579 + 79047	673.904 + 93.316	
hexanal 7.45 1103 1089 A B C $4022485 + 216150 4520207 + 544959 5031063 + 843644 3673208 + 378407 3097018 + 617795 5259611 + 659729 4121559 + 584621 4549167 + 56027 4549167 + 56027 4549167 + 56027 4549167 + 56027 4549167 + 56027 4549167 + 56027 4549167 + 56027 4549167 + 56027 4549167 + 56027 4549167 + 56027 4549167 + 56027 4549167 + 56027 4549167 + 56027 4549167 + 56027 4549167 + 56027 4549167 + 56027 4549167 + 56027 4549167 + 56027 4549167 + 56027 4549167 + 56027 4549167 + 56027 4549167 + 56027 4549167 + 56027 4549167 + 56027 4549167 + 56027 4549167 + 56027 4549167 + 56027 4549167 + 56027 4549167 + 56027 4549167 + 56027 4549167 + 56027 4549167 + 56027 4549167 + 56027 4549167 + 56027 4549167 + 56027 4549167 + 56027 4549167 + 56027 4549167 + 56027 4549167 + 56027 4549167 + 56027 4549167 + 56027 4549167 + 56027 4549167 + 56027 4549167 + 56027 4549167 + 56027 4549167 + 56027 4549167 + 56027 4549167 + 56027 4549167 + 56027 4549167 + 56027 4549167 + 56027 4549167 + 56027 4549167 + 56027 4549167 + 56027 4549167 + 56027 + 56027 + 56027 + 56027 + 56027 + 56027 + 56027 + 56027 + 56027 + 56027 + 56027 + 56027 + 56027 + 55027 + 56027 + 56027 + 56027 + 56027 + 56027 + 56027 + 56027 + 56027 + 56027 + 56027 + 56027 + 56027 + 56027 + 56027 + 56027 + 56027 + 56027 + 56027 + 56027 + 56027 + 56027 + 56027 + 56027 + 56027 + 56027 + 56027 + 56027 + 56027 + 56027 + 56027 + 56027 + 56027 + 56027 + 56027 + 56027 + 56027 + 56027 + 56027 + 56027 + 56027 + 56027 + 56027 + 56027 + 56027 + 56027 + 56027 + 56027 + 56027 + 56027 + 56027 + 56027 + 56027 + 56027 + 56027 + 56027 + 56027 + 56027 + 56027 + 56027 + 56027 + 56027 + 56027 + 56027 + 56027 + 56027 + 56027 + 56027 + 56027 + 56027 + 56027 + 56027 + 56027 + 56027 + 56027 + 56027 + 56027 + 56027 + 56027 + 56027 + 56027 + 56027 + 56027 + 56027 + 56027 + 56027 + 56027 + 56027 + 56027 + 56027 + 56027 + 56027 + 56027 + 56027 + 56027 + 56027 + 56027 + 56027 + 56027 + 56027 + 56027 + 56027 + 56027 + 56027 + 56027 + 56027 + 56027 + 56027 + 56027 + 56027 + 56027 + 56027 + 56027 + 56027 + 56027 $	2, h	evanal	7 45	1103	1089	A B C	4022.485 ± 216.150	$4520\ 207\ +\ 544\ 959$	5031063 + 843644	3673.208 + 378.407	3097.018 + 617.795	5259.611 ± 659.729	4121559 ± 584621	4549167 ± 560278	
$\frac{1}{1000} = \frac{1}{100} = \frac{1}{100} = \frac{1}{100} = \frac{1}{100} = \frac{1}{100} = \frac{1}{1000} = \frac{1}{100$	tr	cans_3_hevenal	9.87	1156	1146	A B C	135352 + 19993	154.852 + 28.860	170.986 + 55.129	160260 ± 12136	120402 + 13843	182.956 ± 21.239	158967 + 30243	223798 + 14887	
c_{16} -breenal 10.14 1161 1176 A.B.C. 400.700 + 155.630 528.964 + 143.416 482.302 + 232.701 515.478 + 19.008 265.576 + 76.414 338.516 + 107.677 265.574 + 24.479 191.470 + 71.417	ci	is-3-hevenal	10.14	1161	1176	A B C	490.799 + 155.639	528964 + 143416	$482\ 302\ +\ 232\ 701$	515.478 ± 19.908	265526 + 76414	338516 ± 107677	$265\ 264\ +\ 24\ 979$	191409 + 71412	
$\frac{10}{100} = \frac{10}{100} = 1$	<i>tr</i>	and 2 herenal	13 13	1221	1230	A B C	680.295 ± 55.609	750105 + 114749	841 578 + 151 778	$769 178 \pm 46 808$	730967 + 98903	1041885 ± 04303	1000.652 + 165.377	1308.056 ± 140.788	
$\frac{114152-1162011}{1250}$	<i>u</i> -	entanal	13.15	008	08/	RC	54.016 ± 5.609	52372 + 1003	53378 ± 3340	40.010 ± 3.000	18537 ± 1505	71660 ± 13078	62.263 ± 8.450	68.483 ± 11.400	
$\begin{array}{cccccccccccccccccccccccccccccccccccc$	2	nentenal	9.50	1150	1130	B,C	113175 + 11266	135.288 + 6.882	120000 ± 16685	9.910 ± 9.221 91.814 ± 8.546	43.557 ± 4.595 83.413 ± 12.964	133.014 + 32.105	87.532 ± 11.461	08.483 ± 11.449 08.113 + 15.070	
	Renzenoje	-pentenar de	7.0	1150	1150	b,c	115.175 ± 11.200	155.200 ± 0.002	120.009 ± 10.005	J1.014 ± 0.540	05.415 ± 12.704	155.914 ± 52.105	07.552 - 11.401	JO.115 ± 15.077	
benerous $25.02 + 15.42 + 15.45 + R.C = 72.407 + 4.051 + 0.2118 + 20.002 + 22.036 + 12.070 + 4.778 + 11.822 + 0.100 + 4.226 + 13.4.01 + 14.810 + 84.011 + 13.205 + 13.6.762 + 11.067$	benzenou be	anzaldahuda	25.02	15/13	1545	ABC	72.407 + 4.051	93.118 + 20.002	82 036 + 12 070	84 778 + 11 832	90.199 + 4.326	134.401 + 14.810	84.011 + 13.205	136 762 + 11 962	
binzibility 25.72 1945 1945 R_{10} , (2.47) \pm 4.643 274.410 \pm 70.10 \pm 20.002 \pm 12.277 \pm 34.76 \pm 16.122 $-$ 70.476 \pm 16.120 194.401 \pm 16.417 \pm 16.17 \pm 16.17 \pm 17.102 \pm 17.102 \pm 17.102 \pm 17.102 \pm 17.102 \pm 17.102 \pm 17.102 \pm 17.102 \pm 17.102 \pm 17.102 \pm 17.102 \pm 17.102 \pm 17.102 \pm 17.102 \pm 17.102 \pm 17.102 \pm 17.102 \pm 17.102 \pm 17.102 \pm 17.102 \pm 17.102 \pm 17.102 \pm 17.102 \pm 17.102 \pm 17.102 \pm 17.102 \pm 17.102 \pm 17.102 \pm 17.102 \pm 17.102 \pm 17.102 \pm 17.102 \pm	b	enzylalebool	23.92	180/	1896	A B C	72.497 ± 4.031 254.358 + 46.436	274.410 + 74.218	32.030 ± 12.979 250.605 ± 30.665	358500 ± 66802	333634 + 42838	370417 + 32104	360.058 ± 81.172	130.702 ± 11.902 483.104 ± 75.501	
$ \begin{array}{cccccccccccccccccccccccccccccccccccc$		uganal	20.99	2195	2109	A P C	234.338 ± 40.430 2180 ± 0.671	2192 ± 0.919	5226 ± 0.653	6745 ± 1270	6 100 + 1 027	7.082 ± 1.172	8 116 ± 0.075	403.104 ± 73.301 0.262 \pm 1.712	
$ \begin{array}{c} \text{cugenoid} \\ \text{strong baryle} \\ \text{cugenoid} \\ \text{strong baryle} \\ \text{cugenoid} \\ \text{strong baryle} \\ \text$	ci nl	hanathy alaahal	24.86	1020	1006	A,B,C	3.189 ± 0.071 170 421 \pm 20 111	3.103 ± 0.010 220.028 ± 46.570	3.220 ± 0.033	0.743 ± 1.370 212.067 ± 22.225	0.199 ± 1.037	7.963 ± 1.173	3.110 ± 0.973	9.203 ± 1.713 547.257 ± 52.705	
prefetuly-accord 34.00 1727 1700 A, b, $C = 1/7.431 \pm 27.111 = 20.736 \pm 40.3/7 = 204.077 \pm 4.701 = 310.007 \pm 30.223 = 370.035 \pm 21.807 = 40.135 \pm 44.471 = 443.241 \pm 30.202 = 347.237 \pm 35.703$	Votonor	nenetity-alconor	54.80	1929	1900	A,B,C	$1/9.431 \pm 29.111$	230.938 ± 40.379	264.099 ± 4.901	518.007 ± 58.225	578.055 ± 21.807	$430.133 \pm 44.4/1$	443.241 ± 30.202	347.237 ± 33.703	
	Celones	-cyclonentene_1_													
$\begin{array}{cccccccccccccccccccccccccccccccccccc$	2.	ne-2-pentenvl	31.29	1762		С	40.507 ± 3.907	58.873 ± 7.251	70.144 ± 1.644	53.636 ± 9.549	62.741 ± 3.030	115.985 ± 36.098	62.789 ± 8.459	79.930 ± 19.898	
6-methyl-5-hepten-2-	6-	-methyl-5-hepten-2-										(a a a a a a a a a a a a a a a a a a a	40.050		
one 19.82 1355 1338 A,B,C 42.002 ± 4.812 37.422 ± 1.693 37.623 ± 1.042 32.501 ± 3.377 39.717 ± 2.899 63.229 ± 17.443 40.959 ± 4.580 51.420 ± 11.587	01	ne	19.82	1355	1338	A,B,C	42.002 ± 4.812	37.422 ± 1.693	37.623 ± 1.042	32.501 ± 3.377	39.717 ± 2.899	63.229 ± 17.443	40.959 ± 4.580	51.420 ± 11.587	
penten-3-one 5.5 1037 1029 B,C 101.571 \pm 9.600 125.396 \pm 11.623 111.830 \pm 19.168 98.079 \pm 6.003 78.678 \pm 5.733 123.752 \pm 18.326 81.263 \pm 8.884 106.663 \pm 8.040	pe	enten-3-one	5.5	1037	1029	B,C	101.571 ± 9.600	125.396 ± 11.623	111.830 ± 19.168	98.079 ± 6.003	78.678 ± 5.733	123.752 ± 18.326	81.263 ± 8.884	106.663 ± 8.040	
2,2,6- 18.51 1202 1206 P.C. 16.807 + 1.124 10.220 + 1.611 22.470 + 2.766 20.812 + 2.411 22.260 + 1.162 24.276 + 0.664 21.200 + 2.128 24.026 + 2.210	2,	,2,6-	19 51	1222	1206	PC	16 207 + 1 124	10.320 ± 1.611	22.470 ± 2.766	20.812 ± 2.411	22.260 ± 1.162	24.276 ± 0.664	21200 ± 2128	24.026 ± 2.210	
trimetylciclohexanone $16.31 1322 1300 \text{ B}, C 10.80/ \pm 1.124 19.320 \pm 1.011 23.479 \pm 3.700 20.812 \pm 2.411 23.209 \pm 1.102 24.370 \pm 0.004 21.299 \pm 2.138 24.030 \pm 3.319$	tr	imetylciclohexanone	18.31	1322	1500	ь,c	10.807 ± 1.124	19.320 ± 1.011	25.479 ± 5.700	20.812 ± 2.411	25.209 ± 1.102	24.570 ± 0.004	21.299 ± 2.138	24.030 ± 5.319	
Terpenoids	Terpenoia	ds													
α -ionone 33.57 1867 1846 A,B,C 111.881 ± 4.051 150.706 ± 18.430 148.403 ± 18.711 133.548 ± 18.941 167.989 ± 9.868 219.708 ± 31.141 163.594 ± 19.312 206.073 ± 18.921	α-	-ionone	33.57	1867	1846	A,B,C	111.881 ± 4.051	150.706 ± 18.430	148.403 ± 18.711	133.548 ± 18.941	167.989 ± 9.868	219.708 ± 31.141	163.594 ± 19.312	206.073 ± 18.921	
$a-\text{terpinolen} \qquad 17.05 1290 1270 A,B,C \qquad 1.125 \pm 0.145 \qquad 1.165 \pm 0.198 \qquad 1.360 \pm 0.166 \qquad 1.561 \pm 0.122 \qquad 1.795 \pm 0.072 \qquad 1.931 \pm 0.138 \qquad 2.076 \pm 0.322 \qquad 2.476 \pm 0.209 1.561 \pm 0.122 \qquad 1.795 \pm 0.072 1.931 \pm 0.138 \qquad 1.765 \pm 0.122 1.765 \pm 0.128 \pm 0.12$	α-	-terpinolen	17.05	1290	1270	A,B,C	1.125 ± 0.145	1.165 ± 0.198	1.360 ± 0.166	1.561 ± 0.122	1.795 ± 0.072	1.931 ± 0.138	2.076 ± 0.322	2.476 ± 0.209	
β -ciclocitral 28.21 1631 1607 B,C 274.364 ± 16.008 332.129 ± 31.099 369.070 ± 40.089 334.883 ± 40.795 396.714 ± 14.566 483.400 ± 44.698 382.671 ± 47.068 491.022 ± 10.595	β-	-ciclocitral	28.21	1631	1607	B,C	274.364 ± 16.008	332.129 ± 31.099	369.070 ± 40.089	334.883 ± 40.795	396.714 ± 14.566	483.400 ± 44.698	382.671 ± 47.068	491.022 ± 10.595	
β -ionone 35.41 1957 1964 A,B,C 404.602 ± 22.204 493.373 ± 45.142 534.176 ± 55.469 475.511 ± 68.675 617.463 ± 38.015 756.106 ± 81.930 602.043 ± 62.952 737.539 ± 62.732	β-	-ionone	35.41	1957	1964	A,B,C	404.602 ± 22.204	493.373 ± 45.142	534.176 ± 55.469	475.511 ± 68.675	617.463 ± 38.015	756.106 ± 81.930	602.043 ± 62.952	737.539 ± 62.732	
β -ionone-epoxido 36.5 2011 2003 B,C 133.702 ± 8.727 187.619 ± 22.233 177.508 ± 25.057 179.137 ± 19.657 213.177 ± 5.725 282.591 ± 45.283 187.129 ± 17.140 266.648 ± 21.384	β-	-ionone-epoxido	36.5	2011	2003	B,C	133.702 ± 8.727	187.619 ± 22.233	177.508 ± 25.057	179.137 ± 19.657	213.177 ± 5.725	282.591 ± 45.283	187.129 ± 17.140	266.648 ± 21.384	
citral 31.05 1752 1706 A,B,C 22.818 \pm 1.633 27.610 \pm 4.721 30.212 \pm 4.785 31.616 \pm 3.607 31.828 \pm 1.477 43.820 \pm 4.569 38.492 \pm 6.918 47.865 \pm 2.685	ci	itral	31.05	1752	1706	A,B,C	22.818 ± 1.633	27.610 ± 4.721	30.212 ± 4.785	31.616 ± 3.607	31.828 ± 1.477	43.820 ± 4.569	38.492 ± 6.918	47.865 ± 2.685	

d	lihydroactinidolide	43.15	2352	2316	B,C	101.628 ± 6.474	130.446 ± 15.089	155.255 ± 6.924	126.570 ± 10.113	144.441 ± 6.386	217.067 ± 41.110	145.990 ± 22.850	201.133 ± 20.793
f	arnesene	31.35	1765	1745	A,B,C	214.061 ± 87.764	225.343 ± 71.747	189.046 ± 89.001	138.028 ± 45.629	116.093 ± 15.453	207.216 ± 56.171	62.214 ± 12.954	82.890 ± 1.204
g	geranic acid	43.04	2347	2334	A,B,C	1.462 ± 0.215	1.897 ± 0.531	2.217 ± 0.325	2.332 ± 0.448	2.835 ± 0.236	3.205 ± 0.109	3.406 ± 0.672	4.309 ± 0.819
g	geraniol	33.5	1864	1847	A,B,C	101.723 ± 20.025	105.713 ± 18.328	119.261 ± 14.303	127.239 ± 7.653	171.623 ± 6.794	188.650 ± 10.243	184.212 ± 22.913	239.225 ± 23.392
g	geranylacetone	33.69	1873	1883	B,C	41.210 ± 7.426	46.242 ± 3.688	37.649 ± 3.124	36.198 ± 3.285	50.172 ± 0.826	80.059 ± 20.947	53.146 ± 4.106	76.120 ± 10.927
i	sogeraniol	32.74	1828	1828	B,C	17.087 ± 3.706	17.041 ± 4.071	21.517 ± 0.350	24.667 ± 2.920	26.761 ± 1.612	32.750 ± 6.037	31.240 ± 5.928	42.558 ± 5.343
l	inalool	26.47	1564	1553	A,B,C	17.753 ± 1.294	17.894 ± 2.093	16.566 ± 2.762	20.579 ± 4.301	21.871 ± 3.018	22.787 ± 1.533	22.705 ± 3.263	28.736 ± 2.565
n	nerol	32.48	1816	1808	A,B,C	17.835 ± 3.833	18.916 ± 4.690	24.147 ± 1.533	28.674 ± 4.259	29.522 ± 2.432	33.802 ± 2.032	32.753 ± 4.582	46.369 ± 6.257
Others													
1	-acetyl-ciclohexene	9.07	1138		С	0.898 ± 0.483	1.154 ± 0.134	1.555 ± 0.573	0.952 ± 0.010	1.224 ± 0.292	2.875 ± 1.227	1.515 ± 0.521	2.201 ± 0.681
n	n,n-dibutylformamide	31.88	1788	1767	B,C	34.147 ± 3.618	35.240 ± 7.080	25.326 ± 3.336	32.244 ± 2.792	42.998 ± 3.704	37.595 ± 3.550	46.009 ± 7.562	40.896 ± 2.394
2	2-ethyl-furan	3.79	978	962	B,C	146.633 ± 18.778	159.687 ± 41.192	162.461 ± 61.050	125.446 ± 11.069	110.011 ± 18.293	143.947 ± 25.785	126.079 ± 26.523	82.737 ± 17.578
2	2-hexen-1,4-lactone	31.64	1778	1755	B,C	83.447 ± 16.534	89.232 ± 22.023	92.824 ± 35.974	82.318 ± 5.848	61.977 ± 10.889	72.938 ± 15.301	64.002 ± 17.789	45.716 ± 7.223
c	eis-3-hexenylacetate	18.99	1334	1311	B,C	273.597 ± 89.438	335.107 ± 56.094	303.161 ± 50.345	210.678 ± 20.297	90.068 ± 33.861	138.243 ± 24.720	46.454 ± 7.432	27.475 ± 7.079
Unknown	is:												
u	inknown-1	17.28	1294			11.489 ± 1.921	15.076 ± 1.474	11.705 ± 1.956	9.470 ± 0.639	9.292 ± 0.946	14.388 ± 3.733	9.652 ± 0.756	11.225 ± 2.286
u	inknown-2	18.3	1316			5.180 ± 0.196	7.019 ± 1.090	6.683 ± 2.453	5.882 ± 0.742	4.679 ± 1.539	10.239 ± 0.918	6.306 ± 0.614	9.742 ± 0.359
u	inknown-3	20.13	1362			51.232 ± 1.115	65.047 ± 2.829	71.342 ± 3.998	57.686 ± 6.233	57.906 ± 2.953	73.876 ± 5.387	47.328 ± 4.060	56.973 ± 2.843
u	inknown-4	30.36	1721			70.779 ± 1.927	62.711 ± 0.165	48.533 ± 7.392	54.524 ± 8.556	70.722 ± 2.977	90.622 ± 16.102	85.389 ± 15.040	99.151 ± 15.879
u	inknown-5	32.06	1796			13.168 ± 0.511	17.535 ± 1.004	20.520 ± 2.162	20.229 ± 3.381	25.110 ± 0.672	31.093 ± 5.690	19.706 ± 1.174	26.087 ± 2.528

Supplementary Table 2: Quantification of phenols in Bianca grapevine samples in inoculated (I) and not inoculated (NI) at different time points, hours post infection (hpi). The concentrations reported represent the average value of 3 biological replicates ± error standard, expressed as mg/kg of fresh leaves.

			12 hni	24	hni	48	hni	96	hni
Class	Compound	NI	I	NI	I	NI	I	NI	I
Benzoic Aci	d Derivatives								
	gallic acid	0.509 ± 0.122	0.304 ± 0.039	0.419 ± 0.054	0.462 ± 0.086	2.144 ± 0.126	1.912 ± 0.693	0.595 ± 0.111	2.572 ± 0.076
	<i>p</i> -hydroxybenzoic acid	0.181 ± 0.096	0.189 ± 0.048	0.224 ± 0.068	0.221 ± 0.078	0.182 ± 0.097	0.259 ± 0.119	0.111 ± 0.015	0.235 ± 0.149
	vanillin	0.014 ± 0.009	0.037 ± 0.007	0.049 ± 0.018	0.043 ± 0.012	0.048 ± 0.014	0.093 ± 0.051	0.021 ± 0.012	0.076 ± 0.017
	vanillic acid	0.010 ± 0.004	0.013 ± 0.007	0.014 ± 0.009	0.008 ± 0.006	0.009 ± 0.005	0.012 ± 0.006	0.008 ± 0.004	0.011 ± 0.006
Coumarins									
	esculin	0.273 ± 0.041	0.279 ± 0.028	0.344 ± 0.049	0.280 ± 0.027	0.482 ± 0.050	0.763 ± 0.233	0.322 ± 0.024	0.473 ± 0.070
	fraxin	0.006 ± 0.003	0.012 ± 0.006	0.023 ± 0.006	0.007 ± 0.004	0.030 ± 0.005	0.045 ± 0.034	0.019 ± 0.009	0.020 ± 0.012
Phenylprope	anoids								
	trans-coutaric acid	136.118 ± 17.272	150.176 ± 16.665	152.854 ± 16.985	134.460 ± 5.757	214.978 ± 11.171	243.079 ± 67.475	137.786 ± 6.125	221.437 ± 11.505
	caffeic acid	0.199 ± 0.071	0.260 ± 0.055	0.216 ± 0.045	0.174 ± 0.015	0.486 ± 0.097	0.471 ± 0.103	0.292 ± 0.065	0.285 ± 0.065
	caftaric acid	955.650 ± 113.38	1030.847 ± 126.943	1025.786 ± 87.448	948.470 ± 49.137	1294.351 ± 83.802	1468.795 ± 436.708	897.711 ± 42.342	1348.314 ± 56.839
	fertaric acid	20.369 ± 1.903	21.722 ± 2.453	22.911 ± 1.615	20.387 ± 1.287	37.502 ± 2.663	47.519 ± 13.279	23.949 ± 3.003	40.439 ± 2.817
	ferulic acid	0.023 ± 0.007	0.017 ± 0.007	0.021 ± 0.008	0.008 ± 0.002	0.036 ± 0.013	0.074 ± 0.033	0.045 ± 0.029	0.051 ± 0.011
	sinapic acid	0.219 ± 0.020	0.246 ± 0.043	0.228 ± 0.019	0.225 ± 0.027	0.335 ± 0.026	0.401 ± 0.120	0.221 ± 0.004	0.354 ± 0.013
Dihydrocha	lcones								
	phlorizin	$2.450 \hspace{0.2cm} \pm \hspace{0.2cm} 0.324$	2.990 ± 0.357	2.795 ± 0.442	2.415 ± 0.252	3.628 ± 0.131	5.409 ± 1.355	2.736 ± 0.249	4.047 ± 0.148
Flavones									
	luteolin-7-O-glucoside	0.056 ± 0.004	0.072 ± 0.009	0.082 ± 0.002	0.076 ± 0.009	0.113 ± 0.001	0.123 ± 0.030	0.065 ± 0.000	0.136 ± 0.016
Flavan-3-ol.	S								
	catechin	3.427 ± 0.276	3.523 ± 0.303	3.507 ± 0.507	4.097 ± 0.279	5.828 ± 1.011	4.151 ± 1.188	2.932 ± 0.319	5.399 ± 0.895
	epicatechin	2.376 ± 0.410	1.470 ± 0.269	2.479 ± 0.644	1.555 ± 0.377	2.066 ± 0.241	2.452 ± 1.328	1.545 ± 0.364	2.273 ± 0.458
	epicatechin gallate	1.016 ± 0.111	0.835 ± 0.150	1.048 ± 0.153	0.756 ± 0.321	0.915 ± 0.010	1.025 ± 0.413	0.379 ± 0.089	0.788 ± 0.182
	epigallocatechin	0.863 ± 0.154	1.372 ± 0.295	1.457 ± 0.336	1.104 ± 0.129	0.651 ± 0.251	0.930 ± 0.200	0.721 ± 0.139	0.616 ± 0.102
	epigallocatechin gallate	1.757 ± 0.398	1.529 ± 0.280	1.465 ± 0.171	1.546 ± 0.103	1.153 ± 0.230	1.598 ± 0.400	1.007 ± 0.222	1.327 ± 0.085
	gallocatechin	8.064 ± 0.763	9.477 ± 1.210	9.227 ± 1.665	7.349 ± 0.442	6.022 ± 0.855	7.625 ± 1.615	5.283 ± 0.845	5.640 ± 0.326
	procyanidin B1	10.643 ± 1.200	14.200 ± 1.392	13.358 ± 2.395	12.606 ± 1.437	14.036 ± 1.742	16.315 ± 3.533	10.503 ± 1.205	14.566 ± 1.936
	procyanidin B2 + B4 (as B2)	3.616 ± 0.737	4.170 ± 1.079	3.242 ± 0.474	3.301 ± 0.343	4.145 ± 1.020	4.701 ± 1.562	2.565 ± 0.288	3.043 ± 0.528
	procyanidin B3 (as B1)	$1.494 \hspace{0.2cm} \pm \hspace{0.2cm} 0.348$	1.164 ± 0.134	1.743 ± 0.603	1.547 ± 0.388	2.566 ± 1.358	2.818 ± 0.716	1.217 ± 0.330	1.405 ± 0.236
Flavonols									
	isorhamnetin-3-O- glucoside	0.290 ± 0.008	0.318 ± 0.049	0.337 ± 0.009	$0.297 \ \pm \ 0.006$	0.514 ± 0.059	0.645 ± 0.119	0.232 ± 0.022	0.491 ± 0.120
	isorhamnetin-3- rutinoside	0.373 ± 0.041	0.375 ± 0.112	$0.432 \hspace{0.2cm} \pm \hspace{0.2cm} 0.080$	0.371 ± 0.036	0.637 ± 0.067	0.797 ± 0.341	0.376 ± 0.034	0.601 ± 0.124
	kaempferol-3-O- glucoside	1.953 ± 0.077	2.164 ± 0.141	2.417 ± 0.213	2.325 ± 0.578	3.408 ± 0.320	4.730 ± 0.907	2.161 ± 0.211	3.993 ± 0.479
	kaempferol-3-O- glucuronide	33.631 ± 1.192	33.216 ± 3.357	38.743 ± 5.032	33.716 ± 3.765	58.965 ± 4.289	70.698 ± 19.730	35.773 ± 4.200	66.036 ± 2.375
	kaempferol-3-O- rutinoside	0.706 ± 0.030	0.538 ± 0.024	0.649 ± 0.028	0.590 ± 0.067	1.011 ± 0.132	1.246 ± 0.387	0.589 ± 0.077	1.087 ± 0.130

myricetin	0.160 ± 0.021	0.194 ± 0.041	$0.162 \hspace{0.2cm} \pm \hspace{0.2cm} 0.007$	0.169 ± 0.018	0.205 ± 0.103	0.245 ± 0.060	$0.165 \hspace{0.2cm} \pm \hspace{0.2cm} 0.014$	$0.278 \hspace{0.2cm} \pm \hspace{0.2cm} 0.023$
quercetin-3-								
glucoside+quercetin-3-	23.589 ± 1.273	27.049 ± 3.555	29.414 ± 3.234	25.674 ± 4.266	41.481 ± 2.705	56.874 ± 14.051	24.573 ± 2.014	45.477 ± 1.712
glacioside (as que-5-								
guercetin-3-O-								
glucoside-arabinoside	0.212 ± 0.079	0.220 ± 0.075	0.166 ± 0.055	0.185 ± 0.069	0.264 ± 0.021	0.555 ± 0.207	0.116 ± 0.012	0.397 ± 0.088
quercetin-3-O-	858 665 + 56 470	906 107 + 117 221	907 788 + 59 963	829 646 + 50 520	1450 626 + 85 326	1622 592 + 505 698	825 314 + 57 431	1487359 + 16072
glucuronide	4.526 + 0.051	5140 - 0.016	501.000 = 55.505	029.010 = 90.520	0.104 - 0.005	10.050 + 0.154	025.511 = 57.151	1107.555 = 10.072
rutin	$4./26 \pm 0.2/1$	5.149 ± 0.816	5.219 ± 1.000	4.648 ± 0.450	8.184 ± 0.335	$10.059 \pm 3.4/4$	$4.162 \pm 0.4/8$	9.335 ± 1.320
taxifolin	0.066 ± 0.025	0.108 ± 0.017	0.095 ± 0.017	0.078 ± 0.037	0.097 ± 0.038	0.132 ± 0.077	0.074 ± 0.017	0.158 ± 0.019
Stilbenes + Stilbenoids								
α -viniferin	ND	ND	3.418 ± 0.654	2.963 ± 1.745	59.901 ± 4.428	68.092 ± 19.052	65.357 ± 14.101	190.065 ± 46.783
cis-piceid	1.580 ± 0.193	1.773 ± 0.177	1.678 ± 0.248	1.632 ± 0.165	2.662 ± 0.167	5.291 ± 1.280	1.563 ± 0.173	4.892 ± 0.884
$cis + trans-\omega$ -viniferin	0.107 ± 0.107	ND	ND	ND	0.267 ± 0.267	0.671 ± 0.189	0.263 ± 0.138	0.522 ± 0.282
trans- <i>e</i> -viniferin	0.314 ± 0.041	0.505 ± 0.505	1.168 ± 0.480	1.059 ± 0.290	1.192 ± 0.017	3.628 ± 1.090	1.543 ± 0.443	4.477 ± 1.714
trans-piceide	1.316 ± 0.197	2.306 ± 0.152	2.060 ± 0.267	3.707 ± 0.412	3.995 ± 0.288	9.330 ± 1.365	4.040 ± 0.237	11.859 ± 1.192
trans-resveratrol	0.353 ± 0.087	1.675 ± 0.090	0.359 ± 0.015	0.652 ± 0.134	0.564 ± 0.229	0.864 ± 0.151	0.444 ± 0.115	2.036 ± 0.615
ampelopsin D +	0.001 + 0.001	0.128 + 0.041	0.150 + 0.099	0.123 + 0.103	0.354 + 0.082	0.636 + 0.107	0.245 + 0.011	1188 + 0383
quadrangularin A	0.001 ± 0.001	0.128 ± 0.041	0.150 ± 0.077	0.125 ± 0.105	0.554 ± 0.062	0.050 ± 0.107	0.245 ± 0.011	1.100 ± 0.505
ampelopsin H +	1.604 ± 0.802	2.804 ± 0.414	6.491 ± 0.910	4.726 ± 0.516	17.180 ± 3.713	26.207 ± 7.637	11.521 ± 2.572	24.096 ± 0.435
vaticanol C-like isomer	r							
astringin	0.292 ± 0.045	0.372 ± 0.051	0.363 ± 0.052	0.560 ± 0.103	0.885 ± 0.052	1.391 ± 0.441	0.562 ± 0.059	2.190 ± 0.163
<i>E-cis-</i> miyabenol	ND	ND	0.621 ± 0.399	1.023 ± 0.350	1.981 ± 0.218	2.436 ± 0.626	1.199 ± 0.366	3.560 ± 1.565
isohopeaphenol	0.124 ± 0.124	0.603 ± 0.364	2.841 ± 1.135	1.772 ± 0.774	2.886 ± 0.449	8.107 ± 2.828	5.054 ± 1.439	12.963 ± 2.636
isorhapontin	0.139 ± 0.020	0.216 ± 0.059	0.198 ± 0.048	0.190 ± 0.023	0.288 ± 0.035	0.478 ± 0.090	0.266 ± 0.022	0.779 ± 0.105
pallidol	1.285 ± 0.172	3.194 ± 0.475	3.828 ± 0.750	4.664 ± 1.188	10.100 ± 2.320	20.197 ± 6.078	5.253 ± 1.806	24.218 ± 6.375
Z-miyabenol C	ND	ND	ND	0.011 ± 0.011	2.371 ± 1.876	6.708 ± 3.998	1.115 ± 0.510	9.259 ± 2.501
Others								
arbutin	1.218 ± 0.156	1.548 ± 0.203	1.916 ± 0.076	1.600 ± 0.142	3.526 ± 0.140	4.442 ± 1.215	2.789 ± 0.375	5.944 ± 0.361
caffeic acid+catechin	35.435 ± 2.791	43.572 ± 5.193	42.520 ± 9.312	41.494 ± 3.789	55.908 ± 6.001	71.689 ± 16.017	35.751 ± 5.611	49.815 ± 5.191
condensation product								

Metabolite	hpi	statistic
1-Palmitoyl-sn-glycero-3-phosphocholine	24	5.9
2,4-Hexadienal	48	-3.7
3-or-4-Hexenoic-acid	48	-3.2
3-or-4-Hexenoic-acid	96	-4.1
alpha-Viniferin	96	-3.1
Ampelopsin D + quadrangularin A	12	-8.4
Ampelopsin D + quadrangularin A	96	-4.4
Ampelopsin H + vaticanol C-like isomer	96	-3.0
Arabitol	24	8.5
Arbutin	96	-5.4
Aspartic acid	96	-3.7
Astringin	96	-10.4
Benzaldehyde	48	-3.4
Caftaric acid	96	-6.4
Ceramide	12	-9.3
Ceramide	24	-12.7
Ceramide	48	-23.4
Ceramide	96	-8.0
<i>cis</i> -Piceide	96	-5.2
Fertaric acid	96	-3.9
Gallic acid	96	-8.4
Glycine	12	3.1
Isorhamnetin-3-O-glucoside	24	4.0
Isorhapontin	96	-6.6
Kaempferol-3-O-glucoside	96	-3.7
Kaempferol-3-O-glucuronide	96	-4.9
Kaempferol-3-O-rutinoside	12	4.4
Kaempferol-3-O-rutinoside	96	-3.2
Linoleic acid	24	5.5
Linolenic acid	24	3.2
Luteolin-7-O-glucoside	96	-6.6
Maleic acid	48	-4.4
<i>myo</i> -Inositol	48	-4.1
Myricetin	96	-4.5
Myristoleic acid	24	3.0
Oleic acid+cis-Vaccenic acid	24	5.1
Pallidol	12	-4.5
Pallidol	96	-3.0
Palmitic acid	96	-3.2
Phenylalanine	48	-3.4
Phlorizin	96	-4.2

24	6.6
96	-6.6
96	-4.2
96	-8.6
48	-3.5
96	-4.1
96	-11.9
24	4.0
96	-3.0
24	5.3
96	-7.0
48	-3.5
12	-3.7
24	-3.5
48	-4.8
96	-9.2
12	-5.4
96	-3.4
96	-4.3
12	-4.9
12	4.4
12	-4.1
96	-3.8
	24 96 96 48 96 24 96 24 96 48 12 24 48 96 12 96 96 12 12 12 96

"Omics" technologies have advanced significantly in the last few years. Single datasets still offer only one dimension of an organism's activities, while integrated 'omics' analysis can be the key to deciphering complex biological systems. Comprehensive omics studies have been applied to model plant study, contributing enormously to plant science (Shiratake and Suzuki 2016). Their application makes it possible to build up a relationship between transcripts/genes and metabolites presenting a comprehensive view of biological processes and considering the organisms as complex systems. The Jasmine grapevine cultivar is a variety resistant to *Plasmopara viticola*, obtained in 2000 by P. Kozma and S. Hoffmann in Hungary, deriving from a cross between Bianca and SK 77-4/5 and not officially registered for use in wine production. The resistance phenotype of Jasmine is explained by the *Rpv12* locus, located in chromosome 14, introgressed from *Vitis amurensis*.



To obtain a complete picture of resistant grapevine-*P. viticola* interaction, we monitored metabolite and transcript changes in leaf discs of the resistant variety Jasmine after inoculation with a suspension of *P. viticola*.

My personal contribution to this work started with the experimental design; I personally performed the experiment, the extraction, analysis and data processing of chemical compounds.

The following is a manuscript in preparation.

Chapter V

Multi-omics approach in a resistant grapevine inoculated with *Plamopara viticola*

Abstract

The destructive disease downy mildew causes significant economic losses to viticulture. Plasmopara viticola (Berk. And Curt) Berl. and Toni is the causal agent of the disease and its interaction with the grapevine needs to be further investigated. The use of grapevine varieties with durable resistance to downy mildew is a promising strategy to control the disease. Vitis-P. viticola interaction is still poorly understood, so applying a multi-omics approach can extend knowledge of how the plant system is affected by biotic stress. We used the grapevine variety Jasmine with a QTL providing resistance to P. viticola (Rpv12) to investigate the defence response to the pathogen at metabolite and transcriptional levels. Leaf discs were artificially inoculated and sampling took place at different time points at 12, 24, 48 and 96 hours post inoculation (hpi), together with not inoculated controls. We investigated primary and secondary metabolism using methods of identification and quantification for lipids (LC-MS/MS), phenols (LC-MS/MS) and primary compounds from acids, amino acids, amines/others and sugars (GC-MS), and semi-quantification for volatile compounds (GC-MS). The same samples were used for Rna-seq analysis to evaluate transcriptomic perturbation. The two datasets were explored separately to better highlight the single -omics perturbation caused by pathogen attack. Eighty eight metabolites belonging to several classes show values of the t-statistics indicating a different behaviour between the two conditions. At 12 hours we found only some terpenoid metabolite modulation. The last two time points, 48 and 96 hours were characterised by an increase in some lipid compounds (mostly fatty acids) flavonols and phenylpropanoids. At the latest stage we found an increase in amino acids and sugars after pathogen inoculation. The change in the metabolism is a reflection of transcript modulation. Rna-seq analysis showed 432 differentially expressed genes (DEGs) with general down-regulation at 24 hours and reactivation of metabolic processes at 48 and 96 hpi. A global view of transcriptome perturbation showed general down-regulation at 24 hours post infection, probably to save energy that can be used for defence responses. Metabolic processes seemed to be reactivated at the later time points; amino acid, carbohydrate and lipid related genes were up-regulated, together with secondary metabolism. Multiple Co-Inertia Analysis revealed a strong effect of perturbation due to the time course, with a similar trend in both inoculated and not inoculated samples. Good separation of the two condition samples was shown at 96 hpi; at that time point the effect of the pathogen was strongly manifested, with separation of inoculated and not inoculated samples. Future integration analysis is required to better highlight the correlation between our two -omics datasets.

1. Introduction

Downy mildew is one of the most destructive diseases of the grapevine, causing significant limitations for European grape production in the absence of chemical protection of vineyards; it can cause crop losses and health consequences, due to fungicide application as a control measure. Downy mildew is caused by the biotrophic pathogen *Plasmopara viticola* (Berk. And Curt) Berl. and Toni, native to North America and introduced into Europe at the end of the 19th century (Millardet 1881; Viennot-Bourgin 1949). Since European *Vitis vinifera* grapevines do not have natural resistance to the pathogen, the introgression of resistant genes from resistant *V. rupestris, V. amurensis, V. cinerea, V. riparia* and *Muscadinia rotundifolia* varieties can be an alternative to the use of pesticides (Olmo 1971; Brown et al. 1995; Staudt and Kassemeyer 2015).

The pathogen can attack any grapevine green tissue; it is an obligate biotroph and using a specialised structure called the haustoria, it maintains close interaction with the hosts while keeping the plant alive for its own survival (Whisson et al. 2007; Fawke et al. 2015). Plants are able to detect the presence of the pathogen and employ a defence mechanism. P. viticola is able to infect both susceptible and resistant varieties and complete its life cycle; the first phases in the infection seem to be the same, but in resistant Vitis, sporangia are released at lower rates than in susceptible individuals. The similarity in the first phases of infection suggests the presence of post-infection mechanisms of resistance, including callose deposition, cell wall-associated defence processes, accumulation of reactive oxygen species and hypersensitive response (HR) with necrosis (Gindro et al. 2003; Kortekamp and Zyprian 2003; Díez-Navajas et al. 2008; Bellin et al. 2009; Polesani et al. 2010), up-regulation of genes coding for pathogenesis-related proteins, and defencerelated genes with the production of phytoalexins and antimicrobial compounds (Dercks and Creasy 1989; Dai et al. 1995; Pezet et al. 2004). The study of resistant varieties can help on understanding the mechanisms of resistance against the pathogen; biomarkers discovering may be used as tool for breaders to easily select resistant plants in nurseries and for viticulturist to monitor crops.

Metabolomics analysis using Bianca variety after pathogen inoculation has been performed with the identification of 53 metabolites probably involved in resistance (Chitarrini et al. 2017a). We decided to extend the investigation using a resistant variety containing a different source of resistance. Jasmine is a resistant genotype obtained in 2000 by P. Kozma and S. Hoffmann in Hungary, deriving from a cross between Bianca and SK 77-4/5. It is not yet registered in the European Catalogue for wine production. Jasmine introgressed a QTL providing resistance to P. viticola (Rpv12) from Vitis amurensis, which gives it a high degree of resistance to the pathogen and makes it interesting for finding biomarkers of resistance (Venuti et al. 2013). SK77-4/5 was bred at the University of Novi Sad, Serbia and Montenegro (Cindric et al. 2000) by crossing Kumbarát, which originated from hybridisation of V. amurensis and V. vinifera, and V. vinifera 'Traminer'. Bianca is an hybrid between Villard Blanc and Bouvier, obtained in Hungary in 1963 (Csizmazia and Bereznai 1968). In our previous study we focused on metabolites modulation after pathogen inoculation with the aim to identify biomarkers of resistance (Chitarrini et al. 2017a); in the present work we decided to increase the level of investigation with the analysis of two -omics. Technical developments in genomics, transcriptomics, proteomics and metabolomics have become key tools in the development of systems biology. Metabolomics is a widely used approach of great importance, mainly because plants contain a unique metabolome that changes with the environment, development and following pathogen infection (Fernie et al. 2004). In grapevine research, the main focus in metabolomics studies has been on grape growth, development and ripening, with a focus on grapevine berries of a specific cultivar (Smart et al. 2006; Grimplet et al. 2009; Zamboni et al. 2010; Fortes et al. 2011; Cuadros-Inostroza et al. 2016), or a particular kind of stress (Hong et al. 2012). Recent studies have been performed with metabolomics analysis of the grapevine after P. viticola infection involving a specific class of compound or a small number of identified compounds (Ali et al. 2012; Becker et al. 2013; Algarra Alarcon et al. 2015). Transcriptomics refers to the study of the entire RNA derived from genome; transcriptomic technologies have allowed a better and comprehensive understanding of the transcriptional changes occurring during grapevine response to downy mildew (Polesani et al. 2010; Wu et al. 2010). Several studies have been performed involving transcript investigation in response to pathogens, including fungi, oomycetes, viruses and phytoplasma (Malacarne et al. 2011; Giraud et

al. 2012; Abbà et al. 2014; Almagro et al. 2014; Gauthier et al. 2014; Li et al. 2015). Using a single 'omics' approach it is possible to clarify and show only one dimension of an organism's activities, but it may not be sufficient to characterise the complexity of biological systems (Gygi et al. 1999). Comprehensive omics studies have been applied to model plant study, contributing enormously to plant science (Shiratake and Suzuki 2016). Their application makes it possible to build up a relationship between transcripts/genes and metabolites, offering a comprehensive view of biological processes and considering the organisms as complex systems. Some work has been done on the grapevine using an integrated approach after drought stress (Savoi et al. 2016), on berry developmental stage (Zamboni et al. 2010), light exclusion (Guan et al. 2016) or interaction with powdery mildew (Agudelo-Romero et al. 2015). Integrated transcriptomics and metabolomics analysis could be powerful tool for building up the relationship between informative elements - genes/transcripts - and functional elements - metabolites - in cells (Zhang et al. 2010). The aim of this work was to combine metabolomic and transcriptomic approaches to investigate perturbation in the resistant Jasmine grapevine inoculated with P. viticola.

2. Material and methods

2.1 Plant material and artificial inoculation

The Jasmine mother plants were from the Alto Adige region. Own-rooted vines (n=45) were grown in potted soil in controlled greenhouse conditions. At the 12-leaf shoot stage, the plants were sorted into three homogenous groups, each group representing a biological replicate. At the time of the experiment the plants were healthy, with no evidence of foliar diseases. The third, fourth and fifth fully expanded leaves from the apex were detached and rinsed with ultrapure water. 1.1 cm diameter discs from each leaf were excised with a cork borer and distributed randomly in Petri dishes with the abaxial surface up. Petri dishes were divided into two groups: inoculated samples and not inoculated (control) ones. Inoculated samples were treated by spraying a *P. viticola* sporangial suspension at 10^6 sporangia mL⁻¹, while control samples were treated by spraying ultrapure water (mock). Petri dishes were incubated at 21°C until sampling. Samples were

collected at 12, 24, 48 and 96 hours post infection/mock (hpi) for metabolic analysis and immediately frozen with liquid nitrogen. We obtained 4 Petri dishes for each time point for each condition, which have been grounded together to constitute a single sample. Leaf discs of 1.1 cm were excised from the same plants for transcriptomic analysis; inoculated and control samples were treated in the same way as metabolic ones. At 12, 24, 48 and 96 hours post infection/mock, two leaf discs of 1.1 cm in diameter for each condition were placed in a 2 mL Eppendorf, immediately frozen with liquid nitrogen and stored at -80°C for Rna-seq analysis, performed in collaboration with the genomic platform of the Fondazione Edmund Mach. Three biological replicates per treatment and time point were analysed.

2.2 Metabolite analysis

Primary metabolites were determined following the methodology published by Chitarrini et al. (2017a). One μ L of derivatised extract was injected for GC/MS analysis. The analysis was performed using a Trace GC Ultra combined with a TSQ Quantum GC mass spectrometer and an Triplus autosampler (Thermo Electron Corporation, Waltham, MA). A RXI-5-Sil MS w/Integra-Guard® (fused silica) (30 m x 0.25 mm x 0.25 μ m) column was used for compound separation. Data acquisition was performed in full scan mode from 50 to 700 m/z. Data processing was performed using XCALIBURTM 2.2 SOFTWARE.

Lipid compounds were determined accordingly to Della Corte et al. (2015). Chromatographic analysis was carried out using a UHPLC Dionex 3000 (Thermo Fischer Scientific Germany) with a RP Ascentis Express column (15 cm x 2.1 mm; 2.7 µm C18), following a 30 min multistep linear gradient as described in Della Corte et al., (2015) (Della Corte et al. 2015). The UHPLC system was coupled to an API 5500 triple-quadrupole mass spectrometer (Applied Biosystems/MDS Sciex). Compounds were identified based on their reference standard, retention time and qualifier and quantifier ion, and were quantified (expressed as mg/Kg) from linear calibration curves built with standard solutions using Analyst Software.

Phenolic compounds were determined with a method adapted from Vrhovsek et al. (2012). Chromatographic analysis was performed using a Waters Acquity UPLC system (Milford) with a Waters Acquity HSS T3 column (100 mm x 2.1 mm; 1.8 μ m). Mass spectrometry detection was performed on a Waters Xevo triple-quadrupole mass spectrometer detector (Milford) with an electrospray ionization (ESI) source (Vrhovsek et al. 2012). Compounds were identified based on their reference standard, retention time and qualifier and quantifier ion, and were quantified using their calibration curves and expressed as mg/kg of fresh leaves. Data processing was performed using Waters MassLynx V4.1 software.

Volatile compounds were extracted from grapevine leaves using a method adapted from Matarese et al. (2014) and Salvagnin et al. (2016). A Trace GC Ultra gas chromatograph coupled to a Quantum XLS mass spectrometer (Thermo Scientific, Electron Corporation, Waltham, MA) was used. Compounds were separated using a fused silica Stabilwax[®]-DA column (30 m x 0.25 mm i.d. x 0.25 µm) (Restek Corporation, Bellefonte, USA). The headspace was sampled using 2-cm DVB/CAR/PDMS 50/30 µm fibre from Supelco (Bellefonte, PA). Data processing was performed using XCALIBURTM 2.2 SOFTWARE. Identification of volatile compounds was carried out by injecting pure reference standards when available, or comparing retention index and mass spectra using the NIST MS Search 2.0 database. Results were expressed in $\mu g/kg$ with semi-quantification using 1-heptanol. All the metabolite extraction methods and instrumental conditions were adapted for the leaf matrix as previously described (Chitarrini et al. 2017a).

2.3 RNA extraction and RNA sequencing analysis

One-hundred milligrams of tissue were ground to powder with liquid nitrogen. RNA was isolated according to the manufacturer's instructions of Spectrum[™] Plant Total RNA Kit (Sigma-Aldrich, Germany) commercial kit. Total RNA quality was checked on RNA ScreenTape with an Agilent 2200 Tapestation (Agilent Technologies, USA). Starting from 1µg of high quality total RNA, 120 cDNA libraries were constructed according to the KAPA Stranded mRNA-Seq Kit (Kapa Biosystems, Massachusetts, US). Each library was baracoded using the SeqCap Adapter kit A and B (NimbleGen, Roche) and the final size of 250-280bp was confirmed on High Sensitivity D1000 ScreenTape with the

Tapestation 2200 (Agilent Technologies, USA). All the libraries were quantified with a KAPA Library Quantification kit – Illumina (Kapa Biosystems, Massachusetts, US) using the LightCycler 480 (Roche, Mannheim) and multiplexed random in 4 pools in equimolar way. Each pooled library was sequenced on an Illumina HiSeq 2500 platform with paired end runs of 2×50 bps. Base calling and quality control were performed on the Illumina RTA sequence analysis pipeline.

2.4 Data analysis

Statistical analysis and data visualisation were performed with custom R scripts (R Core Team 2017). Any missing values presents in the metabolomics dataset were imputed with a random value between zero and the LOQ. The metabolite concentrations were transformed using the base 10 logarithm, in order to make data distribution more normal-like (van den Berg et al. 2006). Metabolite principal component analysis (PCA) was performed on the obtained multidimensional dataset, after mean centring and unit scaling, using the FactoMineR and Factoextra R packages (Lê S., Josse J., Husson F. 2008; Kassambara A., Mundt F. 2017). The t-statistic was computed using the Stats package (R Core Team 2017), while network visualisation exploited the ggraph package (Pedersen T. L. 2017).

Raw sequenced reads were aligned to the grapevine transcriptome of reference (PN40024 12X CRIBI) (Jaillon et al. 2007; Wang et al. 2015) using Bowtie2 software (version 2.1.0, www.sourceforge.net/projects/bowtie-bio/files/bowtie2) (Langmead 2010). An average of 26,240,191 M 50 nt pair ends reads was generated per sample. The average percentage of reads aligned to the transcriptome reference for all the samples was 84.71%. Differentially expressed genes (DEGs) analysis was performed with the R package DeSeq2 (Love et al. 2014). Transcripts PCAs were performed with the FactoMineR and Factoextra R packages (Lê S., Josse J., Husson F. 2008; Kassambara A., Mundt F. 2017). Gene enrichment analysis was performed using blast2GO (http://www.blast2go.org/). Functional annotations of genes were retrieved from Grimplet et al. (2012), VitisCyc and CRIBI V2.1 version (http://genomes.cribi.unipd.it/DATA/) (Grimplet et al. 2014). MCIA on the two dataset was performed using the omicsade4 R package and the results were visualized using custom R scripts.

3. Results and Discussion

3.1 Impact on the metabolome

To evaluate metabolic perturbation after *P. viticola* infection, targeted metabolite analysis was undertaken at all time points in inoculated and not inoculated samples. A total of 175 metabolites were identified and quantified, belonging to acids, amino acids, amines and others, sugars, carnitines, sterols, fatty acids, glycerolipids, glycerophospholipids, sphingolipids, prenols, benzoic acid derivates, coumarins, phenylpropanoids, dihydrochalcones, flavones, flavan-3-ols, flavonols, stilbenes and stilbenoids and other phenolics. All these were quantified with the relative standard and expressed as mg/kg of fresh leaves, while volatile acids, alcohols, aldehydes, benzenoids, ketones, terpenoids, other VOCs and unknown VOCs were semi-quantified as the equivalent of the internal standard (1-heptanol) and expressed as μ g/kg of fresh leaves (Table V. 1). A complete list of the compounds identified and their concentration is reported in Table V. S1-S2-S3-S4.

	CLASS	#		CLASS	#
	acids	17		carnitines	1
ndŝ	amino acids	13	ds	sterols	3
noc	amines	3	un	fatty acids	13
1 L	sugars	14	bd	glycero lipids	4
/ 00			con	glycero phospholipids	4
าลท			pid	sphingolipids	1
rin			Ë	prenols	1
r			1		
	CLASS	#		CLASS	#
10	benzoic acid derivateds	4	Ś	acids	3
nds	coumarins	2	pu	alchohols	7
nor	phenylpropanoids	6	noc	aldehydes	9
du	dihydrochalcones	1	d u	benzenoids	4
2	flavones	1	00	ketones	4
oua	flavan-3-ols	9	atile	terpenoids	14
Phe	flavonols	11	/ola	other	4
	stilbenes+stilbenoids	14	-	unknown	6
1	other phenolic	2			

Table V. 1: Class and number of compounds identified and quantified in Jasmine samples for primary, lipid, phenol and volatile compounds

The metabolic response was explored by using a PCA (Fig V. 1): the first two dimension explain the 49.1% of the total variance. The three biological replicates are reported as small points (dots correspond to inoculated samples, triangles to non inoculated) and are linked to their mean value (biggest point). The analysis of all the measured compounds revealed a trend based on time (from top left to bottom right), which is highlighted with different colours (from red, corresponding to 12 hpi, to violet, corresponding to 96 hpi) as well as a separation between inoculated samples and not inoculated mostly at 12, 48 and 96 hpi.



Fig. V. 1. Principal component analysis performed on the log 10-transformed metabolite concentration of all analysed compounds. For each time point, three biological replicates (smaller dots) are represented for each condition (circle: inoculated samples; triangle: not inoculated) and linked with their means (larger dots). Each time point is represented with a different colour: red for samples collected at 12 hpi, blue for 24 hpi samples, green for 48 hpi samples and violet for 96 hpi samples.

Analyzing the classes of compounds separately, we noticed a different behaviour for each class (see Fig. V.2). Among primary metabolites (Fig. V. 2A) we notice a separation between the two conditions at 96 hours, which correspond to the violet points very close by. The time trend is captured by the first component, more than the difference between the two conditions. The points at 12 and 48 hpi lying far away from their means indicates high variability among biological replicates. In particular, it is due to a different sugar and amino acid profile in one biological replicate compared to the remaining ones. Lipid compounds (Fig. V. 2B) seem to be mostly different between inoculated samples and not inoculated ones at 24 and 48 hpi. Now the time trend is captured by the second component (from top to botton), while the first axis separates the two conditions. Polyphenols PCA (Fig. V. 2C) shows a separation at 48 and 96 hpi and volatiles (Fig. V. 2D) are mainly perturbed at 12 and then at 96 hours caused by the pathogen infection. Again the time trend can be noted from top to bottom, indicating that it is captured by the second component.



Fig. V. 2. Principal component analysis of the log 10-transformed metabolite concentration of individual classes: A) primary compounds B) lipids C) phenol compounds D) volatile compounds. For each time point, three biological replicates (smaller dots) are represented for each condition (circle: inoculated samples; triangle: not inoculated) and are linked with their means (larger dots). Each time point is represented with a different colour: red for samples collected12 hours post-infection (hpi), blue for 24 hpi samples, green for 48 hpi samples and violet for 96 hpi samples.

In order to focus on the metabolites which are mostly different between inoculated samples and not inoculated, we selected 88 metabolites having absolute value of the t statistic greater than 3 (|t|>3) in at least one time point (Fig. V. S1; Table V. S5) (Fig. V. 3). We summarized the information in the network represented in Fig. V. 3. The network contains 88 nodes, each one representing one metabolite. A link is drawn between two metabolites only if both are among those most different (|t|>3) at the same time point. In the same visualisation the compound classes is highlighted by the color of the nodes and the time course information by color of the link (Fig. V. 3).

The *P. viticola*-Jasmine interaction perturbs the metabolome with a higher number of compounds modulated in comparison with the same experiment performed on Bianca variety, (Chitarrini et al. 2017a). A small number of VOCs and primary compounds appear to be modulated at 12 hpi, as occurred in Bianca; in particular, terpenoid compounds such as α -terpinolen, farnesene, geraniol, dihydroactinolide and β -ionone epoxide were present in higher concentrations in inoculated samples at this early stage. As has been previously reported, our results suggest that volatile compounds may have a role in plant defence against pathogens, interfering at very early stages with pathogen endophytic invasion of mesophyll air space (Gershenzon and Dudareva 2007; Chitarrini et al. 2017a). No high modulation was found at 24 hpi, with the presence of few perturbed VOCs. We identified the largest number of nodes in the network at the 48 hpi time point, indicating that metabolic changes involving in particular phenol and lipid compounds were maximal at that time. Among lipids we found higher concentrations in inoculated samples for some fatty acids, in contrast to Bianca in the same experimental conditions. In Bianca we found a faster decrease in some unsaturated fatty acids after P. viticola inoculation (Chitarrini et al. 2017a). In Jasmine fatty acids such as arachidic acid, oleic acid+cis-vaccenic acid, myristic acid, linolenic acid, stearic acid, lignoceric acid and some glycerolipids and gliycerophospholipids were accumulated after pathogen inoculation. Many functions of fatty acids are connected with the adjustment of membrane fluidity mediated by desaturases. Fatty acids have an important role in plant defence against environmental factors and pathogens, since they are modulators in transduction signal pathways (Walley et al., 2013). In particular, polyunsaturated fatty acids are released from the membrane by lipase in response to biotic stress, with an important role as oxylipins. Of these, trienoic fatty acids are involved in response to

pathogens; linolenic acid in particular (18:3) is reported to directly activate NADPHoxidase and, by extension, to generate reactive oxygen intermediates after inoculation with *Pseudomonas syringae* pv. *tomato* (Yaeno et al. 2004). In the light of our findings, linolenic acid and other fatty acids may have a role in Jasmine response to *P. viticola*. Previous works have found prenols to be the class of lipids involved in the membrane structure and the redox mechanism, and in plant defence mechanisms (Enfissi et al. 2005; Osbourn et al. 2011). In Jasmine and in our previous work with Bianca, the oleanolic acid concentration increased in both conditions, so its modulation is not therefore specific to the defence response (Chitarrini et al. 2017a, b). The progressive accumulation of stressrelated compounds in leaf discs in both conditions can be explained by other stress affecting the tissues as a consequence of leaf removal, punching of the leaf lamina and artificial conditions for leaf disc incubation.

At 48 hpi phenols appeared to be highly modulated in response to the pathogen, particularly flavonols such as isorhamnetin-3-rutinoside, quercetin-3-glucoside+quercetin-3-galactoside, quercetin-3-O-glucuronide and rutin. Stilbenes and stilbenoids such as *trans*-resveratrol, pallidol, *trans*-piceid and astringin were modulated from 24 to 96 hpi. Phenylpropanoids were modulated at 48 hpi, comparing inoculated with not inoculated samples, in particular *trans*-coutaric acid, sinapic acid and caftaric acid. All these compounds showed higher concentrations after pathogen infection. Our results are in agreement with statements in previous works about the importance of phenylpropanoid and flavonoids in resistance against pathogens due to their antimicrobial proprieties (Dixon et al. 2002; Ali et al. 2012; Chitarrini et al. 2017a).

At 96 hours primary metabolism was modulated, in particular in sugars and amino acid compounds with a higher accumulation after *P. viticola* infection. It has been demonstrated that sugar concentration, sucrose in particular, can modulate the expression of genes related to photosynthesis, respiration, nitrogen and defence processes acting as signal molecules in plants (Jang et al. 1997; Ferri et al. 2011).

A large number of connections are presented in the graph and the subgraphs are not clearly separated; this means that a large number of compounds showed differences between the two conditions at more than one time point (connected with different coloured lines). For example, we found that ceramide was connected at all times, indeed it accumulated significantly from very early on and continued along the entire time course in inoculated samples. Ceramide is thought to carry out signalling activity in the activation of defence-related plant programmed cell death (PCD), as recently discussed in the Bianca grapevine study (Kachroo and Kachroo 2009; Berkey et al. 2012; Chitarrini et al. 2017a). We also found *trans*-resveratrol, pallidol, serine and benzaldehyde to have differences at more than one time point, probably having a key role in plant response to the pathogen.

The picture for metabolite changes appears to be complex in Jasmine; we indeed found a high number of metabolites modulated after pathogen inoculation. Furthermore, we found earlier activation of metabolite changes as compared to Bianca, with the largest number of modulated metabolites at 48 hpi, including phenols and lipid compounds (Chitarrini et al. 2017a).



Fig. V. 3: Network plot representing the 88 most different metabolites in inoculated and not inoculated samples, corresponding to compounds having an absolute value of the tstatistic greater than 3 (|t| > 3). Each of the 88 compounds is represented with a dot; dots belonging to different classes are in different colours (green: lipids; brown: polyphenols; violet: primary compounds; pink: volatile compounds). Metabolites with differences after infection modulated at the same time point are linked using the colour link of the specific time point (red: 12 hpi; blue: 24 hpi; green: 48 hpi; violet: 96 hpi).

3.2 Impact on the transcriptome

We compared the transcriptome of inoculated samples and the not inoculated one (mock) at different time point (12, 24, 48 and 96 hours post infection/mock). We focused on transcript having absolute value of the log2 of the Fold Change greater than 1 (|log2FC|>1) and adjusted p-value<0.05. The choice should ensure to focus on the total number of differentially expressed genes (DEGs) between inoculated samples and control (not inoculated). We found 432 DEGs including alternative transcripts for 35 genes (Table V. S6). The entire transcriptome was taken into account for the PCAs in which the first two dimension allow the separation of 12 and 96 hours samples from the other time points based on the time; using the second and third dimension we can observe a better separation of all time points in both condition, without separation between inoculated and not inoculated (Fig. V. 4 A-B).



Fig. V. 4: Principal component analysis performed on the entire transcriptome. A: dimension 1 (27.3%) dimension 2 (11.4%); B: dimension 2 (11.4%), dimension 3 (5.9%) For each time point, three biological replicates (smaller dots) are represented for each condition (circle: inoculated samples; triangle: not inoculated) and linked with their means (larger dots). Each time point is represented with a different colour: red for samples collected at 12 hpi, blue for 24 hpi samples, green for 48 hpi samples and violet for 96 hpi samples.

PCA obtained using only DEGs showed clear separation between inoculated and not inoculated at 96 hours along the diagonal of the first two dimensions; using the second and third dimensions we also obtained slight separation of the two conditions at 24 and 48 hours (Fig. V. 5 A-B).



Fig. V. 5: Principal component analysis performed on the DEGs. A dimension 1 (48.4%) dimension 2 (20.6%); B dimension 2 (20.6%), dimension 3 (11.3%) For each time point, three biological replicates (smaller dots) are represented for each condition (circle: inoculated samples; triangle: not inoculated) and linked with their means (larger dots). Each time point is represented with a different colour: red for samples collected at 12 hpi, blue for 24 hpi samples, green for 48 hpi samples and violet for 96 hpi samples.

P. viticola infection modulated the expression of only 2 genes up-regulated at 12 hpi; 70 genes (17 up-regulated; 53 down-regulated) at 24 hpi; 50 genes (41 up-regulated; 9 down-regulated) at 48 hpi and 340 genes (326 up-regulated; 14 down-regulated) at 96 hpi. A global view of the transcriptome perturbation shows a general down regulation at 24 hours post infection and an up regulation at 48 and 96 hours. Some genes were differentially regulated in unison among two or three time points as reports the Venn diagram (Fig. V. 6).



Fig. V. 6: Common and unique DEGs at 12, 24, 48 and 96 hpi are represented in the Venn diagram.

We focused our attention on the top 100 enriched GO terms (biological process) present in our DEGs. The 20 most meaningful GO categories are shown in Table V. 2.

GO.ID	Term	Annotated	Significant	Expected	
GO:0050832	defense response to fungus	814	30	5.98	1.10E-12
GO:0010200	response to chitin	639	28	4.7	2.00E-12
GO:0050691	regulation of defense response to virus by host	9	6	0.07	1.24E-11
GO:0009811	stilbene biosynthetic process	172	13	1.26	5.79E-10
GO:0009751	response to salicylic acid	848	30	6.23	7.99E-09
GO:0009805	coumarin biosynthetic process	217	13	1.6	9.77E-09
GO:0009611	response to wounding	765	23	5.62	1.62E-08
GO:0009625	response to insect	77	8	0.57	1.05E-07
GO:0009809	lignin biosynthetic process	274	13	2.01	1.51E-07
GO:0009862	systemic acquired resistance, salicylic acid mediated signaling pathwat	336	14	2.47	2.45E-07
GO:0009867	jasmonic acid mediated signaling pathway	417	17	3.07	8.17E-07
GO:0042742	defense response to bacterium	1062	24	7.81	1.43E-06
GO:0010112	regulation of systemic acquired resistance	49	6	0.36	1.61E-06
GO:0018879	biphenyl metabolic process	32	5	0.24	3.55E-06
GO:0010363	regulation of plant-type hypersensitive response	513	15	3.77	7.40E-06
GO:0000165	MAPK cascade	783	15	5.76	8.57E-06
GO:0042184	xylene catabolic process	40	5	0.29	1.11E-05
GO:0042203	toluene catabolic process	40	5	0.29	1.11E-05
GO:0009595	detection of biotic stimulus	191	10	1.4	1.18E-05
GO:0046417	chorismate metabolic process	41	5	0.3	1.25E-05

Table V. 2: Top 20 enriched GOs categories presents in our DEGs.

At 12 hpi only 2 genes were differentially expressed (|log2FC|>1 and padj<0.05). Indole-3-acetic acid amido synthetase (IAA) *VIT_207s0129g00660.1* was over-expressed at 12; blue copper protein gene *VIT_209s0002g06890.1* was up-regulated and differentially expressed in a significant manner at 12, 24 and 48 hpi in inoculated samples compared to the control (Figure V. 7). On manually exploring DEGs belonging to the "*metabolic process*" (GO:0008152), we found general down-regulation at 24 hpi. It has been suggested that the energy saved by down-regulation of primary metabolism can be used for defence responses (Rojas et al. 2014). In our study the "*tyrosine metabolic process*" (GO:0006570) and "*L-phenylalanine metabolic process*" (GO:0006558) were down-regulated at 24 hpi. Secondary metabolism was also down-regulated at 24 hpi, in particular the "*stilbene biosynthetic process*" (GO:0009811), "*coumarin biosynthetic process*" (GO:0009805), "*flavonol biosynthetic process*" (GO:0051555) and "*carotenoid biosynthetic process*" (GO:0016117).



Fig. V. 7: Heatmap representation of log2 fold change (inoculated/not inoculated) interesting genes. Blue and red boxes represent down and up regulation of the gene under pathogen attack. Asterisks identify significant differences (p<0.05) between inoculated and control. For VIT 216s0098g00860 we reported two alternatives transcripts (.1 and .2). IAA: indole-3-acetic acid; CH: chitinase; PR: pathogenesis-related protein; BGS: beta glucanase; F3DO: flavanone-3-dioxygenase; CaMBP: calmodulin binding protein; TLP: thaumatin like protein; CAB: chlorophyll a-b binding protein; LHCB2: light harvesring complex ii protein.

In Jasmine inoculated samples, we found up-regulation of the "*plant hypersensitive response*" (GO:0009626) at 48-96 hpi. It is known that biotrophic pathogens mainly activate the SA-dependent signalling pathway in the host; in our DEGs we found GO

specific categories such as the "salicylic acid biosynthetic process" (GO:0009697), "systemic acquired resistance, salicylic acid mediated signalling pathway" (GO:0009862) and "response to salicylic acid stimulus" (GO:0009751) up-regulated at 48 and 96 hpi. The plant hypersensitive response led to activation of signalling transduction pathways in inoculated samples. Indeed, we found activation of the "MAPK cascade" category (GO:0000165) present in the top 20 enriched GOs (Table V. 2) previously identified as a signalling cascade fundamental for physiological functions involved in hormonal responses, cell cycle regulation, abiotic stress signalling, and defence mechanisms (Tena et al. 2001). For instance, up-regulation of a gene encoding the calmodulin-binding protein VIT 209s0002g04560.1 was significant at 48 and 96 hpi in inoculated samples (Fig. V. 7). Calmoduline-binding proteins play crucial roles in cellular signalling cascades through the regulation of numerous target proteins (Ranty et al. 2006). We also observed the activation of the pathogenesis related protein (PR-1) gene *VIT 203s0088g00910.1* at 24, 48 and 96 hpi in inoculated samples (Fig. V. 7). As already well-described, PR genes play a crucial role in plant resistance to various pathogens (van Loon et al. 2006; Liu et al. 2017). Members of PR-5 class proteins are also called thaumatin-like proteins (TLPs) because of their sequence similarity with the sweet-tasting protein thaumatin from Thaumatococcus danielli. In our DEGs we found up-regulation of the thaumatin-like protein VIT 202s0025g04330.1 at 48 and 96 hpi after P. viticola inoculation (Fig. IV. 7). Once resistance is induced, the plant expresses a number of inducible defence responses, including the production of cell wall lytic enzymes such as chitinases and 1,3-B-glucanases (Lawton and Lamb 1987). Interestingly, after P. viticola inoculation we found enrichment of the "response to chitin" (GO:0010200), "chitin catabolic process" (GO:0006032) and "cellular response to chitin" (GO:0071323) GO categories and up-regulation of chitinase VIT 204s0008g00140.1 at 24, 48 and 96 hpi after pathogen inoculation. Oomycetes differ from true fungi in terms of the presence of cellulose in the oomycete cell wall, as opposed to chitin in true fungi. Despite this, Werner et al. (2002) demonstrated chitin synthesis during growth and asexual propagation of *P.viticola*, with the presence of chitin on the cell walls of the hyphae, sporangiophores and sporangia of the grape downy mildew pathogen (Werner et al. 2002). In addition to this, we also found up-regulation of the "cell wall macromolecule catabolic process" (GO:0016998). At 24 and 48 hpi we also found up-regulation of betaglucanases

VIT_208s0007g06040.1 and *VIT_208s0007g06060.1* (Fig. V. 7). Metabolic processes seem to be reactivated at later time points; amino acid, carbohydrate and lipids were upregulated, together with secondary metabolites such as the "*coumarin biosynthetic process*" (GO:0009805), and "*stilbene biosynthetic process*" (GO:0009811) present in the top 20 GO categories (Table V. 2) and flavanone 3-dioxygenase-like gene *VIT_216s0098g00860*, expressed with two alternative transcripts significantly upregulated at 48 and 96 hpi (Fig. V. 7). Although the photosynthesis process can produce Reactive Oxygen Species useful for localised cell death against the pathogen, its down-regulation could be a mechanism to alleviate plant energy expenditure (Zurbriggen et al. 2010; Rojas et al. 2014). We found significant down-regulation of chlorophyll a-b binding protein *VIT_210s0003g02900.1*, chlorophyll a-b binding protein 4 precursor homolog *VIT_217s0000g06350.1* and light-harvesting complex ii protein lhcb *VIT_210s0003g02890.1*, involved in photosynthesis and photosynthetic electron transport chain processes at 96 hpi.

3.3 MCIA of metabolites and transcripts

Among multivariate dimension reduction approaches, we choose MCIA, an exploratory data analysis method that identifies co-relationships between multiple high dimensional datasets (Meng et al. 2014) to integrate the two datasets and explore them jointly. In particular, MCIA can be applied when the same samples are measured using different omics techniques. It projects both the datasets into the same dimensional space and then solves an optimization problem using a covariance criterion. The results of the analysis are reported in Fig. V. 8. Transcripts and metabolites are represented as grey filled square and black filled dot respectively. The projection of both datasets is represented; inoculated samples (big coloured circles) and not inoclulated samples (big coloured triangles) are identified with different colours based on the time (red for samples collected at 12 hpi, blue for 24 hpi samples, green for 48 hpi samples and violet for 96 hpi samples). Close to each sample we can observe the small filled square (transcript dataset) and unfilled circle (metabolite dataset) that represents the respective omic projection. The distance between the symbol and the relative sample shows us how the two datasets are coherent. In view of this, we found very good consistency in not inoculated samples at 96 hours,

which did not occur in inoculated ones, for which the two datasets appear in the opposite position to the sample. The MCIA is dominated by the time course. It is worth noting here that the MCIA is computed on all the transcriptomic data, which gives us a global picture of what is happening in the cell and is not determined only by the pathogen interaction. The analysis shows that at 96 hpi the effect of the pathogen is strongly manifested with a separation of inoculated from not inoculated samples. Anyway future integration analysis are required to better highlit the correlation between our two –omics datasets.



Fig. V. 8: MCIA analysis of two –omics datasets. Each transcript is represented with a grey filled square and each metabolite with a black filled dot. For each time point, three biological replicates are represented for each condition (circle: inoculated samples; triangle: not inoculated). Each time point is represented with a different colour: red for samples collected at 12 hpi, blue for 24 hpi samples, green for 48 hpi samples and violet for 96 hpi samples. Two connections with each sample are shown: a small filled square represents the sample position taking into account only the metabolomic dataset; a small unfilled dot represents the sample position taking into account only the transcriptomic dataset. The distance between the two symbols indicates the similarity between the two –omics.

4. Conclusions

In this study a combined metabolomics and transcriptomics approach was applied to generate a global picture of the defence reaction mounted by Jasmine in response to *P*. *viticola* inoculation. Activation of the defence response induced the accumulation of volatile compounds (12-24 hpi) and lipids (48 hpi). The primary metabolism was down-regulated during the first hours post inoculation, concomitant with the up-regulation of genes related to specific defence mechanisms, hypersensitive response and signal transduction, in order to quickly counteract pathogen attack. Afterwards, genes belonging to GOs typical of primary and secondary metabolism were activated or over-expressed, together with the accumulation of important chemical compounds with potential antimicrobic properties, such us lipids and phenol compounds. Despite the advancements made in hightroughput technologies, integration of these data remains a challenge. In this study two –omics were combined to elucidate the interaction of a resistant variety Jasmine with *P. viticola*. Applying MCIA analysis we were able to integrate the entire two datasets but further integration analysis is needed to better correlate the two –omics.

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Supplementary Figure V. S1: t-statistic values of our metabolites. For network analysis we took into account only metabolites with |t| > 3.

	12	hpi	24	hpi	4	8 hpi	96	hpi
Class Compound Acids	NI	Ι	NI	I	NI	Ι	NI	I
malonic acid	3.461 ± 0.118	4.334 ± 0.232	5.452 ± 0.213	4.987 ± 0.066	4.523 ± 0.177	4.988 ± 0.902	5.466 ± 1.055	5.503 ± 0.332
benzoic acid	1.708 ± 0.279	3.332 ± 0.300	2.762 ± 0.487	1.010 ± 0.252	1.457 ± 0.468	2.198 ± 0.146	1.820 ± 0.481	2.535 ± 0.719
maleic acid	10.435 ± 1.311	11.902 ± 0.771	11.285 ± 1.111	13.664 ± 0.540	10.390 ± 1.497	16.244 ± 3.795	10.916 ± 1.369	13.571 ± 2.816
succinic acid	8.372 ± 2.852	13.101 ± 1.154	13.849 ± 1.135	13.703 ± 1.614	13.289 ± 0.610	13.503 ± 4.675	11.808 ± 0.566	15.859 ± 2.371
glyceric acid	615.344 ± 126.663	892.702 ± 49.620	706.903 ± 66.100	671.659 ± 91.900	492.773 ± 62.079	614.516 ± 123.439	320.407 ± 36.779	486.195 ± 39.645
fumaric acid	2.202 ± 0.360	2.077 ± 0.085	2.266 ± 0.113	2.278 ± 0.052	2.158 ± 0.078	2.593 ± 0.074	2.403 ± 0.310	2.760 ± 0.063
glutaric acid	1.279 ± 0.094	1.471 ± 0.061	1.456 ± 0.006	1.518 ± 0.085	1.394 ± 0.053	1.508 ± 0.019	1.506 ± 0.140	1.711 ± 0.034
citramalic acid	1.251 ± 0.175	1.295 ± 0.158	1.468 ± 0.256	1.428 ± 0.178	1.331 ± 0.117	2.006 ± 0.043	0.988 ± 0.201	1.819 ± 0.247
malic acid pyroglutamic	1408.263 ± 230.090	1743.283 ± 68.365	1828.623 ± 138.859	1785.078 ± 130.553	1380.851 ± 83.993	1765.676 ± 381.167	1238.247 ± 37.877	1762.726 ± 164.347
acid	55.446 3.682	78.031 2.539	51.222 2.355	58.982 0.704	47.525 2.138	74.704 ± 4.069	58.828 2.393	88.918 5.988
threonic acid	25.248 ± 11.153	34.008 ± 3.756	29.017 ± 6.115	26.884 ± 5.701	17.607 ± 2.135	14.783 ± 4.841	13.128 ± 1.535	16.662 ± 3.449
tartaric acid	8391.930 ± 1430.072	8876.637 ± 526.568	9309.959 ± 362.995	8488.098 ± 498.571	8154.106 ± 431.643	10911.878 ± 1234.669	9511.884 ± 495.736	10533.904 ± 533.399
shikimic acid	51.583 ± 10.909	69.291 ± 20.105	63.848 ± 10.030	53.986 ± 14.178	46.968 ± 8.915	54.321 ± 3.700	36.589 ± 9.210	40.319 ± 7.120
citric acid 5-ketogluconic	66.171 ± 4.089	75.140 ± 5.559	90.555 ± 1.800	91.691 ± 2.090	91.770 ± 6.727	123.886 ± 16.147	126.811 ± 1.043	186.254 ± 20.367
acid	161.999 - 55.732	278.957 - 40.382	252.990 - 17.576	252.715 - 41.301	210.123 - 22.664	216.977 76.097	197.950 - 24.866	258.258 - 40.964
ascorbic acid	121.628 ± 34.543	181.751 ± 12.589	176.811 ± 7.598	173.131 ± 14.897	146.928 ± 3.109	170.686 ± 57.515	130.318 ± 5.627	171.200 ± 13.971
abscisic acid	111.774 ± 6.251	144.573 ± 8.707	34.020 ± 17.113	116.717 ± 25.910	31.241 ± 16.006	18.540 ± 18.405	0.179 ± 0.154	22.476 ± 22.394
Amino acids	4.406	0.500 + 0.611		0.114 1.1.765	22 222 1 4 450	24.007 1 2.201	57 (04) 5 100	104.050 0.000
valine	4.496 ± 0.289	3.520 ± 0.611	8.112 ± 1.326	9.114 ± 1.765	22.333 ± 4.458	34.897 ± 3.301	$5/.694 \pm 5.108$	104.950 ± 8.889
louoine	18.537 ± 4.681	$3/.45/ \pm 0.030$	41.718 ± 7.204	$3/.808 \pm 4.021$	54.240 ± 5.024	56.325 ± 22.975	$105.154 \pm 9.09/$	134.509 ± 7.493
icolouoino	1.910 ± 0.350	2.063 ± 0.043	3.297 ± 0.676	4.844 ± 0.707	7.543 ± 1.035	10.882 ± 0.365	16.858 ± 1.164	35.469 ± 4.477
isoleucille	0.432 ± 0.737	0.912 ± 0.093	$9.149 \pm 1.1/6$	10.142 ± 1.137 10.550 ± 2.230	15.409 ± 1.970 25.550 ± 2.256	22.908 ± 0.246	53.729 ± 2.934 53.722 ± 1.064	05.243 ± 5.837
threenine	11.032 ± 1.323 0.016 + 1.222	16.932 ± 0.804 16.285 ± 0.520	16.099 ± 0.147 16.202 ± 0.241	19.339 ± 2.330 15.481 ± 1.478	25.550 ± 5.250 18 445 ± 1.055	31.009 ± 9.131 22.130 ± 6.372	33.272 ± 1.904 20.252 + 2.255	50.724 ± 0.411 50.810 ± 7.856
proline	9.910 ± 1.232	0.285 ± 0.329	10.505 ± 0.541 12.788 + 0.677	13.461 ± 1.478 14.615 ± 0.407	18.445 ± 1.055 18.047 ± 1.646	122.130 ± 0.372 18 186 + 5 288	39.333 ± 2.333 36.854 ± 1.323	57.819 ± 7.850 52.001 ± 4.530
glycine	0.080 = 1.103 0.884 ± 0.070	1.051 + 0.058	13.788 - 0.077 1.393 + 0.465	1319 ± 0.050	10.947 = 1.040 1.070 ± 0.107	10.100 - 5.500	1768 ± 0.467	1426 ± 0.015
aspartic acid	58.891 + 1.670	106256 ± 6815	88490 + 7026	74369 + 5888	70.658 ± 0.958	98985 + 11789	119494 + 7781	139581 + 16112
β -alanine y-aminobutyric	0.316 ± 0.040	0.464 ± 0.025	0.749 ± 0.046	0.740 ± 0.075	0.967 ± 0.148	1.057 ± 0.085	1.476 ± 0.030	1.957 ± 0.122
acid	$11.840 \stackrel{\pm}{} 0.977$	$15.254 \stackrel{\pm}{=} 0.447$	$19.732 \stackrel{\pm}{=} 0.615$	$17.633 \stackrel{\pm}{=} 1.595$	26.025 ± 2.181	25.546 ± 6.658	$26.894 \stackrel{\pm}{=} 1.860$	32.215 ± 0.555
phenylalanine	44.598 ± 2.591	52.393 ± 1.017	65.167 ± 1.708	72.773 ± 3.028	83.013 ± 4.343	110.163 ± 11.220	152.434 ± 4.167	230.893 ± 20.719
lysine	7.226 ± 0.257	9.396 ± 0.243	10.703 ± 0.139	10.249 ± 0.597	11.742 ± 0.602	11.492 ± 0.865	16.580 ± 0.554	16.019 ± 0.888
Amines/Others								
uracil	0.691 ± 0.060	0.665 ± 0.038	0.820 ± 0.048	0.736 ± 0.022	0.806 ± 0.056	0.876 ± 0.043	0.828 ± 0.036	0.963 ± 0.033
pyridoxal	6.292 ± 1.935	8.805 ± 0.459	9.310 ± 0.663	8.873 ± 0.122	7.439 ± 0.762	8.850 ± 2.602	8.684 ± 0.791	10.406 ± 0.805
adenosine Sugars	18.681 ± 1.696	21.714 ± 0.521	20.803 ± 0.057	21.481 ± 0.691	19.339 ± 1.366	22.219 ± 0.381	21.565 ± 0.701	23.793 ± 0.588
threitol	0.988 ± 0.104	1.238 ± 0.039	1.180 ± 0.199	1.264 ± 0.061	1.135 ± 0.087	1.389 ± 0.089	1.052 ± 0.037	0.940 ± 0.470
meso-erythriol	1.799 ± 0.450	1.502 ± 0.124	1.367 ± 0.268	1.493 ± 0.128	1.288 ± 0.064	2.104 ± 0.212	1.270 ± 0.048	1.713 ± 0.191
xylose	7.636 ± 0.888	8.270 ± 0.649	8.963 ± 0.823	8.504 ± 0.615	10.305 ± 0.290	11.087 ± 0.482	13.219 ± 0.932	14.066 ± 0.728
ribose	4.153 ± 0.259	4.239 ± 0.384	4.337 ± 0.469	3.616 ± 0.099	4.542 ± 0.119	4.629 ± 1.093	4.831 ± 0.049	6.900 ± 0.182
a-rhamnose	5.280 ± 0.571	6.733 ± 0.061	6.595 ± 0.328	6.636 ± 0.065	6.505 ± 0.294	7.131 ± 1.051	7.076 ± 0.355	8.981 ± 0.164

Supplementary Table V. 1: Quantification of primary compounds in Jasmine grapevine samples in inoculated (I) and not inoculated (NI) at different time points, hours post infection (hpi). The concentrations reported represent the average value of 3 biological replicates ± error standard, expressed as mg/kg of fresh leaves.

arabitol	0.651	±	0.106	0.627	± (0.059	0.726	±	0.075	0.814	±	0.092	1.026	±	0.161	0.893	±	0.117	1.603	\pm	0.133	1.293	\pm	0.068
adonitol	5.913	\pm	0.557	6.975	± (0.262	6.698	\pm	0.576	6.993	\pm	0.286	6.356	±	0.483	7.539	\pm	0.642	6.504	±	0.509	8.444	±	0.195
fucose	10.732	\pm	2.795	9.054	± (0.206	9.270	\pm	0.820	9.623	\pm	0.338	9.208	±	0.636	13.773	\pm	1.591	9.020	±	0.405	12.877	±	0.310
fructose	289.451	±	110.549	405.362	± 5	5.746	398.711	±	40.610	405.322	\pm	11.005	342.667	±	28.232	425.019	\pm	116.760	337.797	±	34.184	469.591	±	32.038
glucose	904.094	±	359.954	504.495	± 2	26.259	502.028	±	82.238	495.693	\pm	38.249	424.360	±	22.719	977.183	\pm	403.782	367.617	±	20.741	516.602	±	33.601
sorbitol	1.927	±	0.090	2.296	± (0.039	2.019	±	0.167	2.050	\pm	0.127	1.934	. ±	0.100	2.513	\pm	0.166	2.478	±	0.716	2.547	±	0.182
myo-inositol	2346.249	±	492.114	2205.945	± 5	54.240	2138.925	±	262.663	2167.144	\pm	168.634	1872.523	±	82.951	3001.778	\pm	384.577	1631.444	±	130.741	2548.347	±	67.722
sucrose	1519.587	±	351.054	2087.952	±]	16.397	1916.012	±	202.611	1845.283	\pm	147.489	1491.428	±	40.826	1731.516	\pm	651.124	1027.878	±	106.681	1657.326	±	20.040
lactose	79.804	±	12.515	74.604	± 2	2.502	76.776	±	1.843	76.552	±	3.412	74.201	±	2.614	98.584	±	13.103	73.000	±	2.220	89.889	±	5.245

	12	hpi	24	hpi	48 hj	oi	96 hpi	
Class Compound	NI	Ι	NI	I	NI	Ι	NI I	
Carnitines								
palmitoyl-L-carnitine hydrochloride	0.012 ± 0.001	0.011 ± 0.001	0.007 ± 0.000	0.011 ± 0.002	0.008 ± 0.00005	0.009 ± 0.001	0.008 ± 0.002 0.011 ±	0.002
Sterols								
desmosterol	2.050 ± 0.814	1.796 ± 0.982	1.047 ± 0.450	1.493 ± 0.873	0.834 ± 0.339	2.590 ± 0.883	1.195 ± 0.503 0.831 ±	0.424
lanosterol	15.500 ± 1.468	25.841 ± 6.117	21.390 ± 6.728	29.500 ± 7.463	22.131 ± 3.404	32.097 ± 6.827	37.937 ± 8.298 49.016 ±	4.838
uvaol	0.629 ± 0.092	0.595 ± 0.024	0.849 ± 0.056	0.849 ± 0.113	0.977 ± 0.014	1.715 ± 0.070	1.241 ± 0.128 1.889 ±	0.091
Fatty acids								
arachidic acid	2.332 ± 0.148	2.472 ± 0.040	1.856 ± 0.086	2.891 ± 0.450	2.210 ± 0.076	2.996 ± 0.076	2.917 ± 0.447 3.146 ±	0.431
behenic acid	2.329 ± 0.112	2.283 ± 0.351	2.127 ± 0.274	2.495 ± 0.260	2.389 ± 0.118	3.240 ± 0.156	2.675 ± 0.464 3.381 ±	0.499
erucic acid	0.764 ± 0.013	0.725 ± 0.052	0.557 ± 0.049	0.824 ± 0.164	0.604 ± 0.024	0.700 ± 0.068	0.733 ± 0.068 0.652 ±	0.054
heptadecanoic acid	0.442 ± 0.024	0.384 ± 0.023	0.300 ± 0.030	0.322 ± 0.039	0.462 ± 0.112	0.421 ± 0.003	$0.299 \pm 0.026 0.364 \pm$	0.043
lignoceric acid	3.788 ± 0.162	3.856 ± 0.438	3.391 ± 0.375	4.454 ± 0.417	3.775 ± 0.054	5.240 ± 0.111	4.306 ± 0.724 5.468 ±	0.372
linoleic acid	0.700 ± 0.030	0.801 ± 0.100	0.635 ± 0.094	0.517 ± 0.030	0.425 ± 0.030	0.511 ± 0.020	$0.413 \pm 0.028 0.388 \pm$	0.028
linolenic acid	1.128 ± 0.084	1.688 ± 0.339	0.892 ± 0.175	0.682 ± 0.120	0.545 ± 0.042	0.832 ± 0.065	0.644 ± 0.092 0.652 ±	0.042
myristic acid	1.421 ± 0.186	1.330 ± 0.056	1.214 ± 0.172	2.210 ± 0.502	1.176 ± 0.044	1.535 ± 0.062	1.101 ± 0.038 2.007 ±	0.223
myristoleic acid	0.207 ± 0.021	0.202 ± 0.009	0.174 ± 0.010	0.233 ± 0.025	0.160 ± 0.006	0.190 ± 0.018	$0.163 \pm 0.009 0.213 \pm$	0.018
oleic acid+cis-vaccenic acid	1.356 ± 0.191	1.935 ± 0.513	1.225 ± 0.309	0.480 ± 0.260	ND	0.250 ± 0.129	0.233 ± 0.116 ND	
palmitic acid	11.273 ± 2.477	17.069 ± 0.871	13.421 ± 1.100	14.502 ± 3.514	6.825 ± 0.660	9.956 ± 1.826	7.937 ± 0.373 8.074 ±	0.821
palmitoleic acid	0.879 ± 0.052	1.026 ± 0.105	1.061 ± 0.193	0.749 ± 0.178	0.500 ± 0.088	0.619 ± 0.103	0.485 ± 0.009 0.744 ±	0.100
stearic acid	11.420 ± 2.098	12.416 ± 1.887	9.199 ± 0.992	16.470 ± 5.133	9.012 ± 0.344	13.295 ± 0.770	10.222 ± 1.076 10.415 ±	0.553
Glycerolipids								
1,2,3-tripentadecanoylglycerol	0.022 ± 0.004	0.024 ± 0.004	0.016 ± 0.002	0.015 ± 0.004	0.011 0.003	0.015 ± 0.002	0.024 ± 0.020 0.012 ±	0.002
1-oleoyl-rac-glycerol	0.053 ± 0.006	0.044 ± 0.004	0.035 ± 0.003	0.035 ± 0.003	0.024 ± 0.004	0.046 ± 0.005	0.029 ± 0.009 0.034 ±	0.005
glyceryl trioleate	ND	ND	ND	ND	ND	ND	0.077 ± 0.045 ND	
glyceryl tripalmitoleate	0.072 ± 0.013	0.092 ± 0.014	0.053 ± 0.008	0.065 ± 0.005	0.045 ± 0.010	0.048 ± 0.002	$0.025 \pm 0.002 0.039 \pm$	0.002
Glycerophospholipids								
1,2-dilinoleoyl-sn-glycero-3-phosphocholine	34.996 ± 1.854	33.479 ± 1.949	26.462 ± 1.158	37.566 ± 5.028	28.750 ± 0.444	34.151 ± 2.122	33.076 ± 5.881 32.837 ±	1.994
1,2-dioleoyl-sn-glycero-3-phosphocholine	27.198 ± 1.047	27.171 ± 1.810	21.489 ± 2.320	28.986 ± 0.903	20.067 ± 0.091	30.004 ± 1.292	17.057 ± 1.622 22.388 ±	1.352
1,2-dioleoyl-sn-glycero-3-phospho-rac-(1-	0.228 + 0.030	0.215 + 0.007	+	+	+	+	+ +	
glycerol)sodium salt	0.220 2 0.050	0.210 1 0.007	0.161 0.011	0.284 0.036	0.211 0.023	0.343 0.028	0.288 0.040 0.561	0.031
l-palmitoyl-sn-glycero-3-phosphocholine	0.553 ± 0.013	0.630 ± 0.064	0.504 ± 0.073	0.562 ± 0.042	0.513 ± 0.005	0.658 ± 0.037	$0.608 \pm 0.070 0.621 \pm$	0.061
Sphingolipids								
ceramide	0.028 ± 0.001	0.109 ± 0.009	0.027 ± 0.001	0.137 ± 0.002	0.028 ± 0.002	0.203 ± 0.020	$0.033 \pm 0.003 0.198 \pm$	0.009
Prenols								
oleanolic acid	1.151 ± 0.125	1.169 ± 0.086	2.498 ± 0.312	2.162 ± 0.353	3.978 ± 0.306	7.413 ± 0.271	6.986 ± 1.302 10.838 ±	1.250

Supplementary Table V. 2: Quantification of lipids in Jasmine grapevine samples in inoculated (I) and not inoculated (NI) samples at different time points, hours post infection (hpi). The concentrations reported represent the average value of 3 biological replicates ± error standard, expressed as mg/kg of fresh leaves.

Class Compound	12	hni	24	hni	48	hni		hni
Benzoic Acid Derivatives	NI	I	NI	I	NI	I	NI	I
gallic acid	0.265 ± 0.152	0.296 ± 0.048	0.227 ± 0.046	0.134 ± 0.070	0.149 ± 0.012	0.254 ± 0.013	0.105 ± 0.024	0.138 ± 0.037
<i>n</i> -hydroxybenzoic acid	0.205 = 0.152 0.247 ± 0.053	0.290 = 0.040 0.381 ± 0.070	0.227 ± 0.032	0.134 = 0.070 0.239 ± 0.051	0.149 = 0.012 0.278 ± 0.038	0.234 = 0.015 0.228 ± 0.025	0.105 = 0.024 0.136 ± 0.015	0.136 ± 0.096
vanillin	0.247 = 0.005 0.186 ± 0.007	0.381 = 0.070 0.186 ± 0.013	0.242 = 0.032 0.153 ± 0.021	0.257 = 0.051 0.153 ± 0.017	0.278 ± 0.031	0.220 = 0.023 0.241 ± 0.037	0.130 = 0.019 0.135 ± 0.010	0.210 = 0.000 0.141 ± 0.020
vanillic acid	0.100 = 0.007 0.010 ± 0.005	0.100 = 0.013 0.024 ± 0.012	0.029 ± 0.006	0.133 = 0.017 0.018 ± 0.005	0.024 ± 0.006	0.241 = 0.007 0.014 ± 0.008	0.133 ± 0.004	0.141 = 0.020 0.021 ± 0.003
Coumarins	0.010 - 0.005	0.024 - 0.012	0.027 - 0.000	0.010 - 0.005	0.024 - 0.000	0.014 - 0.000	0.050 - 0.004	0.021 - 0.005
esculin	0.189 ± 0.021	0.180 ± 0.033	0.112 ± 0.022	0.155 ± 0.032	0.127 ± 0.017	0.241 ± 0.019	0.122 ± 0.013	0.158 ± 0.011
fraxin	0.109 = 0.021 0.020 ± 0.003	0.100 = 0.000	0.012 = 0.022 0.024 ± 0.004	0.133 ± 0.032	0.127 = 0.017 0.015 ± 0.008	0.241 = 0.012 0.032 ± 0.012	0.122 = 0.013 0.032 ± 0.012	0.150 = 0.011 0.016 ± 0.003
Phenylpropanoids	0.020 0.005	0.027 0.010	0.021 0.001	0.015 0.005	0.015 0.000	0.052 0.012	0.052 0.012	0.010 0.005
trans-coutaric acid	84.769 ± 8.149	86920 ± 9332	66963 ± 1807	67.014 ± 3.378	73.072 ± 2.404	99652 ± 2527	55977 ± 6309	77525 ± 10951
caffeic acid	0.305 ± 0.062	0.388 ± 0.063	0.367 ± 0.071	0.377 ± 0.045	0.411 ± 0.042	0.481 ± 0.060	0.305 ± 0.053	0.494 ± 0.025
caftaric acid	1136.44 + 89.19	110853 ± 10063	894.07 + 18.39	917.94 + 61.14	953.00 + 53.65	1312.65 + 75.77	720.18 + 66.25	974.46 ± 106.88
fertaric acid	1150.44 = 87.17 16.806 ± 0.707	15767 ± 0.861	13267 + 1013	13,860 + 1,246	14640 + 1788	1312.03 = 73.77 21.058 ± 1.283	12100 ± 0.07	15787 + 1766
ferulic acid	10.000 = 0.707 0.054 ± 0.008	0.008 ± 0.001	13.207 = 1.013 0.010 + 0.011	$13.80^{\circ} = 1.240^{\circ}$	0.015 ± 0.004	0.028 ± 0.013	0.016 ± 0.002	13.787 = 1.700 0.013 ± 0.003
sinanie acid	0.034 ± 0.008 0.175 ± 0.012	0.008 ± 0.004	0.019 ± 0.011 0.126 ± 0.007	0.022 ± 0.000	0.013 ± 0.004 0.125 ± 0.002	0.028 ± 0.013	0.010 ± 0.002 0.107 ± 0.008	0.013 ± 0.003
Dihydrochalconas	0.175 ± 0.012	0.110 ± 0.017	0.130 ± 0.007	0.142 - 0.000	0.133 ± 0.003	0.182 ± 0.003	0.107 ± 0.008	0.124 ± 0.012
nhlorizin	0.612 + 0.067	0.699 + 0.077	0.640 + 0.135	0.569 ± 0.065	0.606 + 0.020	0.067 + 0.077	0.436 + 0.022	0.662 + 0.026
Flavores	0.012 ± 0.007	0.088 ± 0.077	0.049 ± 0.133	0.308 ± 0.003	0.090 ± 0.039	0.967 ± 0.077	0.430 ± 0.022	0.002 ± 0.020
luteolin 7 O glucoside	0.052 ± 0.005	0.060 ± 0.019	0.059 ± 0.011	0.059 ± 0.010	0.047 ± 0.005	0.065 ± 0.000	0.055 ± 0.012	0.044 ± 0.001
Elayar 2 ola	0.055 ± 0.005	0.069 ± 0.018	0.038 ± 0.011	0.038 ± 0.019	0.047 ± 0.003	0.005 ± 0.009	0.055 ± 0.012	0.044 ± 0.001
riuvan-3-ols	12.270 ± 1.022	11.021 ± 1.002	0.081 ± 0.760	Q (12 ± 0.9(0	0.211 ± 0.005	10.242 ± 1.400	6.006 ± 0.170	6.970 ± 0.090
catechin	$13.2/9 \pm 1.022$	11.921 ± 1.092	9.081 ± 0.769	8.643 ± 0.860	9.211 ± 0.905	10.243 ± 1.490	$6.996 \pm 0.1/0$	$6.8/9 \pm 0.989$
epicatechin collete	$1.8/6 \pm 0.288$	1.292 ± 0.386	1.038 ± 0.287	$1.0/1 \pm 0.1/5$	1.984 ± 0.632	1.759 ± 0.691	1.210 ± 0.357	1.390 ± 0.314
epicatecnin ganate	0.337 ± 0.173	0.481 ± 0.030	0.429 ± 0.079	0.336 ± 0.084	0.327 ± 0.175	$0.2/3 \pm 0.148$	0.234 ± 0.023	$0.315 \pm 0.10/$
epigallocatechin	1.420 ± 0.462	1.523 ± 0.518	0.810 ± 0.113	0.818 ± 0.231	0.869 ± 0.295	$1.164 \pm 0.34/$	0.528 ± 0.099	$0.8/6 \pm 0.221$
epigallocatechin gallate	0.667 ± 0.066	0.654 ± 0.011	0.459 ± 0.040	0.422 ± 0.051	0.545 ± 0.043	0.643 ± 0.108	0.235 ± 0.118	0.401 ± 0.038
gallocatechin	7.465 ± 0.889	8.444 ± 1.449	5.571 ± 0.289	6.202 ± 0.630	4.483 ± 0.540	7.458 ± 1.150	3.555 ± 0.475	5.284 ± 0.764
procyanidin BI	19.821 ± 2.061	17.934 ± 3.389	13.277 ± 1.614	13.507 ± 0.428	14.121 ± 0.594	21.741 ± 3.301	11.903 ± 2.413	15.910 ± 2.923
procyanidin $B2 + B4$ (as $B2$)	2.995 ± 0.700	3.376 ± 0.360	2.772 ± 0.206	2.181 ± 0.226	2.519 ± 0.493	4.481 ± 0.547	1.731 ± 0.271	2.432 ± 0.475
procyanidin B3 (as B1)	1.459 ± 0.361	1.826 ± 0.074	2.526 ± 0.346	1.173 ± 0.138	1.198 ± 0.350	1.294 ± 0.210	1.431 ± 0.267	1.564 ± 0.203
Flavonols								
isorhamnetin-3-O-glucoside	0.144 ± 0.019	0.184 ± 0.063	0.155 ± 0.045	0.113 ± 0.031	0.187 ± 0.027	0.114 ± 0.003	0.125 ± 0.029	0.225 ± 0.043
isorhamnetin-3-rutinoside	1.272 ± 0.087	1.266 ± 0.176	0.958 ± 0.040	0.979 ± 0.107	1.132 ± 0.068	1.633 ± 0.033	0.820 ± 0.100	1.171 ± 0.188
kaempferol-3-O-glucoside	0.089 ± 0.009	0.089 ± 0.020	0.120 ± 0.009	0.119 ± 0.019	0.125 ± 0.013	0.147 ± 0.025	0.102 ± 0.011	0.157 ± 0.013
kaempferol-3-O-glucuronide	3.379 ± 0.277	3.378 ± 0.161	2.597 ± 0.305	2.947 ± 0.148	2.959 ± 0.247	4.656 ± 0.600	2.348 ± 0.311	3.425 ± 0.180
kaempferol-3-O-rutinoside	0.327 ± 0.023	0.341 ± 0.014	0.289 ± 0.103	0.256 ± 0.029	0.201 ± 0.016	0.488 ± 0.125	0.268 ± 0.015	0.283 ± 0.046
myricetin	0.186 ± 0.014	0.049 ± 0.049	0.158 ± 0.010	0.094 ± 0.047	0.159 ± 0.021	0.126 ± 0.064	0.113 ± 0.002	0.136 ± 0.002
quercetin-3-glucoside+quercetin-3-galactoside (as	7 (05 ± 0.205	7.556 ± 0.600	5.766 ± 0.550	(101 [±] 0.22((002 ± 0.521	0.70([±] 0.721	5 (00 [±] 0 (52	7.422 ± 0.201
que-3-glc)	7.695 0.385	7.556 0.622	5.766 0.550	6.481 0.226	6.893 0.531	9.796 0.731	5.600 0.653	7.432 0.291
quercetin-3-O-glucoside-arabinoside	0.322 ± 0.022	0.249 ± 0.035	0.205 ± 0.061	0.176 ± 0.039	0.264 ± 0.043	0.345 ± 0.047	0.153 ± 0.048	0.306 ± 0.081
quercetin-3-O-glucuronide	572.489 ± 57.94	565.487 ± 37.220	446.57 ± 37.08	469.21 ± 21.55	512.68 ± 26.526	670.994 ± 32.489	392.109 ± 29.462	504.063 ± 44.140
rutin	6.803 ± 0.864	7.423 ± 0.775	5.477 ± 0.568	5.767 ± 0.128	6.179 ± 0.146	9.444 ± 0.838	5.053 ± 0.514	6.435 ± 0.654
taxifolin	0.074 ± 0.039	0.136 ± 0.022	0.072 ± 0.026	0.062 ± 0.013	0.101 ± 0.022	0.082 ± 0.049	0.057 ± 0.057	0.058 ± 0.015
Stilbenes + Stilbenoids								
α-viniferin	ND	ND	5.427 ± 3.041	11.932 ± 2.639	51.358 ± 3.827	97.510 ± 19.660	152.806 ± 17.912	144.671 ± 22.523
<i>cis</i> -piceide	1.409 ± 0.092	1.295 ± 0.379	1.159 ± 0.246	1.516 ± 0.062	1.297 ± 0.123	2.750 ± 0.232	1.313 ± 0.213	1.992 ± 0.339

Supplementary Table V. 3: Quantification of phenols in Jasmine grapevine samples in inoculated (I) and not inoculated (NI) at different time points, hours post infection (hpi). The concentrations reported represent the average value of 3 biological replicates ± error standard, expressed as mg/kg of fresh leaves.

$cis + trans-\omega$ -viniferin	0.154 ± 0.154	0.134 ± 0.134	0.408 ± 0.235	0.151 ± 0.151	0.191 ± 0.191	0.399 ± 0.209	0.375 ± 0.027	0.473 ± 0.083
trans-e-viniferin	1.309 ± 0.549	3.184 ± 0.293	2.451 ± 0.410	3.041 ± 0.962	4.049 ± 0.229	5.002 ± 0.244	2.876 ± 0.361	2.800 ± 0.514
trans-piceide	1.033 ± 0.077	1.154 ± 0.170	0.816 ± 0.174	1.890 ± 0.181	1.317 ± 0.074	3.960 ± 0.471	2.006 ± 0.233	3.633 ± 0.602
trans-resveratrol	1.042 ± 0.166	2.491 ± 0.209	0.979 ± 0.170	1.776 ± 0.258	0.835 ± 0.152	2.615 ± 0.352	0.747 ± 0.205	0.911 ± 0.258
ampelopsin D + quadrangularin A	0.079 ± 0.042	0.187 ± 0.011	0.109 ± 0.060	0.320 ± 0.042	0.495 ± 0.161	0.598 ± 0.050	0.348 ± 0.099	0.292 ± 0.091
ampelopsin H + vaticanol C-like isomer	ND	ND	1.113 ± 1.113	2.265 ± 1.182	1.362 ± 1.362	4.338 ± 0.199	2.782 ± 0.238	2.233 ± 1.157
astringin	0.269 ± 0.067	0.363 ± 0.033	0.182 ± 0.101	0.430 ± 0.026	0.272 ± 0.051	0.692 ± 0.046	0.405 ± 0.043	0.666 ± 0.050
E-cis-miyabenol	0.799 ± 0.188	0.412 ± 0.412	1.873 ± 0.858	2.286 ± 0.625	5.203 ± 0.928	7.801 ± 0.791	5.055 ± 0.329	5.034 ± 0.204
isohopeaphenol	1.167 ± 0.704	3.381 ± 1.569	5.446 ± 1.671	7.007 ± 1.020	11.580 ± 1.463	15.751 ± 1.382	11.916 ± 1.557	11.635 ± 0.779
isorhapontin	0.162 ± 0.013	0.108 ± 0.054	0.176 ± 0.040	0.160 ± 0.019	0.180 ± 0.040	0.250 ± 0.085	0.206 ± 0.033	0.413 ± 0.007
pallidol	2.739 ± 0.282	5.544 ± 0.967	5.001 ± 1.608	7.868 ± 1.067	7.341 ± 1.593	18.833 ± 3.811	6.918 ± 1.794	8.547 ± 1.622
Z-miyabenol C	ND	ND	0.756 ± 0.539	0.592 ± 0.396	4.322 ± 0.589	6.358 ± 1.270	4.848 ± 0.239	7.587 ± 1.374
Others								
arbutin	1.306 ± 0.077	1.219 ± 0.120	1.143 ± 0.166	1.273 ± 0.061	1.679 ± 0.308	2.910 ± 0.367	2.052 ± 0.092	2.888 ± 0.154
caffeic acid+catechin condensation product	34.057 ± 1.834	32.716 ± 3.353	23.177 ± 2.385	29.027 ± 1.642	31.063 ± 1.768	42.318 ± 3.477	19.432 ± 2.102	33.677 ± 2.925

Supplementary Table V. 4: Semi-quantification of volatile compounds in Bianca grapevine samples in inoculated (I) and not inoculated (NI) samples at different time points, hours post infection (hpi). The concentrations reported represent the average value of 3 biological replicates \pm error standard, expressed as $\mu g/kg$ of fresh leaves using 1-heptanol as internal standard. The identification confidence is reported with the letter "A" to compare mass spectra and retention time with those of the pure standard, "B" for retention index match on a similar phase column, and "C" for identification with the mass spectral database.

			12	hpi	24	hpi	48	hpi	96	hpi
			NI	I	NI	I	NI	I	NI	I
Class	Compound	ident.								
Acids										
	3-or-4-hexenoic-acid	B,C	0.0220 ± 0.0017	0.0372 ± 0.0061	0.0238 ± 0.0037	0.0322 ± 0.0031	0.0344 ± 0.0059	0.0595 ± 0.0052	0.0433 ± 0.0082	0.0964 ± 0.0199
	nonanoic-acid	A,B,C	0.0084 ± 0.0009	0.0119 ± 0.0023	0.0068 ± 0.0018	0.0060 ± 0.0013	0.0095 ± 0.0038	0.0097 ± 0.0038	0.0057 ± 0.0013	0.0067 ± 0.0014
	octanoic	A,B,C	0.0352 ± 0.0032	0.0438 ± 0.0038	0.0279 ± 0.0062	0.0319 ± 0.0030	0.0375 ± 0.0125	0.0339 ± 0.0067	0.0331 ± 0.0061	0.0340 ± 0.0016
Alcoho	ls									
	cyclobutanemethanol	С	0.0200 ± 0.0033	0.0233 ± 0.0029	0.0184 ± 0.0020	0.0252 ± 0.0029	0.0189 ± 0.0032	0.0392 ± 0.0047	0.0207 ± 0.0035	0.0230 ± 0.0025
	1-hexanol	A,B,C	0.0698 ± 0.0065	0.0913 ± 0.0103	0.0659 ± 0.0080	0.0782 ± 0.0019	0.0754 ± 0.0089	0.1137 ± 0.0172	0.0991 ± 0.0184	0.1426 ± 0.0035
	1-hexanol-2-ethyl	A,B,C	0.0765 ± 0.0127	0.0851 ± 0.0072	0.0822 ± 0.0069	0.1145 ± 0.0052	0.1465 ± 0.0257	0.1605 ± 0.0156	0.2053 ± 0.0403	0.2460 ± 0.0074
	3-hexen-1-ol-cis/trans	B,C	0.1415 ± 0.0120	0.1863 ± 0.0248	0.1296 ± 0.0112	0.1492 ± 0.0034	0.1483 ± 0.0178	0.2075 ± 0.0204	0.1899 ± 0.0413	0.2602 ± 0.0239
	1-nonanol	A,B,C	0.1076 ± 0.0033	0.1189 ± 0.0056	0.1073 ± 0.0092	0.1351 ± 0.0044	0.1418 ± 0.0258	0.1631 ± 0.0051	0.1354 ± 0.0186	0.1789 ± 0.0382
	3-nonen-1-ol	B,C	0.1459 ± 0.0147	0.1782 ± 0.0057	0.1441 ± 0.0053	0.1742 ± 0.0048	0.1729 ± 0.0177	0.2206 ± 0.0223	0.2013 ± 0.0311	0.2530 ± 0.0125
	1-octanol	A,B,C	0.0668 ± 0.0044	0.0914 ± 0.0061	0.0689 ± 0.0045	0.0961 ± 0.0076	0.0885 ± 0.0141	0.1257 ± 0.0186	0.0954 ± 0.0190	0.1470 ± 0.0205
Aldehy	des									
	2,4-heptadienal	B,C	0.0574 ± 0.0037	0.0818 ± 0.0059	0.0519 ± 0.0017	0.0664 ± 0.0010	0.0583 ± 0.0058	0.1097 ± 0.0174	0.0905 ± 0.0175	0.1127 ± 0.0078
	2-heptenal	B,C	0.0285 ± 0.0019	0.0391 ± 0.0049	0.0253 ± 0.0035	0.0339 ± 0.0031	0.0321 ± 0.0057	0.0470 ± 0.0030	0.0332 ± 0.0041	0.0413 ± 0.0014
	2,4-hexadienal	B,C	0.3086 ± 0.0331	0.4381 ± 0.0225	0.4486 ± 0.0781	0.5288 ± 0.0733	0.4576 ± 0.0196	0.7525 ± 0.1118	0.4774 ± 0.0738	0.7847 ± 0.0219
	hexanal	A,B,C	2.5978 ± 0.1630	3.9854 ± 0.3181	3.1208 ± 0.5092	3.6751 ± 0.4462	2.9886 ± 0.2082	5.3970 ± 0.8107	3.0470 ± 0.1941	4.5586 ± 0.0461
	trans-3-hexenal	A,B,C	0.0942 ± 0.0074	0.1143 ± 0.0075	0.0975 ± 0.0172	0.1190 ± 0.0013	0.1015 ± 0.0086	0.1367 ± 0.0206	0.1060 ± 0.0140	0.1428 ± 0.0052
	cis-3-hexenal	A,B,C	0.3482 ± 0.0644	0.3058 ± 0.0447	0.3833 ± 0.1075	0.3898 ± 0.0532	0.3127 ± 0.0474	0.2247 ± 0.0356	0.1348 ± 0.0181	0.1520 ± 0.0291
	trans-2-hexenal	A,B,C	0.4192 ± 0.0223	0.5939 ± 0.0317	0.4865 ± 0.0807	0.5834 ± 0.0160	0.5271 ± 0.0140	0.8134 ± 0.0971	0.7040 ± 0.0637	1.0444 ± 0.0256
	pentanal	B,C	0.0185 ± 0.0018	0.0263 ± 0.0030	0.0150 ± 0.0029	0.0211 ± 0.0020	0.0170 ± 0.0036	0.0286 ± 0.0025	0.0252 ± 0.0045	0.0276 ± 0.0044
	2-pentenal	B,C	0.0949 ± 0.0042	0.1363 ± 0.0102	0.0857 ± 0.0107	0.1051 ± 0.0056	0.0737 ± 0.0105	0.1637 ± 0.0294	0.0824 ± 0.0144	0.1005 ± 0.0109
Benzen	oids									
	benzaldehyde	A,B,C	0.0576 ± 0.0040	0.1595 ± 0.0065	0.0588 ± 0.0087	0.2142 ± 0.0389	0.0642 ± 0.0084	0.3804 ± 0.0614	0.1394 ± 0.0505	0.4601 ± 0.0425
	benzylalchool	A,B,C	0.4844 ± 0.0755	0.6056 ± 0.0749	0.4912 ± 0.1044	0.5517 ± 0.1679	0.5269 ± 0.0333	0.7349 ± 0.1420	0.6281 ± 0.0706	0.8140 ± 0.1077
	eugenol	A,B,C	0.0523 ± 0.0098	0.0835 ± 0.0043	0.0576 ± 0.0086	0.0744 ± 0.0315	0.0593 ± 0.0079	0.0827 ± 0.0191	0.0965 ± 0.0115	0.1132 ± 0.0196
	phenethy-alcohol	A,B,C	0.2247 ± 0.0353	0.2801 ± 0.0104	0.2354 ± 0.0269	0.2839 ± 0.0364	0.2847 ± 0.0138	0.4453 ± 0.0671	0.3440 ± 0.0505	0.5180 ± 0.0206
Ketone	S									
	2-cyclopentene-1-one-2-pentenyl	С	0.0312 ± 0.0035	0.0504 ± 0.0043	0.0508 ± 0.0065	0.0596 ± 0.0154	0.0451 ± 0.0053	0.0964 ± 0.0217	0.0411 ± 0.0064	0.0574 ± 0.0027
	6-methyl-5-hepten-2-one	A,B,C	0.0273 ± 0.0006	0.0344 ± 0.0032	0.0228 ± 0.0028	0.0271 ± 0.0020	0.0248 ± 0.0038	0.0381 ± 0.0028	0.0277 ± 0.0036	0.0366 ± 0.0005
	penten-3-one	B,C	0.0568 ± 0.0003	0.0754 ± 0.0070	0.0463 ± 0.0068	0.0598 ± 0.0015	0.0452 ± 0.0055	0.0882 ± 0.0178	0.0509 ± 0.0080	0.0648 ± 0.0033
	2,2,6-trimetylciclohexanone	B,C	0.0067 ± 0.0003	0.0087 ± 0.0007	0.0073 ± 0.0008	0.0091 ± 0.0009	0.0082 ± 0.0010	0.0101 ± 0.0006	0.0069 ± 0.0008	0.0085 ± 0.0003
Terpen	oids									
	α-ionone	A,B,C	0.0724 ± 0.0082	0.0931 ± 0.0061	0.0850 ± 0.0098	0.0987 ± 0.0198	0.0877 ± 0.0175	0.1353 ± 0.0253	0.0965 ± 0.0070	0.1177 ± 0.0067
	α-terpinolen	A,B,C	0.0065 ± 0.0003	0.0085 ± 0.0006	0.0081 ± 0.0010	0.0081 ± 0.0015	0.0069 ± 0.0012	0.0082 ± 0.0004	0.0079 ± 0.0006	0.0092 ± 0.0012
	β -ciclocitral	B,C	0.1345 ± 0.0112	0.1898 ± 0.0106	0.1489 ± 0.0120	0.1864 ± 0.0222	0.1745 ± 0.0236	0.2353 ± 0.0157	0.1746 ± 0.0212	0.2079 ± 0.0067
	β-ionone	A,B,C	0.2198 ± 0.0327	0.3091 ± 0.0212	0.2650 ± 0.0188	0.3283 ± 0.0546	0.2999 ± 0.0538	0.4390 ± 0.0526	0.2790 ± 0.0422	0.3415 ± 0.0202
	β -ionone-epoxido	B,C	0.0672 ± 0.0072	0.0972 ± 0.0026	0.0821 ± 0.0111	0.0971 ± 0.0194	0.0894 ± 0.0156	0.1338 ± 0.0173	0.0959 ± 0.0146	0.1128 ± 0.0098
	citral	A,B,C	0.0595 ± 0.0041	0.0806 ± 0.0061	0.0691 ± 0.0102	0.0809 ± 0.0156	0.0689 ± 0.0099	0.0895 ± 0.0063	0.0786 ± 0.0071	0.0953 ± 0.0122
	dihydroactinidolide	B,C	0.0588 ± 0.0043	0.0906 ± 0.0053	0.0707 ± 0.0081	0.0812 ± 0.0090	0.0772 ± 0.0116	0.1176 ± 0.0081	0.0793 ± 0.0114	0.0925 ± 0.0097
	farnesene	A,B,C	0.1336 ± 0.0048	0.2003 ± 0.0219	0.1182 ± 0.0265	0.1591 ± 0.0369	0.1172 ± 0.0445	0.1960 ± 0.0424	0.0536 ± 0.0081	0.0886 ± 0.0199

	geranic acid	A,B,C	0.0191 ± 0.005	$50 0.0273 \ \pm \ 0.0026$	0.0244 ± 0.0062	0.0264 ± 0.0105	0.0246 ± 0.0065	0.0252 ± 0.0051	0.0279 ± 0.0049	0.0303 ± 0.0055
	geraniol	A,B,C	0.2582 ± 0.003	0.3214 ± 0.0057	0.2718 ± 0.0288	0.3068 ± 0.0337	0.2831 ± 0.0309	0.3443 ± 0.0207	0.3488 ± 0.0380	0.4101 ± 0.0164
	geranylacetone	B,C	0.0586 ± 0.005	$58 0.0783 \ \pm \ 0.0032$	0.0581 ± 0.0059	0.0678 ± 0.0122	0.0637 ± 0.0093	0.0852 ± 0.0112	0.0752 ± 0.0096	0.0940 ± 0.0028
	isogeraniol	B,C	0.0161 ± 0.001	$5 0.0202 \pm 0.0009$	0.0140 ± 0.0007	0.0167 ± 0.0015	0.0169 ± 0.0020	0.0223 ± 0.0020	0.0186 ± 0.0022	0.0254 ± 0.0020
	linalool	A,B,C	0.0584 ± 0.000	0.0741 ± 0.0059	0.0629 ± 0.0087	0.0681 ± 0.0149	0.0561 ± 0.0102	0.0666 ± 0.0039	0.0713 ± 0.0072	0.0774 ± 0.0099
	nerol	A,B,C	0.0899 ± 0.008	0.1228 ± 0.0096	0.0929 ± 0.0111	0.1169 ± 0.0301	0.1024 ± 0.0135	0.1319 ± 0.0149	0.1260 ± 0.0088	0.1551 ± 0.0107
Others										
	n,n-dibutylformamide	B,C	0.0293 ± 0.002	0.0324 ± 0.0026	0.0321 ± 0.0046	0.0337 ± 0.0014	0.0304 ± 0.0030	0.0342 ± 0.0066	0.0383 ± 0.0084	0.0394 ± 0.0037
	2-ethyl-furan	B,C	0.0733 ± 0.006	$64 0.0857 \pm 0.0069$	0.0865 ± 0.0208	0.0874 ± 0.0010	0.0796 ± 0.0071	0.0991 ± 0.0133	0.0466 ± 0.0065	0.0551 ± 0.0052
	2-hexen-1,4-lactone	B,C	0.0570 ± 0.004	$19 0.0628 \ \pm \ 0.0063$	0.0651 ± 0.0161	0.0665 ± 0.0048	0.0563 ± 0.0055	0.0535 ± 0.0073	0.0301 ± 0.0038	0.0352 ± 0.0061
	cis-3-hexenylacetate	B,C	0.0761 ± 0.007	0.1297 ± 0.0235	0.0329 ± 0.0043	0.0366 ± 0.0032	0.0286 ± 0.0091	0.0502 ± 0.0114	0.0189 ± 0.0073	0.0321 ± 0.0056
Unkno	wns:									
	unknown-1		0.0067 ± 0.000	0.0081 ± 0.0010	0.0044 ± 0.0008	0.0062 ± 0.0002	0.0044 ± 0.0011	0.0098 ± 0.0020	0.0060 ± 0.0011	0.0070 ± 0.0008
	unknown-2		0.0041 ± 0.000	0.0060 ± 0.0006	0.0035 ± 0.0004	0.0051 ± 0.0008	0.0052 ± 0.0016	0.0080 ± 0.0006	0.0052 ± 0.0015	0.0063 ± 0.0015
	unknown-3		0.0479 ± 0.002	0.0602 ± 0.0012	0.0552 ± 0.0027	0.0623 ± 0.0062	0.0458 ± 0.0010	0.0754 ± 0.0017	0.0417 ± 0.0012	0.0626 ± 0.0078
	unknown-4		0.0106 ± 0.001	$6 0.0184 \ \pm \ 0.0028$	0.0108 ± 0.0030	0.0148 ± 0.0035	0.0148 ± 0.0050	0.0223 ± 0.0019	0.0161 ± 0.0030	0.0166 ± 0.0025
	unknown-10		0.0331 ± 0.000	0.0441 ± 0.0005	0.0285 ± 0.0034	0.0367 ± 0.0032	0.0304 ± 0.0045	0.0464 ± 0.0044	0.0462 ± 0.0085	0.0485 ± 0.0028
	unknown-11		0.0043 ± 0.000	0.0067 ± 0.0007	0.0055 ± 0.0008	0.0074 ± 0.0016	0.0061 ± 0.0010	0.0096 ± 0.0008	0.0067 ± 0.0011	0.0086 ± 0.0008

Metabolite	TIME	t statistic
1-Hexanol-2-ethyl	24	-3.61387
1-Octanol	12	-3.34523
1-Octanol	24	-3.23149
1-Oleoyl-rac-glycerol	48	-3.34292
1-Palmitoyl-sn-glycero-3-phosphocholine	48	-4.28257
1,2-Dioleoyl-sn-glycero-3-phospho-rac-(1-glycerol)sodium	24	-3.79309
1,2-Dioleoyl-sn-glycero-3-phospho-rac-(1-glycerol)sodium	48	-3.63764
1,2-Dioleoyl-sn-glycero-3-phospho-rac-(1-glycerol)sodium	96	-4.65067
1,2-Dioleoyl-sn-glycero-3-phosphocholine	48	-9.42845
2-Cyclopentene-1-one-2-pentenyl	12	-3.51536
2-Pentenal	12	-4.2407
2-Pentenal	48	-3.20558
2,4-Heptadienal	12	-3.72442
2,4-Heptadienal	24	-6.90372
2,4-Heptadienal	48	-3.18871
2,4-Hexadienal	48	-3.00331
2,4-Hexadienal	96	-3.12204
3-Nonen-1-ol	24	-4.15736
Abscisic	12	-3.13694
Adonitol	96	-3.23759
alpha-Rhamnose	96	-4.60048
alpha-Terpinolen	12	-3.28411
Arachidic	48	-7.06007
Arbutin	96	-4.91811
Aspartic	12	-8.54443
Astringin	48	-4.52882
Astringin	96	-3.79455
Behenic	48	-4.49188
Benzaldehyde	12	-12.5345
Benzaldehyde	24	-5.03102
Benzaldehyde	48	-8.95184
Benzaldehyde	96	-3.65251
Benzoic	12	-3.79761
beta-Alanine	96	-4.16601
beta-Ciclocitral	12	-3.52972
beta-Ionone-epoxido	12	-3.34095
Caffeic acid+catechin condensation	48	-3.12576
Caffeic acid+catechin condensation	96	-3.84275
Caftaric	48	-3.90984
Ceramide	12	-14.605
Ceramide	24	-27.497
Ceramide	48	-17.1615
Ceramide	96	-15.1155
<i>cis</i> -Piceid	48	-5.8127

Citramalic	48	-4.67451
Citric	96	-3.55806
Cyclobutanemethanol	48	-3.78747
Desmosterol	96	4.918915
Dihydroactinidolide	12	-4.62477
Esculin	48	-4.31966
Farnesene	12	-3.30054
Ferulic	12	3.829484
Fucose	96	-6.93236
Fumaric	48	-3.96194
Gallic	48	-5.43171
Geraniol	12	-9.46357
Glucose	96	-3.94365
Glyceryl trioleate	96	7.908166
Glyceryl tripalmitoleate	96	-4.42801
Hexanal	12	-4.04782
Hexanal	48	-3.18381
Hexanal	96	-6.47276
Isoleucine	96	-5 46325
Isorhamnetin-3-O-glucoside	48	3 347461
Isorhamnetin-3-rutinoside	48	-5 73167
Isorhapontin	96	-4 63637
Kaempferol-3-O-glucoside	96	-3 2905
Kaempferol-3-O-rutinoside	48	-3 33013
Lactose	96	-3 1892
Leucine	96	-5 18945
Lignoceric	48	-12 8028
Linolenic	48	-3 76781
I vsine	10	-6.02651
Malic	96	-3 60199
Malonic	12	-3 59885
meso-Frythrial	48	-4 18336
muo-Inositol	48	-3 3675
myo-Inositol	96	-5.13669
Myricetin	96	-7 3156
Myrictic	18	-/ 88568
Myristic	96	-4.88508
Oleanolic	18	-7 508
Oleic acid+cis Vaccanic	48	3 1132
Pallidol	40	-5.1152
Pallidal	12	-3.14032
Deputation	48	-3.12783
Dhlorizin	90 48	-4.37333
Phlorizin	48	-5.54079
r monzili Droguonidin D2 og D1	90 24	-0.02349
Proline	24	4.213//4
	90	-3./3808
ryiogiulamic	12	-4.54/43
Pyrogiutamic	24	-3.06419

	10	6 1 - 1 - 6
Pyroglutamic	48	-6.45426
Pyroglutamic	96	-5.10183
Quercetin-3-glucoside+quercetin-3-galactoside (as que-3-glc)	48	-3.25247
Quercetin-3-O-glucuronide	48	-3.81953
Ribose	96	-12.7551
Rutin	48	-4.32333
Serine	12	-4.06745
Serine	96	-3.48392
Sinapic	48	-7.98779
Sorbitol	12	-3.48311
Sorbitol	48	-3.08914
Stearic	48	-5.64894
Sucrose	96	-4.63331
Threonine	12	-3.75152
trans-2-Hexenal	12	-4.50551
trans-2-Hexenal	48	-3.26061
trans-2-Hexenal	96	-4.14686
trans-Coutaric	48	-7.55452
trans-Piceid	24	-3.80608
trans-Piceid	48	-7.75943
trans-Resveratrol	12	-5.10929
trans-Resveratrol	48	-5.04094
unknown-10	12	-11.7843
unknown-3	12	-4.46376
unknown-3	48	-15.648
unknown-3	96	-3.18951
Uvaol	48	-13.1134
Uvaol	96	-3.88727
Valine	96	-5.00539

DEGs 12 hpi

VIT_209s0002g06890.1 VIT_207s0129q00660.1

DEGs 24 hpi

VIT 200s0187q00010.1 VIT_200s0270g00120.1 VIT_200s0301g00080.1 VIT_200s0346g00110.1 VIT 200s0346q00110.2 VIT_200s0615g00010.1 VIT_200s0615g00010.3 VIT 201s0146q00480.1 VIT_201s0146g00480.2 VIT_201s0146g00480.5 VIT_202s0012g00390.1 VIT_202s0012g01630.2 VIT_202s0012g01630.3 VIT_202s0025g00750.1 VIT_202s0025g00760.1 VIT_202s0033g01030.1 VIT_202s0033g01050.1 VIT_202s0033g01060.1 VIT_203s0088g00710.1 VIT_203s0088g00810.1 VIT 203s0088q00910.1 VIT_204s0008g00140.1 VIT_204s0008g07150.1 VIT 205s0020q03190.3 VIT_205s0020g03190.5 VIT_205s0020g03190.6 VIT 205s0020q04780.1 VIT 205s0020q04780.3 VIT_207s0031g00530.1 VIT 207s0129q00390.1 VIT_207s0129g00760.1 VIT 207s0129q00800.1 VIT_208s0007g00580.1 VIT 208s0007q01930.1 VIT_208s0007g01940.1 VIT_208s0007g01940.2 VIT 208s0007q04800.4 VIT_208s0007g06040.1 VIT_208s0007g06060.1 VIT 208s0007q07990.1

VIT_209s0002g00890.3 VIT 209s0002q00890.4 VIT 209s0002q01320.1 VIT_209s0002g02990.1 VIT_209s0002g03020.1 VIT 209s0002q05400.1 VIT_209s0002g06890.1 VIT_210s0042g00840.1 VIT_210s0042g00860.1 VIT 210s0042q00870.1 VIT_210s0042g00880.1 VIT_210s0042g00890.1 VIT_210s0042g00920.1 VIT_211s0016g03250.1 VIT_212s0034g01930.1 VIT_214s0060g02170.1 VIT_214s0108g00630.1 VIT 214s0108q00690.1 VIT_218s0001g06090.1 VIT_218s0001g06120.1 VIT_218s0001g10200.1 VIT_218s0041g02010.1 VIT_218s0041g02270.1 VIT_218s0122g00620.1 VIT_218s0166g00050.1 VIT 219s0015q02680.1 VIT_219s0015g02730.1 VIT_219s0015g02890.1 VIT 219s0093q00110.1 VIT_219s0093g00190.1 DEGs 48 hpi

VIT_200s0187g00020.2 VIT_200s0259g00100.1 VIT_201s0127g00560.1 VIT_202s0025g00750.1 VIT_202s0025g00760.1 VIT_202s0025g04270.1 VIT_203s0088g00700.1 VIT_203s0088g00710.1 VIT_203s0088g00810.1 VIT_203s0088g00910.1 VIT_204s0008g00140.1 VIT_204s0008g01070.1 VIT_204s0008g02510.1 VIT 204s0008q07150.1 VIT 205s0049q00570.1 VIT_207s0031g01070.1 VIT_207s0031g01710.1 VIT_207s0197g00130.1 VIT_208s0007g06060.1 VIT_208s0040g01130.1 VIT_209s0002g01310.1 VIT 209s0002q04560.1 VIT_209s0002g06890.1 VIT_211s0016g03250.1 VIT_212s0057g00140.1 VIT_212s0057g01020.1 VIT_213s0019g04380.2 VIT_214s0030g00680.1 VIT_214s0066g01810.1 VIT 214s0066q01840.1 VIT_214s0066g01970.1 VIT_214s0108g01000.1 VIT_215s0024g00410.1 VIT_215s0046g01140.1 VIT_215s0046g02410.3 VIT_216s0013g00070.1 VIT_216s0022g00890.1 VIT 216s0050q01890.1 VIT_216s0098g00510.1 VIT_216s0098g00860.1 VIT 216s0100q00940.1 VIT_216s0100g01000.1 VIT_217s0000g03370.1 VIT 217s0000q03370.2 VIT 217s0000q03370.4 VIT 217s0000q04880.1 VIT 218s0001q06170.1 VIT_218s0122g00620.1

DEGs 96 hpi

VIT_215s0048g02990.1 VIT_217s0000g08160.1 VIT_214s0060g02270.2 VIT_219s0090g01540.1 VIT_219s0090g00600.1 VIT_211s0016g01810.1 VIT_208s0007g08040.1

VIT_215s0046g01140.3 VIT 212s0059q00830.1 VIT 208s0040q00930.2 VIT_208s0040g00930.3 VIT_208s0040g00930.1 VIT_202s0025g04250.1 VIT_206s0004g06840.2 VIT_203s0091g00310.1 VIT_203s0091g00310.2 VIT 201s0011q04630.1 VIT_201s0127g00700.1 VIT_210s0003g04190.1 VIT_201s0010g03040.1 VIT_216s0100g00830.1 VIT_204s0008g06140.1 VIT_202s0025g04260.1 VIT_202s0025g04270.1 VIT 214s0066q01590.3 VIT_205s0077g00500.1 VIT_201s0011g04650.1 VIT_202s0025g01720.1 VIT_201s0011g03420.1 VIT_209s0002g08420.1 VIT_216s0100g00950.1 VIT_202s0025g04280.1 VIT 215s0046q01570.1 VIT_214s0083g00920.1 VIT_212s0028g03020.1 VIT_213s0067g02710.1 VIT_202s0025g00760.1 VIT_208s0007g05580.1 VIT 208s0040q00540.1 VIT 209s0018q00240.1 VIT 209s0018q00240.4 VIT 209s0018q00240.5 VIT_201s0011g05470.2 VIT 212s0134q00170.1 VIT_204s0044g01430.1 VIT 203s0038q04050.2 VIT_218s0001g10200.1 VIT_211s0052g01200.1 VIT 217s0000q09710.1 VIT_219s0014g05430.1 VIT_215s0048g02070.1 VIT_204s0044g00220.1

VIT_218s0122g00190.1	VIT_201s0011g00820.3	VIT_213s0074g00350.1	VIT_203s0063g02260.3
VIT_208s0105g00180.1	VIT_201s0011g00820.2		VIT_202s0025g04330.1
VIT_211s0052g01260.1	VIT_201s0011g00820.4		VIT_216s0050g01260.1
VIT_212s0034g00130.1	VIT_210s0523g00020.5	VIT_214s0036g00920.3	VIT_215s0024g01170.1
VIT_201s0011g03070.1	VIT_210s0523g00020.4	VIT_214s0036g00920.2	VIT_206s0061g01300.1
VIT_208s0007g05790.1	VIT_210s0523g00020.2	VIT_214s0036g00920.1	VIT_204s0023g01080.1
VIT_218s0072g00550.1	VIT_208s0056g00180.2	VIT_217s0000g08380.1	VIT_204s0008g06570.4
VIT_201s0026g00980.1	VIT_208s0056g00180.1	VIT_203s0088g00910.1	VIT_204s0008g06570.1
VIT_208s0058g00990.1	VIT_212s0059g00920.1	VIT_206s0009g01600.1	VIT_204s0008g06570.2
VIT_215s0021g02060.1	VIT_203s0038g01450.1	VIT_204s0008g06930.1	VIT_204s0008g06570.3
VIT_208s0007g03530.1	VIT_201s0127g00710.11	VIT_214s0083g00670.1	VIT_204s0008g05510.1
VIT_208s0007g03530.2	VIT_201s0127g00710.10	VIT_213s0067g03070.1	VIT_203s0091g00690.1
VIT_209s0002g00150.1	VIT_201s0127g00710.15	VIT_215s0021g02790.1	VIT_208s0032g01150.1
VIT_209s0002g00150.2	VIT_201s0127g00710.14	VIT_215s0021g00470.3	VIT_206s0009g01620.1
VIT_209s0002g00150.5	VIT_201s0127g00710.16	VIT_219s0138g00090.1	VIT_215s0046g02110.1
VIT_209s0002g00150.7	VIT_214s0066g02610.1	VIT_216s0100g01010.1	VIT_205s0077g01970.1
VIT_209s0002g00150.6	VIT_206s0004g04010.1	VIT_205s0020g04990.1	VIT_211s0016g03190.1
VIT_212s0028g03010.1	VIT_216s0013g00070.1	VIT_216s0100g00770.1	VIT_211s0118g00800.3
VIT_217s0000g02050.2	VIT_202s0025g04420.1	VIT_208s0058g01390.1	VIT_211s0118g00800.4
VIT_217s0000g02050.1	VIT_204s0008g05760.1	VIT_208s0007g07990.1	VIT_211s0016g01060.1
VIT_200s0181g00120.1	VIT_204s0008g05760.2	VIT_217s0000g02490.1	VIT_205s0020g03190.2
VIT_203s0091g00810.1	VIT_204s0008g05760.3	VIT_213s0067g03140.1	VIT_201s0011g04460.3
VIT_211s0016g00660.1	VIT_203s0017g01410.3	VIT_211s0037g00940.1	VIT_212s0035g00330.1
VIT_204s0008g02510.3	VIT_203s0017g01410.2	VIT_214s0066g02060.1	VIT_203s0038g03430.2
VIT_211s0016g04650.1	VIT_203s0017g01410.1	VIT_217s0000g01630.1	VIT_219s0014g04650.1
VIT_205s0020g03710.1	VIT_216s0050g00410.1	VIT_211s0016g05780.1	VIT_214s0066g01970.1
VIT_200s0199g00310.1	VIT_218s0001g07320.1	VIT_211s0016g00710.2	VIT_201s0026g01030.1
VIT_205s0077g01600.1	VIT_205s0077g02350.1	VIT_217s0000g09190.2	VIT_207s0031g00530.1
VIT_207s0191g00230.1	VIT_201s0026g00910.1	VIT_217s0000g09190.1	VIT_216s0098g00510.1
VIT_201s0011g05250.1	VIT_217s0000g07420.1	VIT_212s0034g01900.1	VIT_201s0127g00710.9
VIT_215s0021g00470.6	VIT_200s0253g00020.1	VIT_216s0013g01110.1	VIT_201s0127g00710.5
VIT_215s0021g00470.5	VIT_204s0008g00140.1	VIT_201s0011g05180.1	VIT_201s0127g00710.4
VIT_207s0129g01010.1	VIT_211s0118g00250.1	VIT_200s0615g00030.1	VIT_205s0020g00710.1
VIT_213s0019g02990.1	VIT_213s0158g00100.1	VIT_215s0024g00410.1	VIT_207s0129g00240.1
VIT_205s0020g02270.1	VIT_213s0156g00590.1	VIT_215s0024g00410.2	VIT_207s0005g00110.1
VIT_209s0002g06400.1	VIT_216s0013g01090.1	VIT_217s0000g02480.1	VIT_207s0151g00210.1
VIT_218s0001g09040.1	VIT_217s0000g04750.1	VIT_213s0067g00260.1	VIT_212s0057g00613.1
VIT_216s0098g01150.1	VIT_213s0064g01220.1	VIT_216s0022g00700.1	VIT_203s0038g03570.1
VIT_211s0052g01190.1	VIT_213s0019g01130.1	VIT_208s0058g00550.1	VIT_204s0044g01090.6
VIT_218s0001g10180.1	VIT_205s0020g04000.1	VIT_208s0105g00190.1	VIT_212s0035g00350.1
VIT_219s0090g01530.1	VIT_211s0118g00815.1	VIT_218s0001g15130.1	VIT_200s0399g00020.1
VIT_200s0620g00010.1	VIT_207s0031g01130.1	VIT_216s0022g00890.1	VIT_216s0013g00410.1
VIT_202s0025g04230.1	VIT_201s0011g05240.1	VIT_208s0007g08010.1	VIT_218s0001g15140.1
VIT_211s0052g01180.1	VIT_217s0000g02020.1	VIT_206s0004g06940.1	VIT_216s0013g02150.1
VIT_201s0011g00820.1	VIT_215s0045g01416.1	VIT_203s0063g02260.2	VIT_200s0181g00080.2

VIT_200s0181g00080.1 VIT 202s0025g00650.2 VIT_207s0005g00720.1 VIT_203s0088g00700.1 VIT 214s0006q02570.1 VIT_213s0156g00150.1 VIT_219s0014g03270.1 VIT 202s0012q01390.1 VIT_216s0050g01890.1 VIT 207s0005q01950.1 VIT 209s0054q00530.1 VIT_211s0052g01300.1 VIT 206s0061q00100.1 VIT_211s0149g00070.2 VIT 208s0040q00770.3 VIT 216s0013q00440.1 VIT 216s0013q00390.1 VIT 202s0025q04310.1 VIT 202s0025q04300.1 VIT 201s0011q05670.2 VIT 209s0070q00160.1 VIT 212s0055q00020.1 VIT_203s0038g00310.1 VIT 214s0066q02350.1 VIT 214s0006q00300.1 VIT 208s0058q00670.1 VIT 201s0011q02470.1 VIT 212s0035q00920.1 VIT 202s0025q02590.1 VIT 216s0013q00480.1 VIT 219s0015q01440.1 VIT 213s0064q01455.1 VIT 213s0019q01980.1 VIT 201s0011q03430.1 VIT 212s0035q00340.1 VIT 208s0007q02360.1 VIT 209s0054q00110.1 VIT 204s0008q06570.5 VIT_210s0116g00530.1 VIT_216s0013g01080.1 VIT 212s0142q00450.2 VIT 200s0463q00025.2 VIT 213s0084g00670.1 VIT 210s0003q00390.1 VIT 201s0011q02790.1 VIT_201s0011g03440.1 VIT 208s0007q01070.1 VIT_201s0011g06140.1 VIT_215s0046g01160.1 VIT 205s0077q01290.2 VIT_215s0045g01370.1 VIT_202s0025g02990.1 VIT 216s0100q00910.1 VIT_214s0006g02555.1 VIT 216s0050q01400.1 VIT 216s0050q02220.1 VIT_206s0004g00150.1 VIT 206s0009q01060.1 VIT_219s0090g00120.1 VIT 217s0000q07730.1 VIT 202s0025q02805.1 VIT 200s0477q00010.1 VIT 208s0056q01310.1 VIT 216s0050q01420.1 VIT 212s0057q00420.2 VIT 202s0234q00130.1 VIT 218s0086q00410.1 VIT 212s0034q00030.1 VIT 218s0122q00230.1 VIT 201s0010q02020.1 VIT 201s0011q00690.1 VIT 201s0011q04460.4 VIT 201s0011q04460.1 VIT 201s0011q04460.2 VIT 218s0122q00190.2 VIT 208s0007q06620.1 VIT 202s0025q00650.1 VIT 203s0038q03570.3 VIT 203s0038q03570.2 VIT 201s0010q02010.1 VIT 202s0012q00890.1 VIT 219s0090q01720.3 VIT 215s0048q00530.1 VIT_209s0002g04560.1 VIT 204s0008q05390.1 VIT 202s0025q01240.1 VIT 208s0007q01560.1 VIT 203s0017q01460.2 VIT 214s0081q00020.1 VIT 212s0057q00420.1

VIT_208s0007g08020.1 VIT 200s1569q00010.1 VIT_207s0151g00130.1 VIT_204s0008g02510.2 VIT 204s0008q02510.1 VIT_206s0061g00120.1 VIT_218s0001g03370.1 VIT 214s0006q02330.1 VIT_200s1380g00030.1 VIT 214s0006q02370.1 VIT 209s0002q01310.1 VIT_208s0007g01550.1 VIT 210s0003q02280.1 VIT_207s0151g00270.1 VIT 210s0003q02390.1 VIT 210s0003q02400.1 VIT 210s0003q02320.1 VIT 210s0003q02350.1

The main aim of this PhD project was to better understand the mechanisms responsible for resistance in vine plants using targeted metabolomic and transcriptomic approaches. In particular, early responses to the pathogen *P. viticola*, occurring within the initial 96 hours post inoculation, were investigated using two interesting resistant varieties, Bianca and Jasmine. The central question of this study was to identify chemical compounds as putative biomarkers of resistance against *P. viticola*, building up the relationship between informative elements – genes/transcripts – and functional elements – metabolites – in cells.

Our goal was to cover the maximum number of compounds from different chemical classes to identify and quantify them in grapevine leaf samples. To do this, the first part of the thesis has been devoted to covering the lack of targeted methods at our disposal for the development of:

- a rapid and versatile method for the extraction, identification and quantification of different classes of grape lipids: fatty acids, sterols, glycerolipids, glycerophospholipids and sphingolipids using liquid chromatographic electrospray ionization tandem mass spectrometry (LC/ESI-MS/MS) (Chapter II);

and the optimisation and validation of:

a GC-MS protocol for the identification and quantification of primary compounds. Specifically, the method was validated for 96 compounds: 29 acids, 17 amino acids, 12 amines and others, 24 sugars and 14 fatty acids (Chapter IV).

The two methods were successfully validated and applied to Bianca and Jasmine leaf discs.

Furthermore, we decided to investigate the influence of mechanical wounding using a leaf disc technique. The use of leaf discs is widely adopted in various kinds of

experiments for different vegetable species, in particular in studies regarding the effect of different types of biotic stress on the biochemical response of the grapevine. Since there was a little knowledge regarding mechanical wounding of grapevine leaves, this part of the thesis was targeted at analysing changes in phenolic, lipid and carotenoid content in Bianca grapevine leaves subjected to mechanical wounding (leaf discs) at 0, 6, 12, 24, 48, 96 and 120 hours after injury, testing two different sizes of leaf discs (1.1 cm and 2.8 cm in diameter) in order to determine the role of these compounds in response to mechanical stress. For the first time, we focused attention on metabolic perturbation due to mechanical wounding in the grapevine. It was shown that compound accumulation needs to be taken into account when performing metabolic investigation. These results also show that when carrying out experiments on discs it is always necessary to have identical control samples for each time point, in order not to underestimate external factors and other stresses not involved in the research (Chapter III).

The last two parts of this PhD project aimed to explore *P.viticola*-grapevine interaction. We used two different grapevine genotypes containing different sources of resistance to *P. viticola*.

Grapevine and *P. viticola* interaction is still poorly understood in terms of metabolites: there is a need to improve knowledge about how the plant system is perturbed after biotic stress. In the third part of my project we used a resistant Bianca grapevine to identify biomarkers of host-pathogen interaction by applying a metabolomics approach. We observed minimal metabolite changes in the host within 12 hpi; most of them were volatile compounds, which may interfere with pathogen endophytic invasion of mesophyll air spaces. The classes of biomarkers specific to 24 and 48 hpi suggested that early host responses to P. viticola were being set in place during those stages with a sharp shift in primary metabolism. Subsequently, secondary metabolism was affected more strongly by the pathogen, with changes in volatile compounds at 48-96 hpi and at the latest at 96 hpi in phenolic compounds. Based on our results, we can argue that all the compounds significantly differentiated in infected samples have a role in Bianca-P. viticola interaction. Specifically, 53 metabolites were identified as putative biomarkers in hybrid Bianca grapevine leaves after P. viticola inoculation. Some of them are known biomarkers of resistance (viniferins). Among the others, some are likely to be putative biomarkers of resistance in Bianca leaf discs after P. viticola infection, such as

benzaldehyde and proline. This is the first time that an extensive metabolomic study has been undertaken using a hybrid grape variety to better understand metabolic perturbation after *P. viticola* infection, finding early stage biomarkers for different chemical classes of metabolites. These results can represent a starting point for better understanding grapevine resistance and can lead to discoveries regarding new mechanisms for plantpathogen interaction between the grapevine and *P. viticola*.

In the fourth part of the project we decided to perform a two -omics investigation to obtain a wider vision, in order to decipher complex biological systems. Using the Jasmine grapevine we investigated metabolic and transcriptomic perturbation. We were able to identify 88 metabolites highly modulated after pathogen inoculation and probably involved in pathogen resistance. As already described in Bianca, we found volatile production at 12 hpi and then a large number of metabolites modulated at later stages. The 48 and 96 hour time points were characterised by an increase in some lipid compounds (mostly fatty acids) flavonols and phenylpropanoids; we also found an increase in amino acids and sugars after pathogen inoculation. The changes in metabolites reflect gene expression modulation. General down-regulation was found in the early stages, with reactivation of the metabolism at 48 and 96 hours. Genes related to plant response and hypersensitive response were over-expressed, starting from 12 hours and throughout the time course. Multiple co-inertial analysis was applied to integrate metabolomics and transcript data. We found evident differences between inoculated and uninoculated samples at 96 hpi and there was evidence of the influence of time in gene expression and metabolite accumulation.

Future prospects for development of this thesis would involve the investigation of different genotypes of resistant grapevines. Different resistance sources could be taken into account to determine whether the type of resistance affects the accumulation of specific chemical compounds. The putative biomarkers should be better investigated and tested against the pathogen to confirm their implication in resistance processes.

Another interesting future perspective could be better integration and networking of metabolomics and transcriptomic data, in order to investigate their correlation in resistance.

Summary of Ph.D. experiences

This Ph.D. project is part of the International Ph.D. Program in the Genomics and Molecular Physiology of Fruits Plants (GMPF) of the Fondazione Edmund Mach International Research School Trentino (FEM-FIRS>T) with partner institution the University of Udine. In July 2013 I was selected for the Ph.D. scholarship to undertake research in "Metabolomics and Transcriptomics: novel approaches to understand resistance in grapevine against *Plasmopara viticola*" starting from November 2013.

My supervisors are Dr. Urska Vrhovsek of the Metabolomics Unit of the Department of Food Quality and Nutrition, Research and Innovation Center of the Fondazione Edmund Mach, San Michele all'Adige, Italy; Dr. Gabriele Di Gaspero, previously of the Department of Agronomical and Environmental Sciences of the University of Udine, Italy and, currently, of the Applied Genomics Institute (IGA) in Udine, Italy.

During my Ph.D. most of the experiments were conducted at the Fondazione Edmund Mach laboratories. The greenhouse experiments were conducted at the Fondazione Edmund Mach with the help of Marco Stefanini and Dr. Luca Zulini of the Grapevine Genetics and Breading Research unit (GMGV). Transcriptomics analyses were performed in collaboration with Genomic platform of Fondazione Edmund Mach managed by Dr. Massimo Pindo. All the bioinformatics and statistics analysis were performed with Dr. Samantha Riccadonna, Dr. Pietro Franceschi and Dr. Alessandro Cestaro. I spent 5 month working on untargeted lipidomics in the University of North Texas, Denton, Texas at the Dr. Vladimir Shulaev laboratory.

During the past three years, I attended the national school in Analytical and Bioanalytical Techniques in Mass Spectrometry held in Parma (Italy) organized by the Società Chimica Italiana; the workshop on "RNA-seq Data analysis" held in Napoli Italy, organized by Elixir Italia and the summer school held in Toulouse (France) sponsored by EU COST Action "The request for tolerant varieties: phenotyping at plant and cellular level (FA1306) and organized by GenoToul Biostat platform, Laboratory of Plantmicrobe Interaction (LIPM) and Plant Science Research Laboratory (LRSV).

I actively participated to national and international symposia and congress, such as the International Conference Plant Health for Sustainable Agriculture-PHSA (Ljubljana 2015); the IX In Vino Analytica Scientia Symposium-IVAS (Mezzocorona, 2015); X International Symposium on Grapevine Physiology and Biotechnology (Verona, 2016); Massa 2016-Società Chimica Italiana (Roma, 2016); oral presentation are accepted in Global Conference on Plant Science and Molecular Biology-GPMB (September 2017) and IPM3.0-The international conference on "Future Integrated Pest Management" (October 2017).

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