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TESI DI DOTTORATO DI RICERCA

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

DOTTORANDA

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

CONTENT

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 plant-microbe 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 I

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 integrated-omics. 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. Multi-omics information from different cultivars could be useful in order to proceed with the best breeding strategies in the future.

Bibliography

Abbà S, Galetto L, Carle P, et al (2014) RNA-Seq profile of flavescence dorée phytoplasma in grapevine. BMC Genomics 15:1088. doi: 10.1186/1471-2164-15-1088

Agudelo-Romero P, Erban A, Rego C, et al (2015) Transcriptome and metabolome reprogramming in Vitis vinifera cv. Trincadeira berries upon infection with Botrytis cinerea. J Exp Bot eru517. doi: 10.1093/jxb/eru517

Allègre M, Daire X, Héloir M-C, et al (2007) Stomatal deregulation in Plasmopara viticola-infected grapevine leaves. New Phytol 173:832–840. doi: 10.1111/j.1469-8137.2006.01959.x

Almagro L, Carbonell-Bejerano P, Belchí-Navarro S, et al (2014) Dissecting the transcriptional response to elicitors in Vitis vinifera cells. PloS One 9:e109777. doi: 10.1371/journal.pone.0109777

Ash G (2000) Downy mildew of grape. Plant Health Instr. doi: 10.1094/PHI-I-2000-1112-01

Bellin D, Peressotti E, Merdinoglu D, et al (2009) Resistance to Plasmopara viticola in grapevine "Bianca" is controlled by a major dominant gene causing localised necrosis at the infection site. Theor Appl Genet 120:163–176. doi: 10.1007/s00122-009-1167-2

Bennett RN, Wallsgrove RM (1994) Secondary metabolites in plant defence mechanisms. New Phytol 127:617–633. doi: 10.1111/j.1469-8137.1994.tb02968.x

Bisson LF, Waterhouse AL, Ebeler SE, et al (2002) The present and future of the international wine industry. Nature 418:696–699. doi: 10.1038/nature01018

Blaeser M. (1978) Untersuchungen zur Epidemiologie des falschen Mehltaus an Weinrebe, Plasmopara viticola (Berk. et de Toni). PhD Thesis Univ. Bonn Ger. 127 pp.

Blaeser M. and H.C. Weltzien (1978) Die Bedeutung von Sporangienbildung, -ausbreitung und -keimung für die Epidemiebildung von Plasmopara viticola. Z. Für Pflanzenkrankh. Pflanzenschutz 155– 161.

Blaeser M, Weltzien HC (1979) Epidemiological studies of Plasmopara viticola for improving determination of spraying dates. In: Z. Pflanzenkrankh. Pflanzenschutz. https://eurekamag.com/research/000/652/000652449.php. Accessed 11 Oct 2016

Busam G, Kassemeyer HH, Matern U (1997) Differential expression of chitinases in Vitis vinifera L. responding to systemic acquired resistance activators or fungal challenge. Plant Physiol 115:1029–1038.

Chen W-J, Delmotte F, Cervera SR, et al (2007) At Least Two Origins of Fungicide Resistance in Grapevine Downy Mildew Populations. Appl Environ Microbiol 73:5162–5172. doi: 10.1128/AEM.00507-07

Dadakova K, Havelkova M, Kurkova B, et al (2015) Proteome and transcript analysis of Vitis vinifera cell cultures subjected to Botrytis cinerea infection. J Proteomics 119:143–153. doi: 10.1016/j.jprot.2015.02.001

Degu A, Hochberg U, Sikron N, et al (2014) Metabolite and transcript profiling of berry skin during fruit development elucidates differential regulation between Cabernet Sauvignon and Shiraz cultivars at branching points in the polyphenol pathway. BMC Plant Biol 14:188. doi: 10.1186/s12870-014-0188-4

Derckel JP, Baillieul F, Manteau S, et al (1999) Differential Induction of Grapevine Defenses by Two Strains of Botrytis cinerea. Phytopathology 89:197–203. doi: 10.1094/PHYTO.1999.89.3.197

Dercks W, Creasy LL (1989a) Influence of fosetyl-Al on phytoalexin accumulation in the Plasmopara viticola-grapevine interaction. Physiol Mol Plant Pathol 34:203–213. doi: 10.1016/0885-5765(89)90044-1

Dercks W, Creasy LL (1989b) The significance of stilbene phytoalexins in the Plasmopara viticola-grapevine interaction. Physiol Mol Plant Pathol 34:189–202. doi: 10.1016/0885-5765(89)90043-X

Di Gaspero G, Cipriani G (2003) Isolation and characterisation of resistance gene analogs (RGAs) in Grape. Acta Hortic 603:419–427.

Di Gaspero G, Cipriani G, Adam-Blondon A-F, Testolin R (2007) Linkage maps of grapevine displaying the chromosomal locations of 420 microsatellite markers and 82 markers for R-gene candidates. Theor Appl Genet 114:1249–1263. doi: 10.1007/s00122-007-0516-2

Dixon RA (2001) Natural products and plant disease resistance. Nature 411:843–847. doi: 10.1038/35081178

Eibach R, Zyprian E, Welter L, Töpfer R (2007) The use of molecular markers for pyramiding resistance genes in grapevine breeding. VITIS - J Grapevine Res 46:120.

Ferreira RB, Monteiro SS, Piçarra-Pereira MA, Teixeira AR (2004) Engineering grapevine for increased resistance to fungal pathogens without compromising wine stability. Trends Biotechnol 22:168–173. doi: 10.1016/j.tibtech.2004.02.001

Ferri M, Tassoni A, Franceschetti M, et al (2009) Chitosan treatment induces changes of protein expression profile and stilbene distribution in Vitis vinifera cell suspensions. Proteomics 9:610–624. doi: 10.1002/pmic.200800386

Fischer BM, Salakhutdinov I, Akkurt M, et al (2004) Quantitative trait locus analysis of fungal disease resistance factors on a molecular map of grapevine. TAG Theor Appl Genet Theor Angew Genet 108:501–515. doi: 10.1007/s00122-003-1445-3

Gabler FM, Smilanick JL, Mansour M, et al (2003) Correlations of Morphological, Anatomical, and Chemical Features of Grape Berries with Resistance to Botrytis cinerea. Phytopathology 93:1263–1273. doi: 10.1094/PHYTO.2003.93.10.1263

Gauthier A, Trouvelot S, Kelloniemi J, et al (2014) The Sulfated Laminarin Triggers a Stress Transcriptome before Priming the SA- and ROS-Dependent Defenses during Grapevine's Induced Resistance against Plasmopara viticola. PLOS ONE 9:e88145. doi: 10.1371/journal.pone.0088145

Gessler C, Pertot I, Perazzolli M (2011) Plasmopara viticola : a review of knowledge on downy mildew of grapevine and effective disease management. Phytopathol Mediterr 50:3–44. doi: 10.14601/Phytopathol_Mediterr-9360

Gika HG, Theodoridis GA, Vrhovsek U, Mattivi F (2012) Quantitative profiling of polar primary metabolites using hydrophilic interaction ultrahigh performance liquid chromatography–tandem mass spectrometry. J Chromatogr A 1259:121–127. doi: 10.1016/j.chroma.2012.02.010

Gindro K, Pezet R, Viret O (2003) Histological study of the responses of two Vitis vinifera cultivars (resistant and susceptible) to Plasmopara viticola infections. Plant Physiol Biochem 41:846–853. doi: 10.1016/S0981-9428(03)00124-4

Giraud E, Ivanova A, Gordon CS, et al (2012) Sulphur dioxide evokes a large scale reprogramming of the grape berry transcriptome associated with oxidative signalling and biotic defence responses. Plant Cell Environ 35:405–417. doi: 10.1111/j.1365-3040.2011.02379.x

Godard S, Slacanin I, Viret O, Gindro K (2009) Induction of defence mechanisms in grapevine leaves by emodin- and anthraquinone-rich plant extracts and their conferred resistance to downy mildew. Plant Physiol Biochem PPB Société Fr Physiol Végétale 47:827–837. doi: 10.1016/j.plaphy.2009.04.003

Gómez-Zeledón J, Zipper R, Spring O (2013) Assessment of phenotypic diversity of Plasmopara viticola on Vitis genotypes with different resistance. Crop Prot 54:221–228. doi: 10.1016/j.cropro.2013.08.015

Harborne JB (1999) The comparative biochemistry of phytoalexin induction in plants. Biochem Syst Ecol 27:335–367. doi: 10.1016/S0305-1978(98)00095-7

Heath MC (2000) Hypersensitive response-related death. In: Lam E, Fukuda H, Greenberg J (eds) Programmed Cell Death in Higher Plants. Springer Netherlands, pp 77–90

Heath MC, Skalamera D (1997) Cellular Interactions between Plants and Biotrophic Fungal Parasites. In: J.H. Andrews ICT and JAC (ed) Advances in Botanical Research. Academic Press, pp 195–225

Jaillon O, Aury J-M, Noel B, et al (2007) The grapevine genome sequence suggests ancestral hexaploidization in major angiosperm phyla. Nature 449:463–467. doi: 10.1038/nature06148

Keller M (2015) The Science of Grapevines: Anatomy and Physiology. Academic Press

Keller M, Viret O, Cole FM (2003) Botrytis cinerea Infection in Grape Flowers: Defense Reaction, Latency, and Disease Expression. Phytopathology 93:316–322. doi: 10.1094/PHYTO.2003.93.3.316

Kortekamp A (2006) Expression analysis of defence-related genes in grapevine leaves after inoculation with a host and a non-host pathogen. Plant Physiol Biochem 44:58–67. doi: 10.1016/j.plaphy.2006.01.008

Langcake P, Lovell PA (1980) Light and electron microscopical studies of the infection of Vitis spp. by Plasmopara viticola, the downy mildew pathogen.

Li X, Wu J, Yin L, et al (2015) Comparative transcriptome analysis reveals defense-related genes and pathways against downy mildew in Vitis amurensis grapevine. Plant Physiol Biochem PPB 95:1–14. doi: 10.1016/j.plaphy.2015.06.016

Malacarne G, Vrhovsek U, Zulini L, et al (2011) Resistance to Plasmopara viticola in a grapevine segregating population is associated with stilbenoid accumulation and with specific host transcriptional responses. BMC Plant Biol 11:114. doi: 10.1186/1471-2229-11-114

Marguerit E, Boury C, Manicki A, et al (2009) Genetic dissection of sex determinism, inflorescence morphology and downy mildew resistance in grapevine. TAG Theor Appl Genet Theor Angew Genet 118:1261–1278. doi: 10.1007/s00122-009-0979-4

Merdinoglu D, Wiedeman-Merdinoglu S, Coste P, et al (2003) Genetic analysis of downy mildew resistance derived from muscadinia rotundifolia. Acta Hortic 451–456. doi: 10.17660/ActaHortic.2003.603.57

Moreira FM, Madini A, Marino R, et al (2010) Genetic linkage maps of two interspecific grape crosses (Vitis spp.) used to localize quantitative trait loci for downy mildew resistance. Tree Genet Genomes 7:153–167. doi: 10.1007/s11295-010-0322-x

Moroldo M, Paillard S, Marconi R, et al (2008) A physical map of the heterozygous grapevine “Cabernet Sauvignon” allows mapping candidate genes for disease resistance. BMC Plant Biol 8:66. doi: 10.1186/1471-2229-8-66

Mulas G, Galaffu MG, Pretti L, et al (2011) NMR analysis of seven selections of vermentino grape berry: metabolites composition and development. J Agric Food Chem 59:793–802. doi: 10.1021/jf103285f

Munson T.V. (1909) Foundations of American grape culture. In: Orange Judd Co. https://scholar.google.com/scholar_lookup?title=Foundations%20of%20American%20grape%20culture&author=TV.%20Munson&publication_year=1909. Accessed 14 Oct 2016

Peixe A, Hegewald H, Bohm J, et al (2004) Preliminary results of trials to improve fungal resistance in Portuguese Vitis vinifera cultivars using classical breeding approaches. In: Acta Hortic. https://eurekamag.com/research/004/279/004279391.php. Accessed 28 Oct 2016

Shiratake K, Suzuki M (2016) Omics studies of citrus, grape and rosaceae fruit trees. Breed Sci 66:122–138. doi: 10.1270/jsbbs.66.122

Slaughter AR, Hamiduzzaman MM, Gindro K, et al (2008) Beta-aminobutyric acid-induced resistance in grapevine against downy mildew: involvement of pterostilbene. Eur J Plant Pathol 122:185–195. doi: 10.1007/s10658-008-9285-2

Teixeira A, Martins V, Noronha H, et al (2014) The first insight into the metabolite profiling of grapes from three Vitis vinifera L. cultivars of two controlled appellation (DOC) regions. Int J Mol Sci 15:4237–4254. doi: 10.3390/ijms15034237

Velasco R, Zharkikh A, Troggio M, et al (2007) A High Quality Draft Consensus Sequence of the Genome of a Heterozygous Grapevine Variety. PLOS ONE 2:e1326. doi: 10.1371/journal.pone.0001326

Viennot-Bourgin G (1949) Les champignons parasites des plantes cultivees. 1 1. Masson, Paris

Welter LJ, Göktürk-Baydar N, Akkurt M, et al (2007) Genetic mapping and localization of quantitative trait loci affecting fungal disease resistance and leaf morphology in grapevine (Vitis vinifera L). Mol Breed 20:359–374. doi: 10.1007/s11032-007-9097-7

Preface to Chapter II

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 plant-microbe 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 (http://www.lipidmaps.org), 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 R^2 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.

References not included in the paper:

Emanuelli F, Lorenzi S, Grzeskowiak L, et al (2013) Genetic diversity and population structure assessed by SSR and SNP markers in a large germplasm collection of grape. BMC Plant Biol 13:39. doi: 10.1186/1471-2229-13-39

European Commission, Document No. SANCO/12495/2011 Validation and quality control procedures for pesticide residues analysis in food and feed, (2011). doc.SANCO/12495/2011,validation and quality control procedures for pesticide residues analysis in food and feed.

Shah J (2005) Lipids, Lipases, and Lipid-Modifying Enzymes in Plant Disease Resistance. Annu Rev Phytopathol 43:229–260. doi: 10.1146/annurev.phyto.43.040204.135951

Wang X (2004) Lipid signaling. Curr Opin Plant Biol 7:329–336. doi: 10.1016/j.pbi.2004.03.012

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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.

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), glycerolipids (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-(3-hexan-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 (CH_3OH, LC–MS grade) and chloroform ($CHCl_3$) were purchased from Sigma-Aldrich. Formic acid (HCOOH) and ammonium formate (NH_4COOH) 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-dioleoyl-sn-glycero-3-phospho-rac-(1-glycerol)sodium salt, 1-palmitoyl-sn-glycero-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 molecular formula, Chemspider ID and molecular weight.

Class	Compound	Chemspider ID	Molecular formula	Molecular weight (MW)
Carnitines	Oleoyl-L-carnitine hydrochloride	21403150	C25H47NO4	425.4
	Palmitoyl-L-carnitine hydrochloride	16737192	C23H46ClNO4	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	4444437	C18H30O2	278.2
	Myristic acid	10539	C14H28O2	228.2
	Myristoleic acid	4444564	C14H26O2	226.2
	Oleic acid + *cis*-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	C57H104O6	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	C24H51NO7P	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 $CHCl_3/CH_3OH$ 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/H_2O 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/H_2O (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 μg mL^{-1} and 1 μg mL^{-1} respectively and used to check sample preparation efficiency.

The stock of standard solutions and the spiking solutions were stored at −20 °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 (±0.005) g from each sample was weighed and 1.5 mL of CH_3OH were added, vortexed for 30 s, 3 mL of $CHCl_3$ 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 (R^2) limit of quantification (LOQ), limit of detection (LOD) and matrix effect, obtained using solutions of standards and 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	0.0002–5.8	$y=1{,}781{,}6884x+1{,}170{,}800$	1.00	$y=14{,}144{,}865x+666{,}169$	1.00	−26.0	0.1	0.3
	Palmitoyl-L-carnitine hydrochloride	0.00002–4.36	$y=39{,}651{,}340x+78{,}003$	1.00	$y=29{,}621{,}992x+3023$	1.00	−33.9	0.01	0.03
Sterols	Desmosterol	0.09–3.845	$y=295{,}822x+65{,}554$	0.95	$y=274{,}231x+108{,}744$	0.90	−7.9	19.3	57.9
	Ergosterol	0.09–1.985	$y=100{,}137x-12$	0.99	$y=87{,}502x-3004$	1.00	−14.4	49.6	148.8
	Lanosterol	0.05–1.0675	$y=107{,}149x-4434$	1.00	$y=85{,}316x-4259$	1.00	−25.8	21.3	63.9
	Uvaol	0.005–1.105	$y=2{,}507{,}521x+86{,}572$	0.99	$y=2{,}295{,}751x+49{,}898$	0.98	−9.2	2.2	6.7
Fatty acids	Arachidic acid	0.003–31.3	$y=1971{,}102x-83{,}094$	1.00	$y=2{,}046{,}868x+264{,}544$	0.99	3.7	1.6	4.7
	Behenic acid	0.008–17.1	$y=2{,}607{,}531x+130{,}259$	1.00	$y=2{,}628{,}503x-52{,}941$	1.00	0.8	3.4	10.2
	cis-11-eicosenoic acid	0.07–15.5	$y=7673x+1005$	0.99	$y=6317x+1060$	0.99	−21.5	31.1	93.3
	Erucic acid	0.001–33.9	$y=4{,}874{,}309x-636{,}823$	0.99	$y=4{,}949{,}227x-656{,}680$	0.99	1.9	0.8	2.5
	Heptadecanoic acid	0.01–27.1	$y=531{,}131x+728{,}127$	0.99	$y=486{,}268x+460{,}828$	0.99	−9.2	11.3	33.9
	Lignoceric acid	0.003–9.2	$y=2{,}712{,}524x+683{,}788$	0.98	$y=2{,}841{,}008x+337{,}484$	0.98	4.5	1.9	5.6
	Linoleic acid	0.007–28.0	$y=2{,}563{,}516x+5{,}753{,}986$	0.97	$y=2{,}170{,}119x+8{,}184{,}061$	0.94	−18.1	7.0	21.0
	Linolenic acid	0.006–69.5	$y=821{,}382x+828{,}911$	0.99	$y=842{,}024x+806{,}833$	0.99	2.5	2.8	8.4
	Myristic acid	0.05–57	$y=365{,}484x+96{,}999$	1.00	$y=334{,}746x+185{,}754$	1.00	−9.2	22.8	68.4
	Myristoleic acid	0.05–56.5	$y=391{,}868x-15{,}488$	1.00	$y=368{,}574x-36{,}527$	1.00	−6.3	11.3	33.9
	Oleic acid+*cis*-Vaccenic acid	0.02–28.3	$y=1{,}975{,}170x+9{,}760{,}208$	0.96	$y=1{,}777{,}976x+3{,}723{,}146$	0.97	−11.1	14.1	42.3
	Palmitic acid	0.1–32	$y=19{,}724x+12{,}835$	1.00	$y=17{,}856x-19{,}368$	0.98	−10.5	32.0	128.2
	Palmitoleic acid	0.02–25.4	$y=601{,}474x+285{,}142$	0.99	$y=534{,}259x+284{,}641$	0.99	−12.6	12.7	38.1
	Stearic acid	0.002–28.4	$y=1{,}476{,}286x+1{,}248{,}339$	0.99	$y=1{,}506{,}209x-142{,}367$	0.99	2.0	1.4	4.3
Glycerolipids	1.2.3-Tripentadecanoylglycerol	0.00007–0.8	$y=119{,}296{,}649x+6{,}912{,}932$	0.96	$y=120{,}666{,}830x+3{,}263{,}178$	0.97	1.1	0.04	0.1
	1-Linoleoyl-rac-glycerol	0.02–14.2	$y=139{,}154x+1410$	1.00	$y=116{,}890x-10{,}601$	0.97	−19.1	14.2	42.6
	1-Monopalmitoleoyl-rac-glycerol	0.02–52.5	$y=1{,}270{,}229x-135{,}275$	1.00	$y=1{,}051{,}850x-109{,}119$	1.00	−20.8	10.5	31.5
	1-Oleoyl-rac-glycerol	0.003–17.9	$y=1{,}951{,}698x-242{,}429$	1.00	$y=1{,}799{,}361x-506{,}911$	1.00	−8.5	1.8	5.3
	Glyceryl trioleate	0.03–7.1	$y=7{,}274{,}665x+2{,}401{,}909$	0.96	$y=6{,}816{,}032x+1{,}467{,}346$	0.97	6.7	14.1	42.3
	Glyceryl tripalmitoleate	0.003–3.5	$y=34{,}292{,}391x+1{,}504{,}691$	1.00	$y=35{,}000{,}163x-39{,}599$	1.00	2.0	1.4	4.3
Glycerophospholipids	1.2-Dilinoleoyl-sn-glycero-3-phosphocholine	0.01–15.6	$y=59{,}249{,}626x+21{,}763{,}259$	0.98	$y=58{,}952{,}735x+19{,}159{,}586$	0.98	−0.5	6.3	18.8
	1.2-Dioleoyl-sn-glycero-3-phosphocholine	0.006–12.6	$y=60{,}549{,}785x+10{,}796{,}759$	0.99	$y=63{,}008{,}040x+11{,}502{,}248$	0.99	3.9	3.1	9.4
	1.2-Dioleoyl-sn-glycero-3-phospho-rac-(1-glycerol) sodium salt	0.01–12.8	$y=41{,}360{,}769x+1{,}615{,}101$	0.99	$y=41{,}001{,}805x+3{,}811{,}341$	0.97	−0.9	6.4	19.2
	1-Palmitoyl-sn-glycero-3-phosphocholine	0.01–19.9	$y=14{,}155{,}899x+678{,}383$	1.00	$y=11{,}959{,}790x+2{,}361{,}447$	0.99	−18.4	8.0	23.9
Sphingolipids	Ceramide	0.0002–14.9	$y=14{,}453{,}621x+5{,}731{,}780$	0.97	$y=15{,}128{,}604x+6{,}550{,}053$	0.96	4.5	0.1	0.4
Prenols	Oleanolic acid	0.04–4.6	$y=1{,}604{,}598x+474{,}133$	0.97	$y=1{,}469{,}483x+592{,}518$	0.95	−9.2	22.8	68.4

Table 3
Mass transitions (MRM) and instrumental parameters optimized for each metabolyte for the analysis by UHPLC–ESI-MS/MS.

Class	Compound	Ionization mode	Precursor ion	Q1 Product ion	DP	EP	CE	CXP	Q2 Product ion	DP	EP	CE	CXP	t_R	Q2/Q1
Carnitines	Oleoyl-L-carnitine hydrochloride	$[M+H]^+$	426.3	367.0	120	11	26	17	265.0	120	11	30	13	6.5	39.0
	Palmitoyl-L-carnitine hydrochloride	$[M+H]^+$	400.3	341.0	173	10	24	14	239.0	173	10	28	14	6.2	33.8
Sterols	Cholesterol (IS)	$[M+1-H_2O]^+$	369.5	147.0	140	8	35	25	135.0	140	8	35	23	15.7	0.6
	Desmosterol	$[M+NH_4]^+$	402.4	367.2	146	10	13	20						14.1	
	Ergosterol	$[M+H]^+$	397.3	69.3	6	10	33	14	379.4	6	10	17	22	14.9	150.1
	Lanosterol	$[M+NH_4]^+$	444.3	409.5	51	10	13	32	191.3	51	10	21	16	16.9	38.3
	Uvaol	$[M+NH_4]^+$	460.4	443.4	100	10	11	22	191.4	100	10	21	10	7.6	40.5
Fatty acids	Arachidic acid	$[M+HCOO]^-$	357.2	311.4	−5	−10	−12	−21	45.1	−5	−10	−40	−5	14.5	46.7
	Behenic acid	$[M+HCOO]^-$	385.4	339.4	−60	−11	−15	−10	321.4	−60	−11	−47	−16	16.0	0.3
	cis-11-Eicosenoic acid	$[M-H]^-$	309.4	291.5	−167	−11	−33	−19	155.0	−167	−11	−37	−10	12.7	16.2
	Docosahexaenoic acid (IS)	$[M+HCOO]^-$	373.3	327.3	−60	−10	−10	−21	283.4	−60	−10	−20	−20	8.4	0.1
	Erucic acid	$[M+HCOO]^-$	383.2	337.3	−95	−10	−12	−23	45.0	−95	−10	−44	−5	14.6	35.6
	Heptadecanoic acid	$[M+HCOO]^-$	315.4	269.3	−45	−13	−10	−17	45.0	−10	−10	−32	−5	11.0	64.6
	Lignoceric acid	$[M+HCOO]^-$	413.2	367.3	−65	−10	−12	−23	45.0	−65	−10	−42	−5	18.6	35.9
	Linoleic acid	$[M+HCOO]^-$	325.2	279.2	−60	−12	−10	−21	261.0	−60	−12	−35	−23	9.0	0.4
	Linolenic acid	$[M+HCOO]^-$	323.3	277.2	−65	−6	−8	−17	259.3	−65	−6	−27	−17	8.2	0.5
	Myristic acid	$[M+HCOO]^-$	273.2	227.2	−5	−10	−18	−15	45.0	−5	−10	−26	−7	8.4	99.2
	Myristoleic acid	$[M+HCOO]^-$	271.3	225.3	−45	−11	−20	−17	45.0	−30	−10	−26	−7	6.6	87.6
	Oleic acid+*cis*-vaccenic acid	$[M+HCOO]^-$	327.2	281.2	−50	−10	−14	−17	45.1	−50	−10	−34	−5	10.8	48.4
	Palmitic acid	$[M+HCOO]^-$	301.2	255.3	−55	−10	−16	−23	44.9	−55	−10	−38	−5	10.1	80.2
	Palmitoleic acid	$[M+HCOO]^-$	299.3	253.3	−55	−10	−18	−15	44.7	−55	−10	−34	−7	8.5	37.6
	Stearic acid	$[M+HCOO]^-$	329.3	283.3	−60	−12	−9	−22	265.5	−60	−12	−41	−16	12.7	0.3
Glycerolipids	1,2,3-Tripentadecanoylglycerol	$[M+NH_4]^+$	782.7	523.5	106	4	31	33	225.4	106	4	49	17	24.1	6.5
	1-Linoleoyl-rac-glycerol	$[M+H]^+$	355.3	337.0	300	3	15	16	263.0	300	3	13	21	7.6	25.0
	1-Monopalmitoleoyl-rac-glycerol	$[M+H]^+$	329.3	121.2	104	7	29	9	219.3	104	7	17.5	17	7.1	179.7
	1-Oleoyl-rac-glycerol	$[M+H]^+$	357.3	265.2	250	10	15	13	283.2	250	10	13	17	9.2	27.2
	Glyceryl trioleate	$[M+NH_4]^+$	902.8	265.0	80	8	55	14	603.0	80	8	60	14	24.4	59.8
	Glyceryl tripalmitoleate	$[M+NH_4]^+$	818.7	547.0	300	4	28	27	237.3	300	4	53	12	23.7	22.3
Glycerophospholipids	1,2-Dilinoleoyl-sn-glycero-3-phosphocholine	$[M+H]^+$	782.6	184.0	100	15	27	23	125.0	100	15	123	35	15.6	78.7
	1,2-Dioleoyl-sn-glycero-3-phosphocholine	$[M+H]^+$	786.6	184.0	116	3	27	14	125.0	116	3	134	14	18.0	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
	1-Palmitoyl-sn-glycero-3-phosphocholine	$[M+H]^+$	496.4	478.4	100	14	27	14	184.0	100	14	35	14	6.5	641.5
Sphingolipids	Ceramide	$[M+H]^+$	566.6	548.6	100	8	18	14	264.0	100	8	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

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 $CHCl_3/CH_3OH/H_20$ 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 N_2. Samples were reconstituted in 300 μL of ACN/IPA/H_2O (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/H_2O (65:30:5 v/v/v) and re-injected 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 × 2.1 mm; 2.7 μm 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 mL min^{-1}, mobile phase A consisted of ACN 40% in water, NH_4COOH 10 mM and HCOOH 0.1% and B consisted of IPA 90%, ACN 10%, NH_4COOH 10 mM and HCOOH 0.1%.

The UHPLC system was coupled directly to an API 5500 triple-quadrupole 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 μg mL^{-1}) in water/ACN (40:60 v/v) with NH_4COOH 10 mM and HCOOH 0.1% into a mass spectrometer at a flow rate of 10 μL 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/H_2O) 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 and inter-day (CV%), recovery (%) accuracy (%RE) and precision (%RSD).

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	3.10	1.23	63.39	−36.61	5.34
	Behenic acid	2.65	4.44	67.38	−32.62	9.75
	cis-11-Eicosenoic acid	6.97	1.47	105.47	5.47	6.39
	Erucic acid	2.70	3.66	68.89	−31.11	3.19
	Heptadecanoic acid	3.89	1.91	82.79	−17.21	6.23
	Lignoceric acid	2.62	6.56	66.29	−33.71	5.10
	Linoleic acid	2.82	6.27	92.24	−7.76	9.63
	Linolenic acid	2.83	2.97	99.25	−0.75	8.05
	Myristic acid	3.22	2.94	79.02	−20.98	3.76
	Myristoleic acid	2.88	6.35	78.87	−21.13	5.18
	Oleic acid + *cis*-Vaccenic acid	3.45	3.56	79.84	−20.16	33.15
	Palmitic acid	18.86	20.35	84.74	−15.26	3.36
	Palmitoleic acid	2.23	3.82	92.25	−7.75	4.42
	Stearic acid	5.11	2.61	72.48	−27.52	14.38
Glycerolipids	1,2,3-Tripentadecanoylglycerol	0.96	5.23	80.63	−19.37	6.80
	1-Linoleoyl-rac-glycerol	4.56	13.47	95.73	−4.27	11.26
	1-Monopalmitoleoyl-rac-glycerol	3.07	5.27	82.06	−17.94	5.86
	1-Oleoyl-rac-glycerol	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

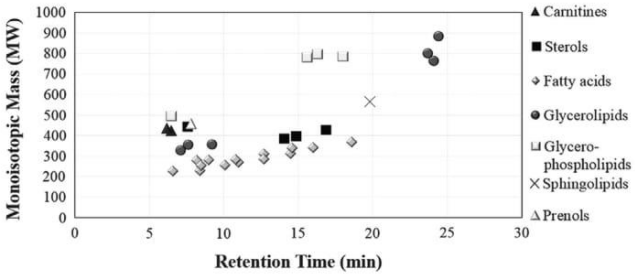

Fig. 1. Division of the different classes of grape lipids on the basis of retention time.

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].

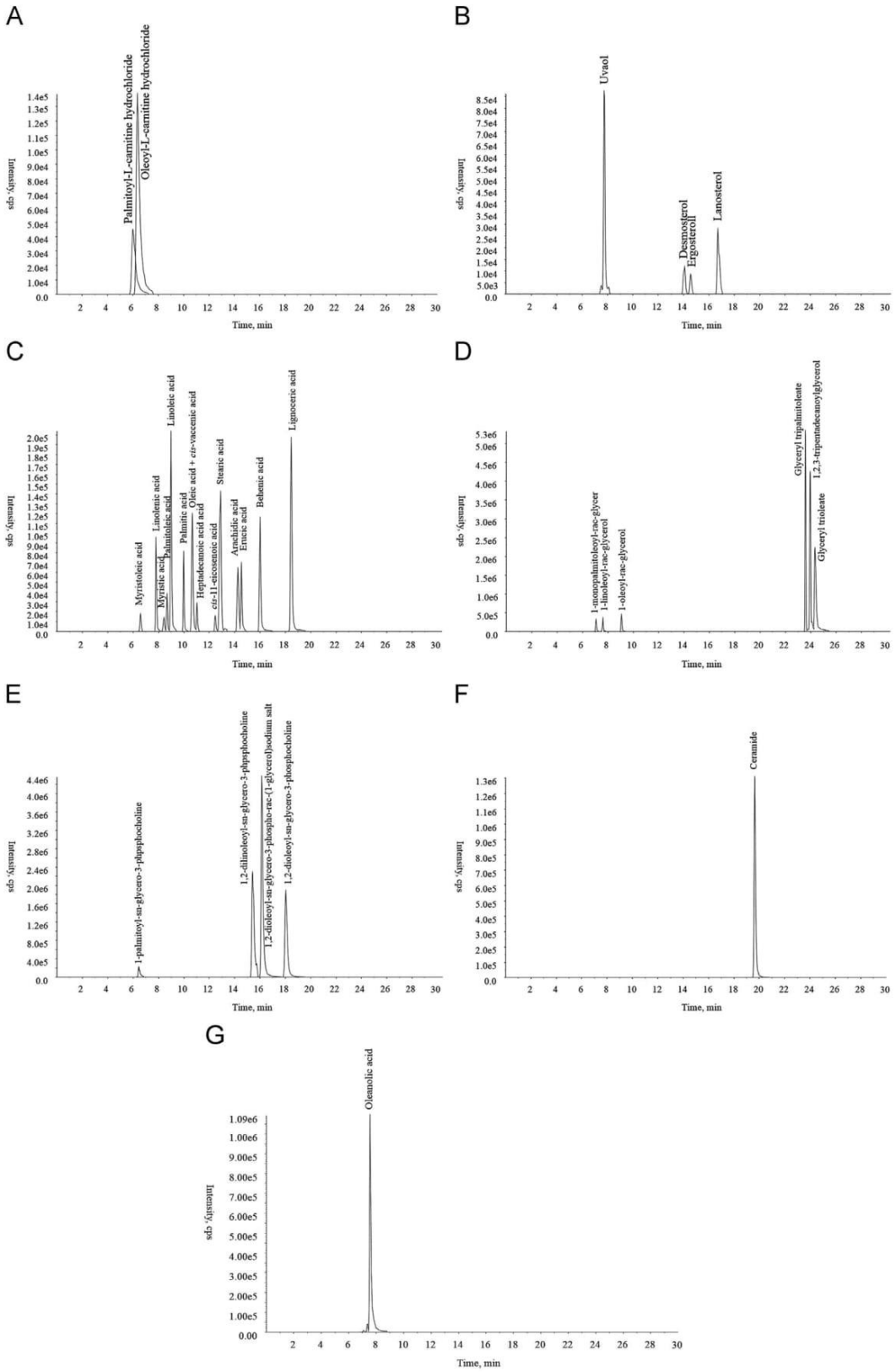

Fig. 2. UHPLC–ESI-MS/MS chromatograms of the quantifier MRM transition for the 33 lipids. (A) Carnitines, (B) sterols, (C) fatty acids, (E) glycerolipids, (F) glycerophospholipids, (G) sphingolipids, and (H) prenols.

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 H_2O 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 ±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 (μg 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 μg 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-dilinoleoyl-sn-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.

References

[1] E. Fahy, D. Cotter, M. Sud, S. Subramaniam, Biochim. Biophys. Acta 1811 (2011) 637.
[2] J. Ohlrogge, J. Browse, Plant Cell Online 7 (1995) 957.
[3] J.J. Reina-Pinto, A. Yephremov, Plant Physiol. Biochem. 47 (2009) 540.
[4] J. Shah, Annu. Rev. Phytopathol. 43 (2005) 229.
[5] R. Welti, W. Li, M. Li, Y. Sang, H. Biesiada, H.-E. Zhou, C.B. Rajashekar, T. D. Williams, X. Wang, J. Biol. Chem. 277 (2002) 31994.
[6] M. Rubio, M. Alvarez-Ortí, A. Alvarruiz, E. Fernández, J.E. Pardo, J. Agric. Food Chem. 57 (2009) 2812.
[7] M. Roufet, C. Bayonove, R. Cordonnier, Vitis 26 (1987) 85.
[8] J.A. Bauman, J.F. Gallander, A.C. Peng, Am. J. Enol. Vitic. 28 (1977) 241.
[9] Y. Lefur, C. Hory, M. Bard, A. Olsson, Vitis 33 (1994) 127.
[10] J.F. Gallander, A.C. Peng, Am. J. Enol. Vitic. 31 (1980) 24.
[11] A. Miele, J. Bouard, A. Bertrand, Am. J. Enol. Vitic. 44 (1993) 180.
[12] L.P. Santos, D.R. Morais, N.E. Souza, S.M. Cottica, M. Boroski, J.V. Visentainer, Food Res. Int. 44 (2011) 1414.
[13] A. Mas, J. Navarro-Pedreño, D.T. Cooke, C.S. James, Phytochemistry 35 (1994) 1249.
[14] A. Sabir, A. Unver, Z. Kara, J. Sci. Food Agric. 92 (2012) 1982.
[15] B. Matthäus, Eur. J. Lipid Sci. Technol. 110 (2008) 645.
[16] J.E. Pardo, E. Fernández, M. Rubio, A. Alvarruiz, G.L. Alonso, Eur. J. Lipid Sci. Technol. 111 (2009) 188.
[17] C. Crews, P. Hough, J. Godward, P. Brereton, M. Lees, S. Guiet, W. Winkelmann, J. Agric. Food Chem. 54 (2006) 6261.
[18] F. De Marchi, R. Seraglia, L. Molin, P. Traldi, M. De Rosso, A. Panighel, A. Dalla Vedova, M. Gardiman, M. Giust, R. Flamini, J. Mass Spectrom. 47 (2012) 1113.
[19] A. Hatanaka, Phytochemistry 34 (1993) 1201.
[20] H. Kobayashi, H. Takase, Y. Suzuki, F. Tanzawa, R. Takata, K. Fujita, M. Kohno, M. Mochizuki, S. Suzuki, T. Konno, J. Exp. Bot. 62 (2011) 1325.
[21] D.L. Capone, D.W. Jeffery, J. Agric. Food Chem. 59 (2011) 4659.
[22] C. Delfini, F. Cervetti, Vitic. Enol. Sci. (1991) 142.
[23] H. Alexandre, T. Nguyen Van Long, M. Feuillat, C. Charpentier, Rev. Fr. Oenologie Cah. Sci. 34 (1994) 11.
[24] P. Ribéreau-Gayon, Handbook of Enology: The Microbiology of Wine and Vinifications, Wiley, Chichester, 2000.
[25] E. Casalta, M. f Cervi, J. m Salmon, J. m Sablayrolles, Aust. J. Grape Wine Res. 19 (2013) 47.
[26] A.R. Borneman, T.A. Gianoulis, Z.D. Zhang, H. Yu, J. Rozowsky, M.R. Seringhaus, L.Y. Wang, M. Gerstein, M. Snyder, Science 317 (2007) 815.
[27] N. Zehethofer, D.M. Pinto, Anal. Chim. Acta 627 (2008) 62.
[28] M. Li, Z.G. Zhou, H.G. Nie, Y. Bai, H.W. Liu, Anal. Bioanal. Chem. (2011) 243.
[29] R.C. Murphy, P.H. Axelsen, Mass Spectrom. Rev. 30 (2011) 579.
[30] X. Han, R.W. Gross, Mass Spectrom. Rev. 24 (2005) 367.
[31] S. Vichi, N. Cortés-Francisco, J. Caixach, J. Mass Spectrom. 47 (2012) 1177.
[32] P.J. Horn, K.D. Chapman, Plant J. 70 (2012) 69.
[33] H.G. Gika, G.A. Theodoridis, U. Vrhovsek, F. Mattivi, J. Chromatogr. A 1259 (2012) 121.
[34] J. Folch, M. Lees, G.H.S. Stanley, J. Biol. Chem. 226 (1957) 497.
[35] C. Hu, J. Van Dommelen, R. Van Der Heijden, G. Spijksma, T.H. Reijmers, M. Wang, E. Slee, X. Lu, G. Xu, J. Van Der Greef, J. Proteome Res. 7 (2008) 4982.
[36] European Commission, Validation and Quality Control Procedures for Pesticide Residues Analysis in Food and Feed, Document No. SANCO/12495/2011, 2011.
[37] US Department of Health and Human Services, Food and Drug Administration, Center for Drug Evaluation and Research (CDER), Centre for Veterinary Medicine (CVM), Rockville, 2001.
[38] H. Kwon, S.J. Lehotay, L. Geis-Asteggiante, J. Chromatogr. A 1270 (2012) 235.
[39] F. Emanuelli, S. Lorenzi, L. Grzeskowiak, V. Catalano, M. Stefanini, M. Troggio, S. Myles, J.M. Martinez-Zapater, E. Zyprian, F.M. Moreira, M.S. Grando, BMC Plant Biol. 13 (2013) 39.
[40] X. Han, K. Yang, R.W. Gross, Mass Spectrom. Rev. 31 (2012) 134.
[41] C.J. DeLong, P.R. Baker, M. Samuel, Z. Cui, M.J. Thomas, J. Lipid Res. 42 (2001) 1959.
[42] E.J. Lesnefsky, M.S. Stoll, P.E. Minkler, C.L. Hoppel, Anal. Biochem. 285 (2000) 246.
[43] R. Taguchi, M. Ishikawa, Search J. Chromatogr. A (2010) 4229.
[44] S. Walorczyk, Talanta 120 (2014) 106.
[45] C. Tesnière, P. Delobel, M. Pradal, B. Blondin, PLoS ONE 8 (2013) e61645.
[46] M. López-Malo, R. Chiva, N. Rozes, J.M. Guillamon, Int. J. Food Microbiol. 162 (2013) 26.

Table S1: Classification of grape samples

Genetic Group Name	Genetic Group Code	Sample Name	Sample Color
Vitis non-vinifera	1	*Vitis* Anderson	Red
		Vitis Champini	Red
Hybrid	3	Alicante Henri Bouschet	Red
		Sauvignon Blanc	White
Muscat	4	Aleatico	Red
		Millardet et Grasset	Red
		Vermentino	White
		Moscato Ottonel X Malvasia bianca di Candia-clone1	White
		Moscato Ottonel X Malvasia bianca di Candia-clone2	White
Vitis vinifera	5	Merlot Noir	Red
		Pinot Noir	Red
		Syrah	Red
		Cabernet Franc	Red
		Sangiovese	Red
		Viogner	White
		Garganega	White
		Peverella	White
		Biancaccia	White

Table S2: Quantification in real samples

Class	Compound	*Vitis* Anderson	*Vitis* Champini	Alicante Henri Bouschet	Sauvignon Blanc	Aleatico	Millardet et Grasset	Vermentino	Moscato Ottonel X Malvasia bianca di Candia clone1	Moscato Ottonel X Malvasia bianca di Candia clone2	Merlot Noir	Pinot Noir	Syrah	Cabernet Franc	Sangiovese	Viogner	Garganega	Peverella	Biancaccia
Carnitines																			
	Oleoyl-L-carnitine hydrochloride	<LOQ	<LOQ	0.0010	<LOQ	0.0012	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	0.0009	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<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	0.26	N.D.	0.51	<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	1.88	4.19	<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	**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 acids																			
	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	0.49	**2.00**	0.65	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<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	**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	0.34	0.19	<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	**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**
Glycerolipids																			

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	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	<LOQ	**2.10**	<LOQ	**2.21**	<LOQ	<LOQ	<LOQ	**2.09**	<LOQ	**2.29**	**1.91**	<LOQ	**7.21**	**1.43**	<LOQ	<LOQ	<LOQ
Glycerophospholipids																		
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**
Sphingolipids																		
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	**0.01**	**0.01**	**0.01**	**0.01**
Prenols																		
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**

Preface to Chapter III

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

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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, Z-miyabenol 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 + *cis*-vaccenic 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-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

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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 μL 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×*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 N_2. Samples were reconstituted in 500 μL 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 N_2. 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 N_2 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

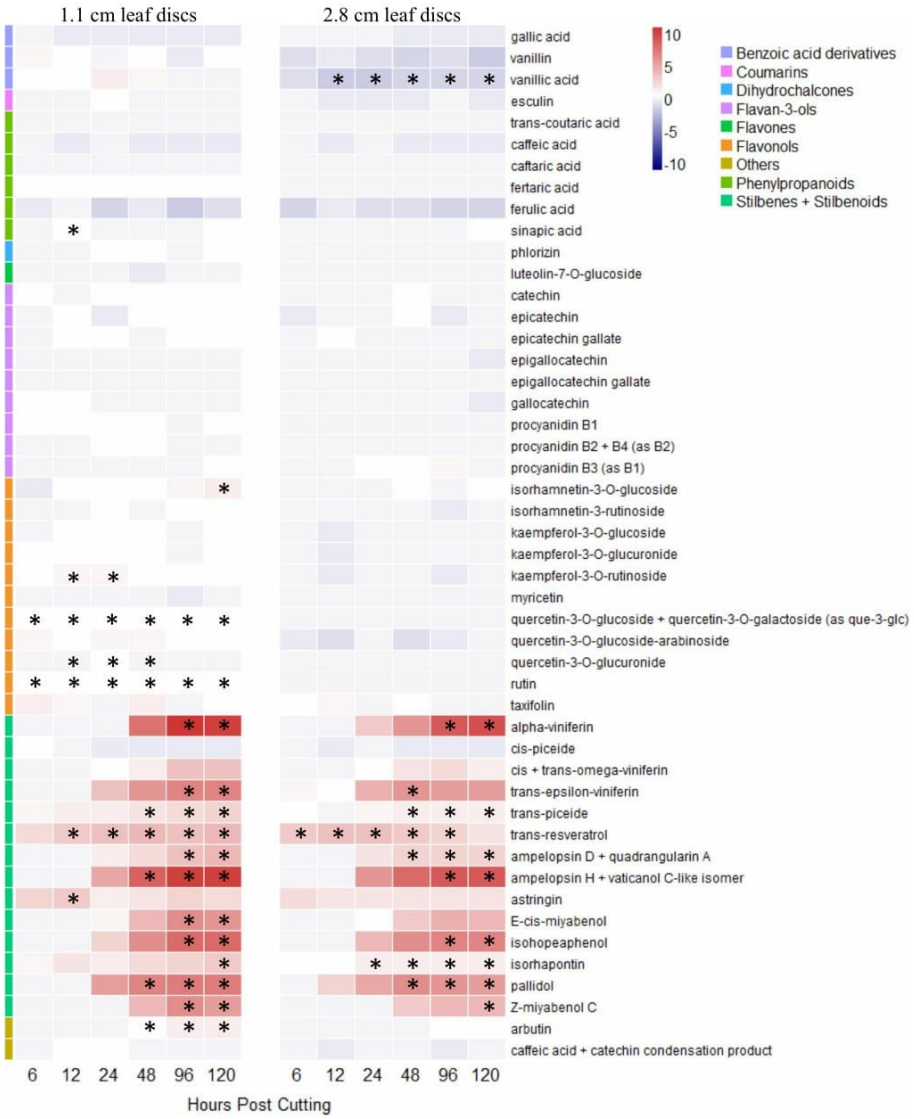

Fig. 1 Effect of mechanical wounding on phenolic metabolites during the first 120 h post cutting. Heatmaps represent $\log_2$ fold change (with zero time point) of phenolic compounds in 1.1 and 2.8-cm-diameter leaf discs 6, 12, 24, 48, 96 and 120 h post cutting. *Blue* and *red boxes* represent lower 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 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

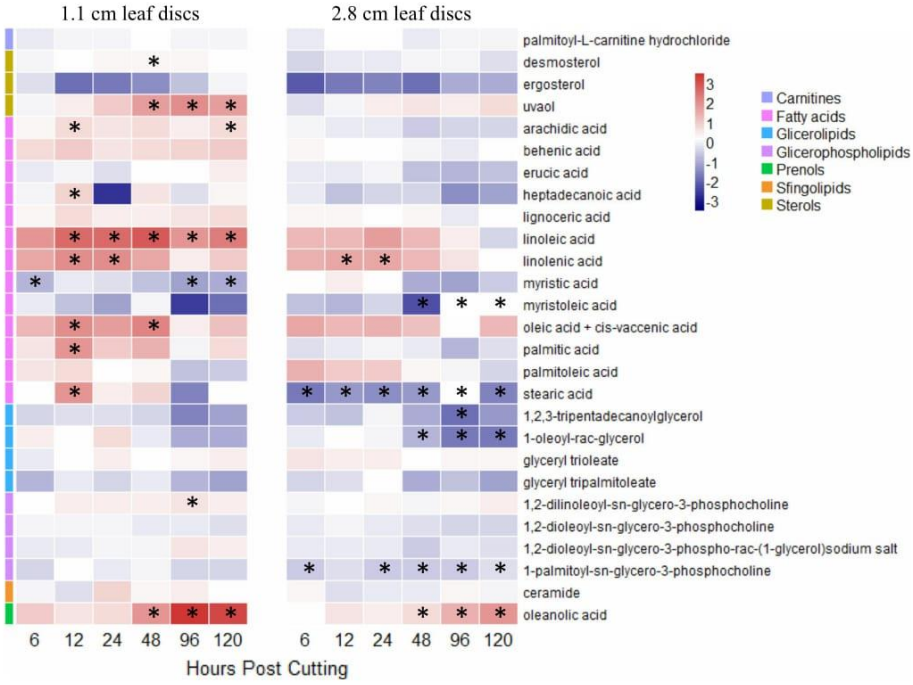

Fig. 2 Effect of mechanical wounding on lipid compounds during the first 120 h post cutting. Heatmaps represent $\log_2$ fold change (with zero time point) of lipid compounds in 1.1 and 2.8-cm-diameter leaf discs 6, 12, 24, 48, 96 and 120 h post cutting. *Blue* and *red boxes* represent lower and higher 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-cm-diameter 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

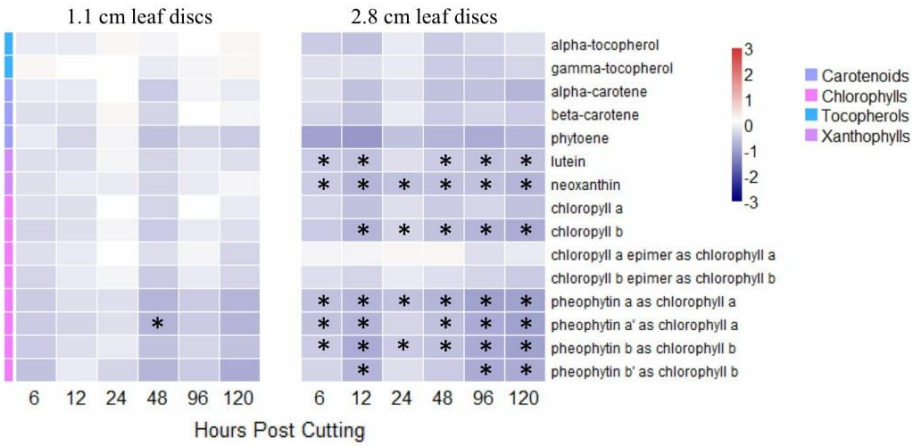

Fig. 3 Effect of mechanical wounding on carotenoid, chlorophyll, xanthophyll and tocopherol compounds during the first 120 h post cutting. Heatmaps represent $\log_2$ fold change (with zero time point) of lipid compounds in 1.1 and 2.8-cm-diameter leaf discs 6, 12, 24, 48, 96 and 120 h post cutting. *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)

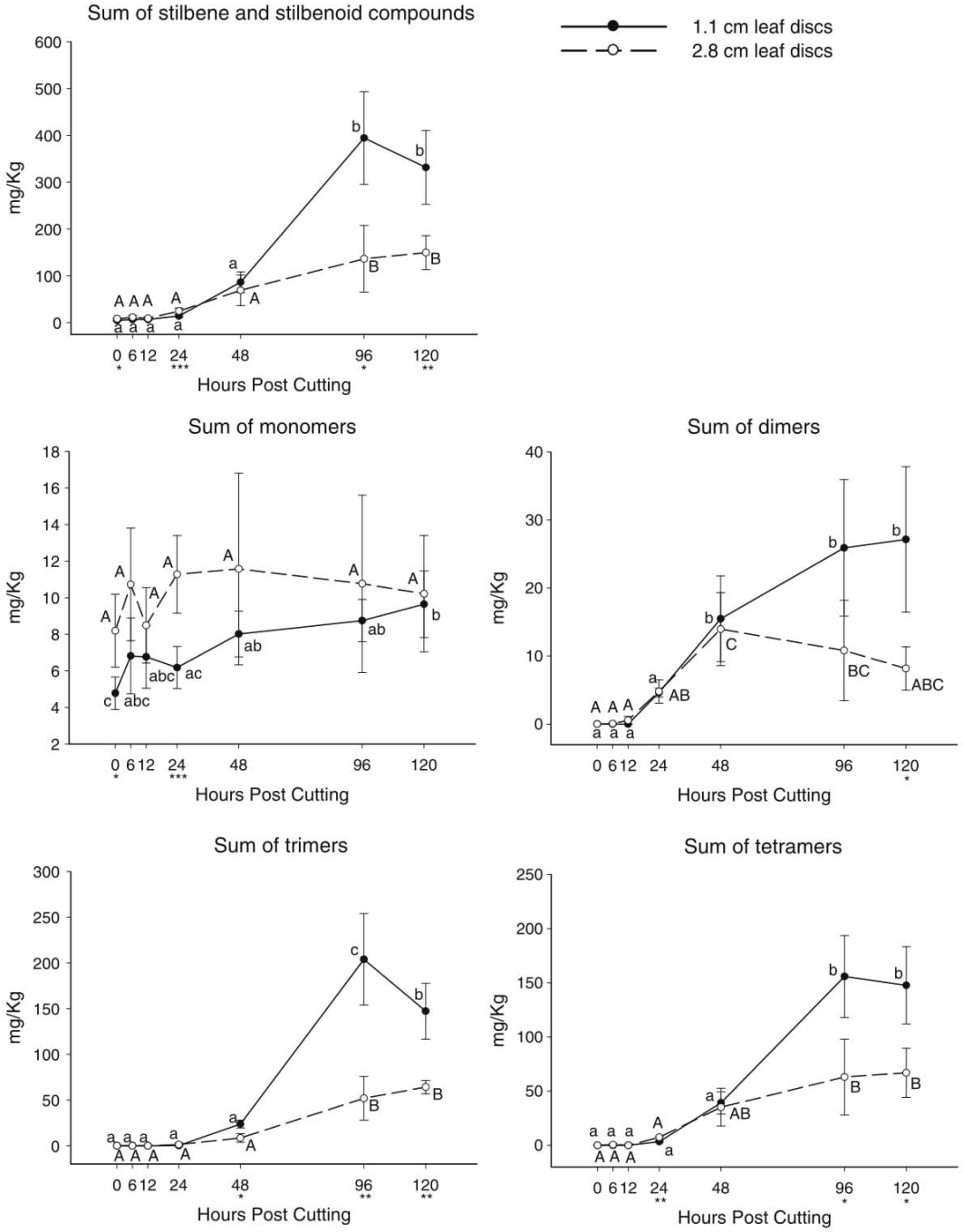

Fig. 4 Effect of mechanical wounding on stilbene + stilbenoid metabolites during the first 120 h post cutting. *Bars* represent ±STDEV $n = 3$. One-way ANOVA followed by Newman-Keuls test was able to determine the differences (*letters in the graph*) over the time points for each of the two different 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 2.8-cm 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

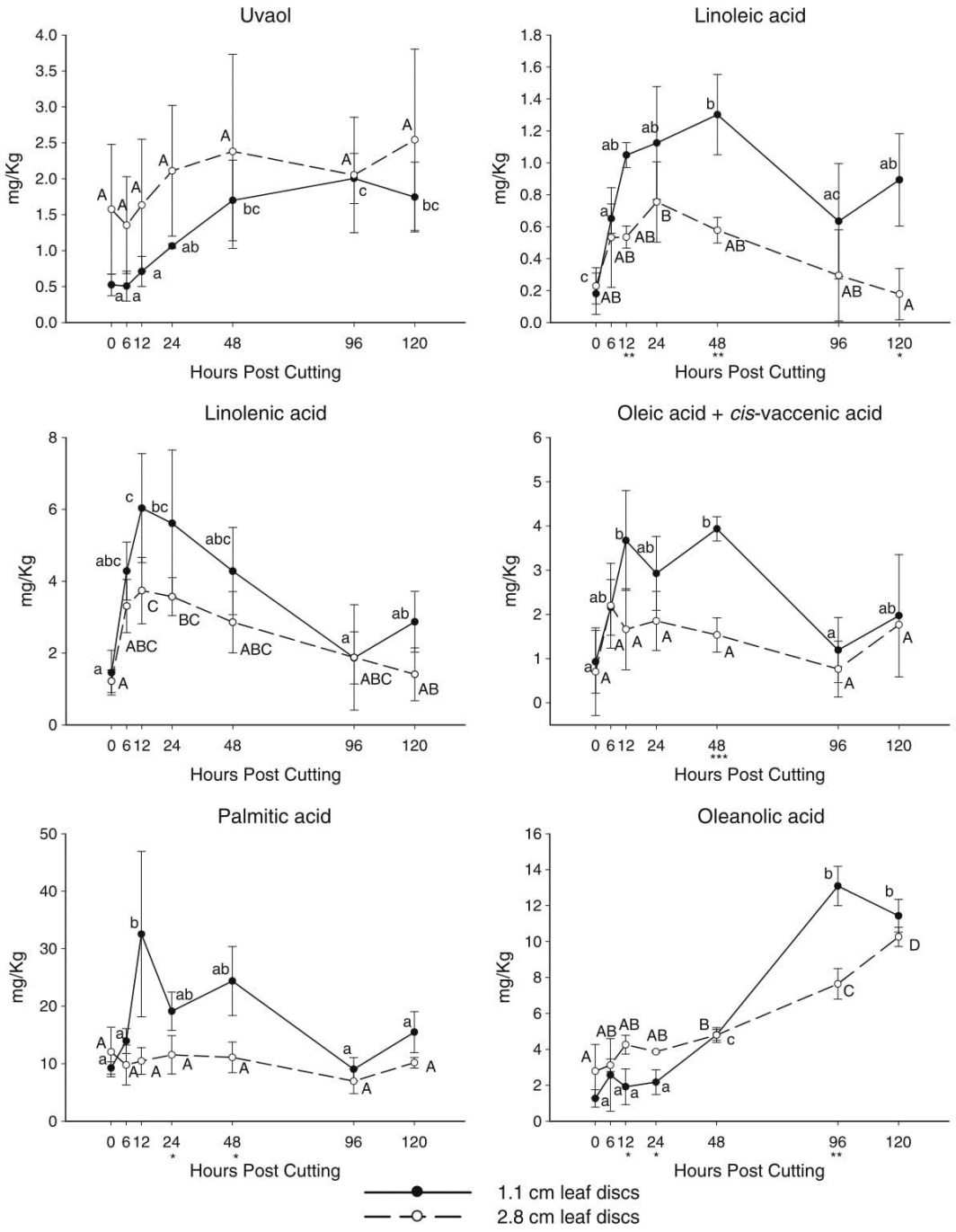

Fig. 5 Effect of mechanical wounding on lipid compounds during the first 120 h post cutting. *Bars* represent ±STDEV $n = 3$. One-way ANOVA followed by Newman-Keuls test was able to determine the differences (*letters in the graph*) over the time points for each of the two different sets (1.1 and 2.8 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

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

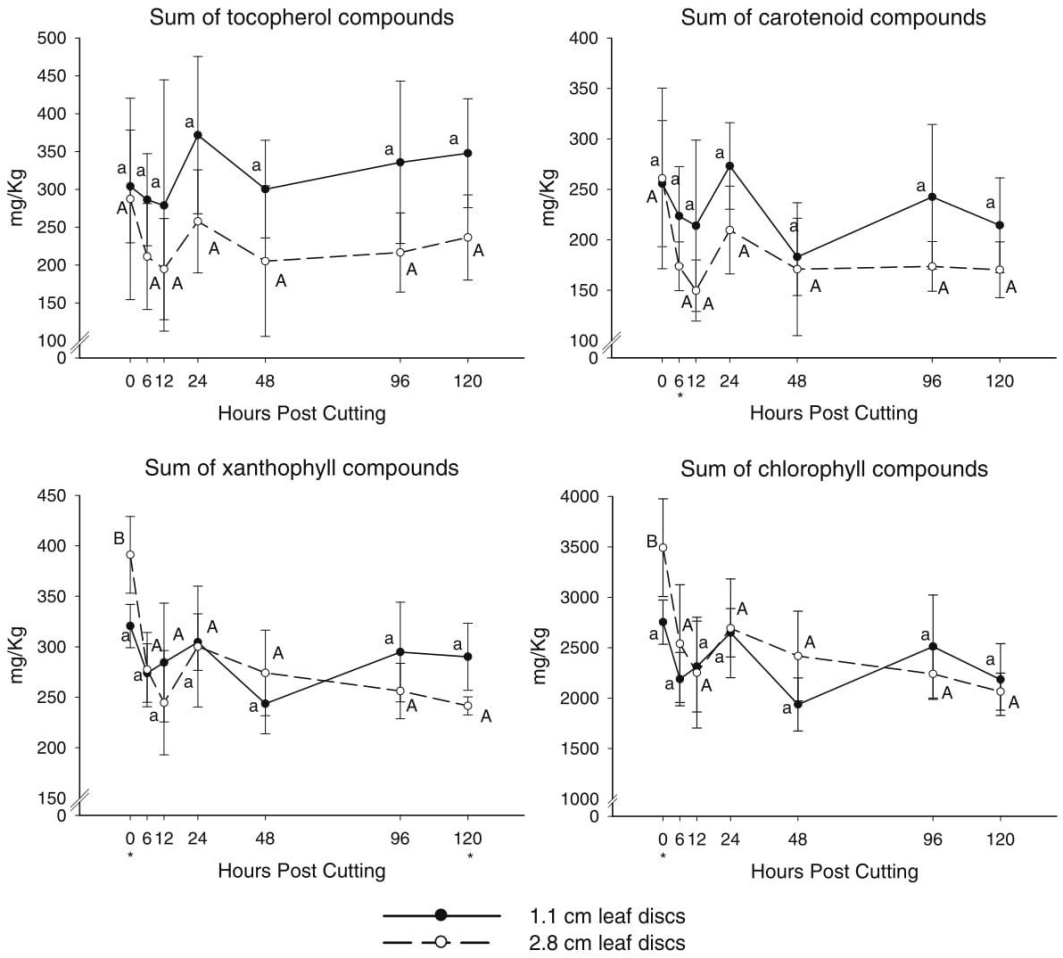

Fig. 6 Effect of mechanical wounding on carotenoid, chlorophyll, xanthophyll and tocopherol compounds during the first 120 h post cutting. *Bars* represent ±STDEV $n = 3$. One-way ANOVA followed by Newman-Keuls test was able to determine the differences (*letters in the graph*) over the time points for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

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.

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 *trans*-piceide, 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-*cis*-miyabenol 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 A_2-mediated release of unsaturated fatty acids from membrane lipids (Farmer and Ryan 1992; Dhondt et al. 2000), and it has been demonstrated that phospholipase A_2 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 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

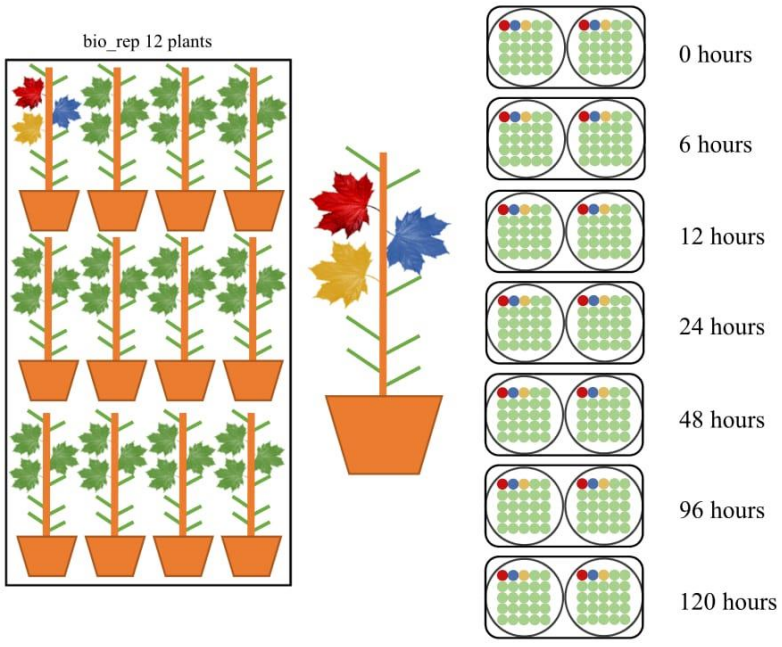

Fig. 7 Randomization scheme for 1.1-cm-diameter leaf discs. One biological replicate was made up of 12 homogeneous plants; the third, fourth and fifth leaves from each plant were detached, and around 20 discs from each leaf were excised. To have sufficient material for metabolic analysis, a minimum of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

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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References

Adrian M, Jeandet P, Veneau J et al (1997) Biological activity of resveratrol, a stilbenic compound from grapevines, against Botrytis cinerea, the causal agent for gray mold. J Chem Ecol 23:1689–1702. doi:10.1023/B:JOEC.0000006444.79951.75

Bellin D, Peressotti E, Merdinoglu D et al (2009) Resistance to Plasmopara viticola in grapevine "Bianca" is controlled by a major dominant gene causing localised necrosis at the infection site. Theor Appl Genet 120:163–176. doi:10.1007/s00122-009-1167-2

Benikhlef L, L'Haridon F, Abou-Mansour E et al (2013) Perception of soft mechanical stress in Arabidopsis leaves activates disease resistance. BMC Plant Biol 13:133. doi:10.1186/1471-2229-13-133

Boso S, Martínez MC, Unger S, Kassemeyer H-H (2006) Evaluation of foliar resistance to downy mildew in different cv. Albariño clones Vitis - J Grapevine Res 45:23–27

Bouvier F, Backhaus RA, Camara B (1998) Induction and control of chromoplast-specific carotenoid genes by oxidative stress. J Biol Chem 273:30651–30659

Brilli F, Ruuskanen TM, Schnitzhofer R et al (2011) Detection of plant volatiles after leaf wounding and darkening by proton transfer reaction "time-of-flight" mass spectrometry (PTR-TOF). PLoS One 6: e20419. doi:10.1371/journal.pone.0020419

Chiron H, Drouet A, Lieutier F et al (2000) Gene induction of stilbene biosynthesis in scots pine in response to ozone treatment, wounding, and fungal infection. Plant Physiol 124:865–872. doi:10.1104/pp.124.2.865

Chong J, Poutaraud A, Hugueney P (2009) Metabolism and roles of stilbenes in plants. Plant Sci 177:143–155. doi:10.1016/j.plantsci.2009.05.012

Chung I-M, Park MR, Chun JC, Yun SJ (2003) Resveratrol accumulation and resveratrol synthase gene expression in response to abiotic

stresses and hormones in peanut plants. Plant Sci 164:103–109. doi:10.1016/S0168-9452(02)00341-2

Conconi A, Miquel M, Browse JA, Ryan CA (1996) Intracellular levels of free linolenic and linoleic acids increase in tomato leaves in response to wounding. Plant Physiol 111:797–803. doi:10.1104/pp.111.3.797

Creelman RA, Mullet JE (1997) Biosynthesis and action of Jasmonates in plants. Annu Rev Plant Physiol Plant Mol Biol 48:355–381. doi:10.1146/annurev.arplant.48.1.355

Csizmazia J, Bereznai L (1968) A szõlõ Plasmopara viticola és a Viteus vitifolii elleni rezisztencia nemesités eredményei. Dans Orszõl Bor Kut Int Evönyve:191–200

Dai GH, Andary C, Cosson-Mondolot L, Boubals D (1994) Polyphenols and resistance of grapevines to downy MILDEW. Acta Hortic 763–766. doi: 10.17660/ActaHortic.1994.381.110

Dai GH, Andary C, Mondolot-Cosson L, Boubals D (1995) Histochemical studies on the interaction between three species of grapevine, *Vitis vinifera*, *V. rupestris* and *V. rotundifolia* and the downy mildew fungus, Plasmopara viticola. Physiol Mol Plant Pathol 46:177–188. doi:10.1006/pmpp.1995.1014

Della Corte A, Chitarrini G, Di Gangi IM et al (2015) A rapid LC-MS/MS method for quantitative profiling of fatty acids, sterols, glycerolipids, glycerophospholipids and sphingolipids in grapes. Talanta 140:52–61. doi:10.1016/j.talanta.2015.03.003

Delye C, Corio-Costet M-F (1998) Origin of primary infections of grape by Uncinula necator: RAPD analysis discriminates two biotypes. Mycol Res 102:283–288. doi:10.1017/S0953756297004632

Dhondt S, Geoffroy P, Stelmach BA et al (2000) Soluble phospholipase A2 activity is induced before oxylipin accumulation in tobacco mosaic virus-infected tobacco leaves and is contributed by patatin-like enzymes. Plant J 23:431–440. doi:10.1046/j.1365-313x.2000.00802.x

Farmer EE, Ryan CA (1992) Octadecanoid precursors of jasmonic acid activate the synthesis of wound-inducible proteinase inhibitors. Plant Cell 4:129–134. doi:10.1105/tpc.4.2.129

Folch J, Lees M, Stanley GHS (1957) A simple method for the isolation and purification of total lipids from animal tissues. J Biol Chem 226:497–509

Höll J, Vannozzi A, Czemmel S et al (2013) The R2R3-MYB transcription factors MYB14 and MYB15 regulate stilbene biosynthesis in *Vitis vinifera*. Plant Cell 25:4135–4149. doi:10.1105/tpc.113.117127

Howe GA, Schilmiller AL (2002) Oxylipin metabolism in response to stress. Curr Opin Plant Biol 5:230–236. doi:10.1016/S1369-5266(02)00250-9

Jailloux F, Thind T, Clerjeau M (1998) Release, germination, and pathogenicity of ascospores of Uncinula necator under controlled conditions. Can J Bot 76:777–781. doi:10.1139/b98-054

Jeandet P, Douillet-Breuil A-C, Bessis R et al (2002) Phytoalexins from the Vitaceae: biosynthesis, phytoalexin gene expression in transgenic plants, antifungal activity, and metabolism. J Agric Food Chem 50:2731–2741

Kachroo A, Kachroo P (2009) Fatty acid-derived signals in plant defense. Annu Rev Phytopathol 47:153–176. doi:10.1146/annurev-phyto-080508-081820

Kennelly MM, Gadoury DM, Wilcox WF et al (2007) Primary infection, lesion productivity, and survival of sporangia in the grapevine downy mildew pathogen Plasmopara viticola. Phytopathology 97:512–522. doi:10.1094/PHYTO-97-4-0512

Keshavarz T, Taheri P (2015) Investigating binucleate Rhizoctonia induced defence responses in kidney bean against Rhizoctonia solani. Biocontrol Sci Tech 25:444–459. doi:10.1080/09583157.2014.984285

Kono A, Sato A, Reisch B, Cadle-Davidson L (2015) Effect of detergent on the quantification of grapevine downy mildew sporangia from leaf discs. Hortscience 50:656–660

Kozma P, Dula T (2003) Inheritance of resistance to downy mildew and powdery mildew of hybrid family Muscadinia x *V. vinifera* x *V. amurensis* x Franco-American hybrid. Acta Hortic 457–463. doi: 10.17660/ActaHortic.2003.603.58

Langcake P, Pryce RJ (1977) A new class of phytoalexins from grapevines. Experientia 33:151–152. doi:10.1007/BF02124034

Lava SS, Zipper R, Spring O (2015) Sunflower white blister rust—host specificity and fungicide effects on infectivity and early infection stages. Crop Prot 67:214–222. doi:10.1016/j.cropro.2014.10.019

Lebeda A, Mieslerová B, Petrželová I et al (2012) Patterns of virulence variation in the interaction between Lactuca spp. and lettuce powdery mildew (Golovinomyces cichoracearum). Fungal Ecol 5:670–682. doi:10.1016/j.funeco.2012.03.005

Lee S, Suh S, Kim S et al (1997) Systemic elevation of phosphatidic acid and lysophospholipid levels in wounded plants. Plant J 12:547–556. doi:10.1046/j.1365-313X.1997.00547.x

León J, Rojo E, Sánchez-Serrano JJ (2001) Wound signalling in plants. J Exp Bot 52:1–9. doi:10.1093/jexbot/52.354.1

Mattivi F, Vrhovsek U, Malacarne G et al (2011) Profiling of resveratrol oligomers, important stress metabolites, accumulating in the leaves of hybrid *Vitis vinifera* (Merzling × Teroldego) genotypes infected with Plasmopara viticola. J Agric Food Chem 59:5364–5375. doi:10.1021/jf200771y

McConn M, Creelman RA, Bell E et al (1997) Jasmonate is essential for insect defense in Arabidopsis. Proc Natl Acad Sci 94:5473–5477

Narváez-Vásquez J, Florin-Christensen J, Ryan CA (1999) Positional specificity of a phospholipase a activity induced by wounding, systemin, and oligosaccharide elicitors in tomato leaves. Plant Cell 11:2249–2260. doi:10.1105/tpc.11.11.2249

Noel JP, Austin MB, Bomati EK (2005) Structure–function relationships in plant phenylpropanoid biosynthesis. Curr Opin Plant Biol 8:249–253. doi:10.1016/j.pbi.2005.03.013

Nyadanu D, Akromah R, Adomako B et al (2012) Inheritance and general combining ability studies of detached pod, leaf disc and natural field resistance to Phytophthora palmivora and Phytophthora megakarya in cacao (*Theobroma cacao* L.) Euphytica 188:253–264. doi:10.1007/s10681-012-0717-x

Park J-W, Balaraju K, Kim J-W et al (2013) Systemic resistance and growth promotion of chili pepper induced by an antibiotic producing Bacillus vallismortis strain BS07. Biol Control 65:246–257. doi:10.1016/j.biocontrol.2013.02.002

Peressotti E, Wiedemann-Merdinoglu S, Delmotte F et al (2010) Breakdown of resistance to grapevine downy mildew upon limited deployment of a resistant variety. BMC Plant Biol 10:147. doi:10.1186/1471-2229-10-147

Pezet R, Gindro K, Viret O, Spring J-L (2004) Glycosylation and oxidative dimerization of resveratrol are respectively associated to sensitivity and resistance of grapevine cultivars to downy mildew. Physiol Mol Plant Pathol 65:297–303. doi:10.1016/j.pmpp.2005.03.002

Prost I, Dhondt S, Rothe G et al (2005) Evaluation of the antimicrobial activities of plant oxylipins supports their involvement in defense against pathogens. Plant Physiol 139:1902–1913. doi:10.1104/pp.105.066274

Reymond P, Weber H, Damond M, Farmer EE (2000) Differential gene expression in response to mechanical wounding and insect feeding in Arabidopsis. Plant Cell 12:707–719. doi:10.1105/tpc.12.5.707

Ryu SB, Wang X (1998) Increase in free linolenic and linoleic acids associated with phospholipase D-mediated hydrolysis of phospholipids in wounded castor bean leaves. Biochim Biophys Acta BBA - Lipids Lipid Metab 1393:193–202. doi:10.1016/S0005-2760(98)00048-4

Savatin DV, Gramegna G, Modesti V, Cervone F (2014) Wounding in the plant tissue: the defense of a dangerous passage. Front Plant Sci. doi:10.3389/fpls.2014.00470

Schnee S, Queiroz EF, Voinesco F et al (2013) *Vitis vinifera* canes, a new source of antifungal compounds against Plasmopara viticola, Erysiphe necator, and Botrytis cinerea. J Agric Food Chem 61: 5459–5467. doi:10.1021/jf4010252

Sun Z-B, Yuan X-F, Zhang H et al (2013) Isolation, screening and identification of antagonistic downy mildew endophytic bacteria from cucumber. Eur J Plant Pathol 137:847–857. doi:10.1007/s10658-013-0293-5

Suthaparan A, Solhaug KA, Stensvand A, Gislerød HR (2016) Determination of UV action spectra affecting the infection process of Oidium neolycopersici, the cause of tomato powdery mildew. J Photochem Photobiol B 156:41–49. doi:10.1016/j.jphotobiol.2016.01.009

Tôyama S (1972) Electron microscope studies on the morphogenesis of plastids. Bot Mag Shokubutsu-Gaku-Zasshi 85:1–10. doi:10.1007/BF02489195

Vannozzi A, Dry IB, Fasoli M et al (2012) Genome-wide analysis of the grapevine stilbene synthase multigenic family: genomic organization and expression profiles upon biotic and abiotic stresses. BMC Plant Biol. doi:10.1186/1471-2229-12-130

Vrhovsek U, Malacarne G, Masuero D et al (2012a) Profiling and accurate quantification of trans-resveratrol, trans-piceid, trans-pterostilbene and 11 viniferins induced by Plasmopara viticola in partially resistant grapevine leaves. Aust J Grape Wine Res 18:11–19. doi:10.1111/j.1755-0238.2011.00163.x

Vrhovsek U, Masuero D, Gasperotti M et al (2012b) A versatile targeted metabolomics method for the rapid quantification of multiple classes of phenolics in fruits and beverages. J Agric Food Chem 60:8831–8840. doi:10.1021/jf2051569

Vu HS, Roston R, Shiva S et al (2015) Modifications of membrane lipids in response to wounding of *Arabidopsis thaliana* leaves. Plant Signal Behav 10:e1056422. doi:10.1080/15592324.2015.1056422

Wehrens R, Carvalho E, Masuero D et al (2013) High-throughput carotenoid profiling using multivariate curve resolution. Anal Bioanal Chem 405:5075–5086. doi:10.1007/s00216-012-6555-9

Willocquet L, Colombet D, Rougier M et al (1996) Effects of radiation, especially ultraviolet B, on conidial germination and mycelial growth of grape powdery mildew. Eur J Plant Pathol 102:441–449. doi:10.1007/BF01877138

Zhang S, Yu S, Zhang F et al (2012) Inheritance of downy mildew resistance at different developmental stages in Chinese cabbage via the leaf disk test. Hortic Environ Biotechnol 53:397–403. doi:10.1007/s13580-012-0057-8

Zhao X, Ren L, Yin H et al (2013) Sensitivity of Pseudoperonospora cubensis to dimethomorph, metalaxyl and fosetyl-aluminium in Shanxi of China. Crop Prot 43:38–44. doi:10.1016/j.cropro.2012.08.002

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	0 hpc			6 hpc			12 hpc			24 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																					
trans-coutaric acid	178.297 (a)	178.371 (A)	***NS***	187.603 (a)	178.201 (A)	***NS***	180.062 (a)	158.013 (A)	***NS***	173.089 (a)	169.767 (A)	***NS***	172.385 (a)	162.847 (A)	***NS***	159.825 (a)	159.529 (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)	1190.112 (A)	***NS***	1251.322 (a)	1228.531 (A)	***NS***	1187.853 (a)	1173.402 (A)	***NS***	1174.417 (a)	1226.218 (A)	***NS***	1211.351 (a)	1219.536 (A)	***NS***	1207.755 (a)	1199.731 (A)	***NS***	1150.476 (a)	1182.607 (A)	***NS***
fertaric acid	62.578 (a)	58.46 (A)	***NS***	85.485 (a)	60.531 (A)	***NS***	76.685 (a)	56.12 (A)	***NS***	74.796 (a)	61.338 (A)	***NS***	79.76 (a)	58.11 (A)	***NS***	76.896 (a)	57.238 (A)	***NS***	77.985 (a)	56.675 (A)	***NS***
ferulic acid	0.043 (a)	0.034 (A)	***NS***	0.03 (a)	0.012 (A)	***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***
sinapic acid	0.401 (a)	0.401 (A)	***NS***	0.468 (ab)	0.458 (A)	***NS***	0.558 (b)	0.423 (A)	***NS***	0.464 (ab)	0.401 (A)	***NS***	0.41 (a)	0.454 (A)	***NS***	0.411 (a)	0.374 (A)	***NS***	0.511 (ab)	0.489 (A)	***NS***
Dihydrochalcones																					
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***
Flavones																					
luteolin-7-O-glucoside	0.11 (a)	0.109 (A)	***NS***	0.105 (a)	0.119 (A)	***NS***	0.105 (a)	0.094 (A)	***NS***	0.097 (a)	0.105 (A)	***NS***	0.093 (a)	0.105 (A)	***NS***	0.102 (a)	0.112 (A)	***NS***	0.106 (a)	0.095 (A)	***NS***
Flavan-3-ols																					
catechin	2.642 (a)	3.604 (A)	***NS***	3.255 (a)	3.256 (A)	***NS***	3.038 (a)	3.493 (A)	***NS***	3.491 (a)	4.019 (A)	***NS***	3.468 (a)	5.19 (A)	***NS***	3.274 (a)	3.431 (A)	***NS***	3.722 (a)	3.347 (A)	***NS***
epicatechin	1.1 (a)	1.357 (A)	***NS***	0.975 (a)	1.115 (A)	***NS***	1.411 (a)	1.214 (A)	***NS***	0.87 (a)	1.481 (A)	***NS***	1.341 (a)	1.696 (A)	***NS***	1.376 (a)	1.009 (A)	***NS***	1.336 (a)	1.314 (A)	***NS***
epicatechin gallate	1.724 (a)	1.951 (A)	***NS***	1.854 (a)	1.937 (A)	***NS***	2.469 (a)	2.341 (A)	***NS***	2.387 (a)	2.073 (A)	***NS***	1.988 (a)	1.995 (A)	***NS***	2.592 (a)	2.107 (A)	***NS***	2.697 (a)	2.202 (A)	***NS***
epigallocatechin	2.917 (a)	3.338 (A)	***NS***	3.112 (a)	2.92 (A)	***NS***	3.009 (a)	3.145 (A)	***NS***	2.722 (a)	3.267 (A)	***NS***	2.921 (a)	3.282 (A)	***NS***	2.608 (a)	2.99 (A)	***NS***	2.641 (a)	2.7 (A)	***NS***
epigallocatechin gallate	5.292 (a)	4.666 (A)	***NS***	5.657 (a)	5.198 (A)	***NS***	5.995 (a)	5.441 (A)	***NS***	5.063 (a)	5.147 (A)	***NS***	5.594 (a)	5.446 (A)	***NS***	5.479 (a)	5.325 (A)	***NS***	5.242 (a)	4.976 (A)	*
gallocatechin	10.909 (a)	11.831 (A)	***NS***	13.771 (a)	11.061 (A)	***NS***	12.777 (a)	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***
procyanidin B1	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)	23.363 (A)	***NS***
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***	5.386 (a)	4.945 (A)	***NS***	5.259 (a)	5.343 (A)	***NS***	3.669 (a)	5.692 (A)	***NS***	4.622 (a)	5.572 (A)	***NS***
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***
Flavonols																					
isorhamnetin-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 (b)	0.91 (A)	***NS***
isorhamnetin-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	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***
quercetin-3-glucoside+quercetin-3-galactoside (as que-3-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)	*
quercetin-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***
quercetin-3-O-glucuronide	734.293 (b)	729.58 (A)	***NS***	825.844 (ab)	739.492 (A)	***NS***	848.788 (a)	729.888 (A)	***NS***	868.634 (a)	774.825 (A)	***NS***	851.071 (a)	715.364 (A)	***NS***	788.2 (ab)	742.961 (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)	**
cis-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***
cis + trans-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***

	trans-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***
Others																						
	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***
	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***	110.043 (a)	99.623 (A)	***NS***	101.426 (a)	85.377 (A)	***NS***	97.111 (a)	80.008 (A)	*	97.563 (a)	87.376 (A)	***NS***

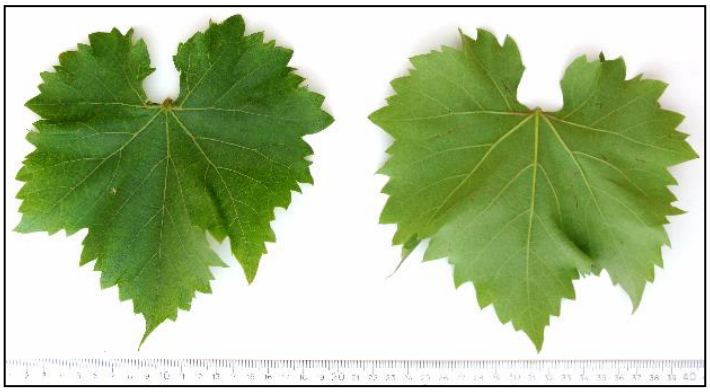
Supplementary Table 1: *Quantification of lipids compounds in 1.1 cm and 2.8 cm diameter leaf discs; the concentration is represented as mg/Kg of fresh leaves. One way ANOVA followed by Newman-Keuls test was able to determine the differences during the time point for 1.1 cm discs (lowercase letters) 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 hpc			12 hpc			24 hpc			48 hpc			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	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***
	1,2-Dioleoyl-sn-glycero-3-phospho-rac-(1-glycerol)sodium 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<0.01 (**), 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*
		0 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																						
	Chloropyll a	1128.057 (a)	1119.233 (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***	1188.498 (a)	839.078 (A)	***NS***	1036.986 (a)	734.455 (A)	***NS***
	Chloropyll b	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***
	Chloropyll a epimer as chlorophyll a	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)	*	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)	1112.644 (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

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

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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 semi-quantify 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 α-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 two-node cuttings of the cultivar "Bianca." The mother plants were held at Fondazione Edmund Mach grape collection, San Michele all'Adige, Italy (46°12′0″N, 11°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.

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 N_2. 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 N_2. 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-*N*-trimethylsilyl-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 × 0.25 mm × 0.25 μm) 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 XCALIBUR™ 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 × (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

toluene (500 mg/L) were 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. 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 N_2. Samples were re-suspended 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. 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 N_2. 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 × 2.1 mm; 1.8 μm) 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 Na_2HPO_4 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 × 0.25 mm i.d. × 0.25 μm) (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 XCALIBUR™ 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),

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 μg/kg of fresh leaves (Supplementary Table S2). The concentration reported represents the average value of three biological replicates ± 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 *myo*-inositol, which slightly exceeded the limit of ±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

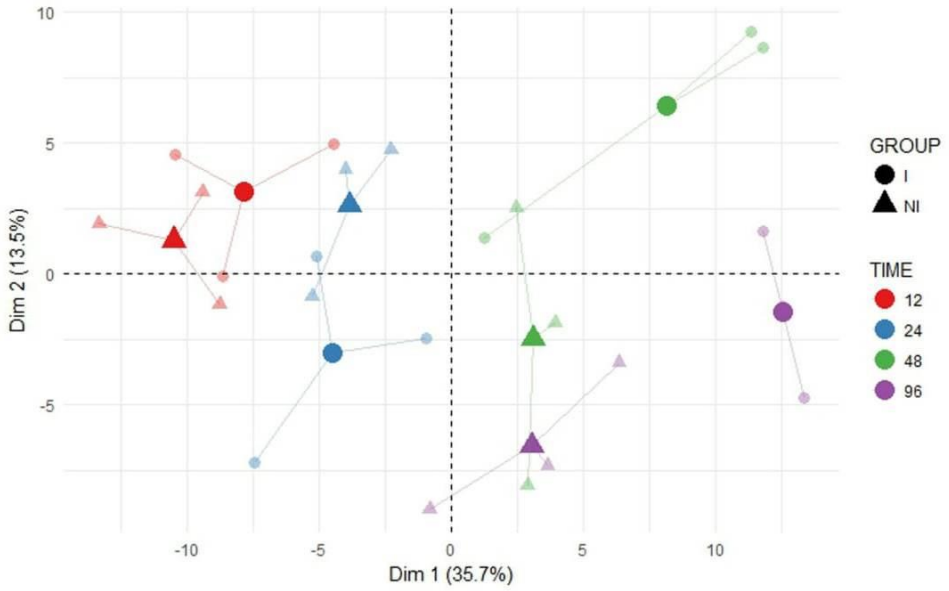

FIGURE 1 | Principal component analysis performed on the log10 of the concentration of all analyzed compounds. For each time point, three biological replicates (smaller dots) are represented for each condition (circle: inoculated samples; triangle: not inoculated) and linked with their means (larger dots). Each time point is represented with a different color: red for samples collected at 12 hpi, blue for 24 hpi samples, green for 48 hpi samples, and violet for 96 hpi samples.

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 *trans*-resveratrol (**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*-ε-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 α-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

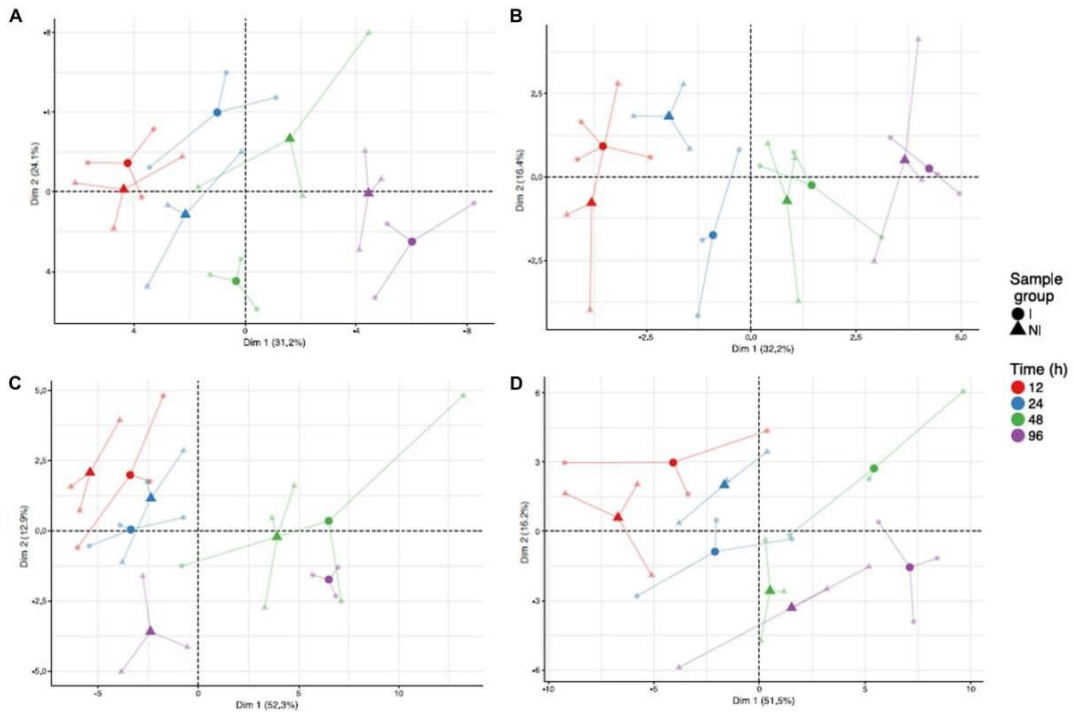

FIGURE 2 | Principal component analysis of the log10-transformed metabolite concentration of individual classes: **(A)** primary compounds, **(B)** lipids, **(C)** phenol compounds, and **(D)** volatile compounds. The analysis was performed on the log10 of the concentration. For each time point, three 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 color: red for samples collected 12 h post-infection (hpi), blue for 24 hpi samples, green for 48 hpi samples, and violet for 96 hpi samples.

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

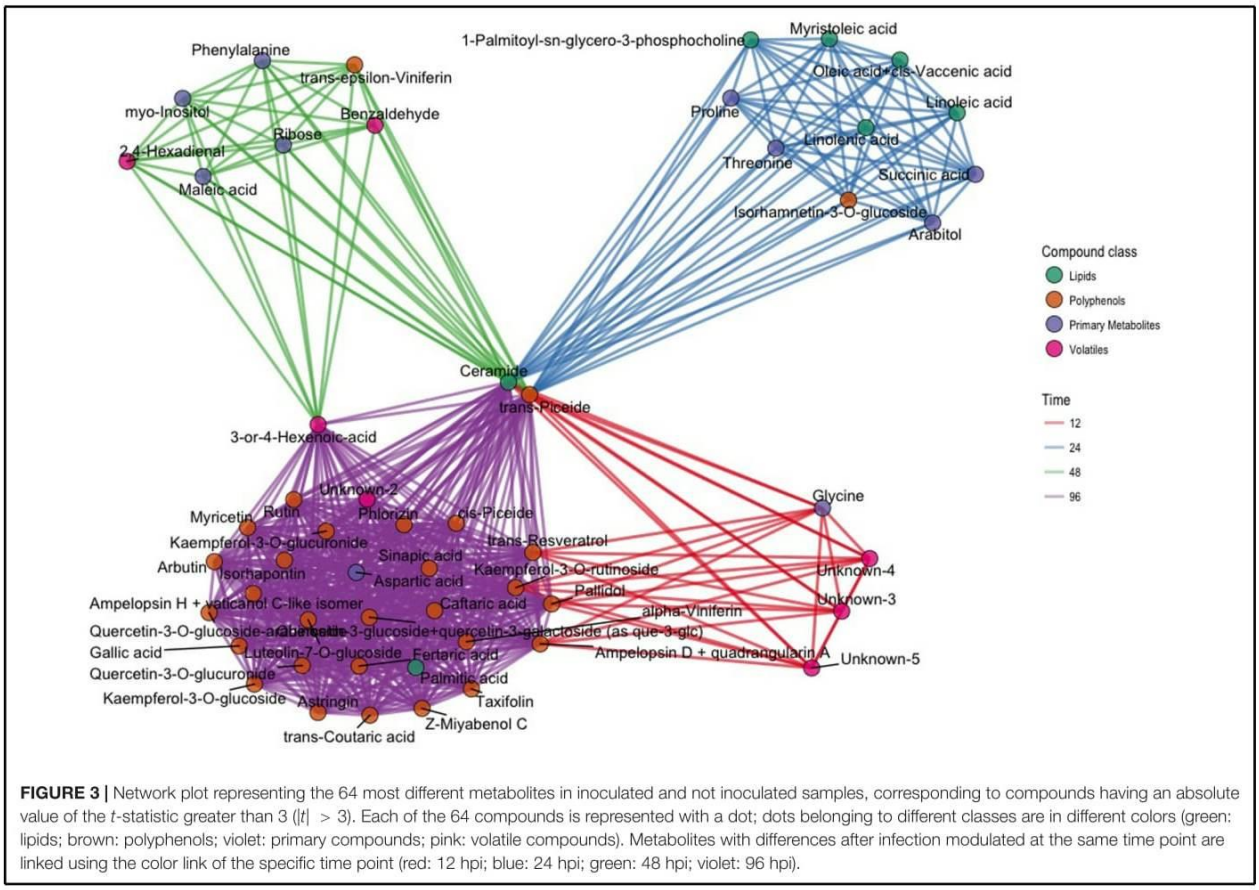

FIGURE 3 | Network plot representing the 64 most different metabolites in inoculated and not inoculated samples, corresponding to compounds having an absolute value of the *t*-statistic greater than 3 ($|t| > 3$). Each of the 64 compounds is represented with a dot; dots belonging to different classes are in different colors (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 color link of the specific time point (red: 12 hpi; blue: 24 hpi; green: 48 hpi; violet: 96 hpi).

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

resistance to the pathogen in the Bianca grapevine. *Trans*-resveratrol 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*-ε-viniferin at 48 hpi and subsequently *trans*- and *cis*-piceid, isoraphontin, ampelopsin H + vaticanol C-like isomer, α-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

REFERENCES

Algarra Alarcon, A., Lazazzara, V., Cappellin, L., Bianchedi, P. L., Schuhmacher, R., Wohlfahrt, G., et al. (2015). Emission of volatile sesquiterpenes and monoterpenes in grapevine genotypes following *Plasmopara viticola* inoculation in vitro. *J. Mass Spectrom.* 50, 1013–1022. doi: 10.1002/jms.3615

Ali, K., Maltese, F., Figueiredo, A., Rex, M., Fortes, A. M., Zyprian, E., et al. (2012). Alterations in grapevine leaf metabolism upon inoculation with *Plasmopara viticola* in different time-points. *Plant Sci. Int. J. Exp. Plant Biol.* 19, 100–107. doi: 10.1016/j.plantsci.2012.04.014

Batovska, D. I., Todorova, I. T., Parushev, S. P., Nedelcheva, D. V., Bankova, V. S., Popov, S. S., et al. (2009). Biomarkers for the prediction of the resistance and susceptibility of grapevine leaves to downy mildew. *J. Plant Physiol.* 166, 781–785. doi: 10.1016/j.jplph.2008.08.008

Bavaresco, L., De Rosso, M., Gardiman, M., Morreale, G., and Flamini, R. (2016). Polyphenol metabolomics of twenty Italian red grape varieties. *BIO Web Conf.* 7:01022. doi: 10.1051/bioconf/20160701022

Bavaresco, L., Mattivi, F., De Rosso, M., and Flamini, R. (2012). Effects of elicitors, viticultural factors, and enological practices on resveratrol and stilbenes in grapevine and wine. *Mini Rev. Med. Chem.* 12, 1366–1381. doi: 10.2174/13895575112091366

Becker, L., Poutaraud, A., Hamm, G., Muller, J.-F., Merdinoglu, D., Carré, V., et al. (2013). Metabolic study of grapevine leaves infected by downy mildew using negative ion electrospray–Fourier transform ion cyclotron resonance mass spectrometry. *Anal. Chim. Acta* 795, 44–51. doi: 10.1016/j.aca.2013.07.068

Bellin, D., Peressotti, E., Merdinoglu, D., Wiedemann-Merdinoglu, S., Adam-Blondon, A.-F., Cipriani, G., et al. (2009). Resistance to *Plasmopara viticola* in grapevine "Bianca" is controlled by a major dominant gene causing localised necrosis at the infection site. *Theor. Appl. Genet.* 120, 163–176. doi: 10.1007/s00122-009-1167-2

Bennett, R. N., and Wallsgrove, R. M. (1994). Secondary metabolites in plant defence mechanisms. *New Phytol.* 127, 617–633. doi: 10.1111/j.1469-8137.1994.tb02968.x

Berkey, R., Bendigeri, D., and Xiao, S. (2012). Sphingolipids and Plant Defense/Disease: The "Death" connection and beyond. *Front. Plant Sci.* 3:68. doi: 10.3389/fpls.2012.00068

Bisson, L. F., Waterhouse, A. L., Ebeler, S. E., Walker, M. A., and Lapsley, J. T. (2002). The present and future of the international wine industry. *Nature* 418, 696–699. doi: 10.1038/nature01018

Bolton, M. D. (2009). Primary metabolism and plant defense—fuel for the fire. *Mol. Plant. Microbe Interact.* 22, 487–497. doi: 10.1094/MPMI-22-5-0487

Brown, J. K. M., and Rant, J. C. (2013). Fitness costs and trade-offs of disease resistance and their consequences for breeding arable crops. *Plant Pathol.* 62, 83–95. doi: 10.1111/ppa.12163

Cecchini, N. M., Monteoliva, M. I., and Alvarez, M. E. (2011). Proline dehydrogenase contributes to pathogen defense in Arabidopsis[1,[C][W][OA]]. *Plant Physiol.* 155, 1947–1959. doi: 10.1104/pp.110.167163

Chitarrini, G., Zulini, L., Masuero, D., and Vrhovsek, U. (2017). Lipid, phenol and carotenoid changes in "Bianca" grapevine leaves after mechanical wounding: a case study. *Protoplasma* doi: 10.1007/s00709-017-1100-5 [Epub ahead of print].

Christen, V., Rusconi, M., Crettaz, P., and Fent, K. (2017). Developmental neurotoxicity of different pesticides in PC-12 cells in vitro. *Toxicol. Appl. Pharmacol.* 325, 25–36. doi: 10.1016/j.taap.2017.03.027

Csizmazia, J., and Bereznai, L. (1968). A szőlő *Plasmopara viticola* és a Viteus vitifolii elleni rezisztencia nemesités eredményei. *Dans Orszöl Bor Kut Int Evönyve* 191–200.

Degu, A., Hochberg, U., Sikron, N., Venturini, L., Buson, G., Ghan, R., et al. (2014). Metabolite and transcript profiling of berry skin during fruit development elucidates differential regulation between Cabernet Sauvignon and Shiraz cultivars at branching points in the polyphenol pathway. *BMC Plant Biol.* 14:188. doi: 10.1186/s12870-014-0188-4

Della Corte, A., Chitarrini, G., Di Gangi, I. M., Masuero, D., Soini, E., Mattivi, F., et al. (2015). A rapid LC–MS/MS method for quantitative profiling of fatty acids, sterols, glycerolipids, glycerophospholipids and sphingolipids in grapes. *Talanta* 140, 52–61. doi: 10.1016/j.talanta.2015.03.003

Derckel, J. P., Baillieul, F., Manteau, S., Audran, J. C., Haye, B., Lambert, B., et al. (1999). Differential induction of grapevine defenses by two strains of *Botrytis cinerea*. *Phytopathology* 89, 197–203. doi: 10.1094/PHYTO.1999.89.3.197

Dercks, W., and Creasy, L. L. (1989). The significance of stilbene phytoalexins in the *Plasmopara viticola*-grapevine interaction. *Physiol. Mol. Plant Pathol.* 34, 189–202. doi: 10.1016/0885-5765(89)90043-X

Deuschle, K., Funck, D., Forlani, G., Stransky, H., Biehl, A., Leister, D., et al. (2004). The role of [Delta]1-pyrroline-5-carboxylate dehydrogenase in proline degradation. *Plant Cell* 16, 3413–3425. doi: 10.1105/tpc.104.023622

European Commission (2011). *Method Validation and Quality Control Procedures for Pesticide Residues Analysis in Food and Feed.* Doc.SANCO/12495/2011. Brussels: European Commission.

Ferri, M., Righetti, L., and Tassoni, A. (2011). Increasing sucrose concentrations promote phenylpropanoid biosynthesis in grapevine cell cultures. *J. Plant Physiol.* 168, 189–195. doi: 10.1016/j.jplph.2010.06.027

Fiehn, O., Wohlgemuth, G., Scholz, M., Kind, T., Lee, D. Y., Lu, Y., et al. (2008). Quality control for plant metabolomics: reporting MSI-compliant studies. *Plant J. Cell Mol. Biol.* 53, 691–704. doi: 10.1111/j.1365-313X.2007.03387.x

Flamini, R., Mattivi, F., De Rosso, M., Arapitsas, P., and Bavaresco, L. (2013). Advanced knowledge of three important classes of grape phenolics: anthocyanins, stilbenes and flavonols. *Int. J. Mol. Sci.* 14, 19651–19669. doi: 10.3390/ijms141019651

Folch, J., Lees, M., and Stanley, G. H. S. (1957). A simple method for the isolation and purification of total lipides from animal tissues. *J. Biol. Chem.* 226, 497–509.

Gabaston, J., Cantos-Villar, E., Biais, B., Waffo-Teguo, P., Renouf, E., Corio-Costet, M. F., et al. (2017). Stilbenes from *Vitis vinifera* L. waste: a sustainable tool for controlling plasmopara viticola. *J. Agric. Food Chem.* 65, 2711–2718. doi: 10.1021/acs.jafc.7b00241

Gershenzon, J., and Dudareva, N. (2007). The function of terpene natural products in the natural world. *Nat. Chem. Biol.* 3, 408–414. doi: 10.1038/nchembio.2007.5

Gessler, C., Pertot, I., and Perazzolli, M. (2011). *Plasmopara viticola*: a review of knowledge on downy mildew of grapevine and effective disease management. *Phytopathol. Mediterr.* 50, 3–44. doi: 10.14601/Phytopathol_Mediterr-9360

Gika, H. G., Theodoridis, G. A., Vrhovsek, U., and Mattivi, F. (2012). Quantitative profiling of polar primary metabolites using hydrophilic interaction ultrahigh performance liquid chromatography–tandem mass spectrometry. *J. Chromatogr. A* 1259, 121–127. doi: 10.1016/j.chroma.2012.02.010

Godard, S., Slacanin, I., Viret, O., and Gindro, K. (2009). Induction of defence mechanisms in grapevine leaves by emodin- and anthraquinone-rich plant extracts and their conferred resistance to downy mildew. *Plant Physiol. Biochem.* 47, 827–837. doi: 10.1016/j.plaphy.2009.04.003

Greenberg, J. T., and Yao, N. (2004). The role and regulation of programmed cell death in plant-pathogen interactions. *Cell. Microbiol.* 6, 201–211. doi: 10.1111/j.1462-5822.2004.00361.x

Heath, M. C. (2000). "Hypersensitive response-related death," in *Programmed Cell Death in Higher Plants*, eds E. Lam, H. Fukuda, and J. Greenberg (Berlin: Springer), 77–90. doi: 10.1007/978-94-010-0934-8_6

Jean-Denis, J. B., Pezet, R., and Tabacchi, R. (2006). Rapid analysis of stilbenes and derivatives from downy mildew-infected grapevine leaves by liquid chromatography–atmospheric pressure photoionisation mass spectrometry. *J. Chromatogr. A* 1112, 263–268. doi: 10.1016/j.chroma.2006.01.060

Jeandet, P., Douillet-Breuil, A.-C., Bessis, R., Debord, S., Sbaghi, M., and Adrian, M. (2002). Phytoalexins from the Vitaceae: biosynthesis, phytoalexin gene expression in transgenic plants, antifungal activity, and metabolism. *J. Agric. Food Chem.* 50, 2731–2741. doi: 10.1021/jf011429s

Jones, J. D. G., and Dangl, J. L. (2006). The plant immune system. *Nature* 444, 323–329. doi: 10.1038/nature05286

Kachroo, A., and Kachroo, P. (2009). Fatty Acid–Derived Signals in Plant Defense. *Annu. Rev. Phytopathol.* 47, 153–176. doi: 10.1146/annurev-phyto-080508-081820

Kassambara, A., and Mundt, F. (2017). *factoextra: Extract and Visualize the Results of Multivariate Data Analyses*. R package version 1.0.3.

Keller, M. (2015). *The Science of Grapevines: Anatomy and Physiology*. Burlington, MA: Academic Press.

Kozma, P., and Dula, T. (2003). Inheritance of resistance to downy mildew and powdery mildew of hybrid family Muscadinia x V. *vinifera x V. amurensis x Franco-American hybrid*. *Acta Hortic* 603, 457–463. doi: 10.17660/ActaHortic.2003.603.58

Kwon, H., Lehotay, S. J., and Geis-Asteggiante, L. (2012). Variability of matrix effects in liquid and gas chromatography–mass spectrometry analysis of pesticide residues after QuEChERS sample preparation of different food crops. *J. Chromatogr. A* 1270, 235–245. doi: 10.1016/j.chroma.2012.10.059

Langcake, P., and Pryce, R. J. (1977). A new class of phytoalexins from grapevines. *Experientia* 33, 151–152. doi: 10.1007/BF02124034

Lê, S., Josse, J., and Husson, F. (2008). FactoMineR: an R package for multivariate analysis. *J. Stat. Softw.* 25, 1–18. doi: 10.18637/jss.v025.i01

Less, H., Angelovici, R., Tzin, V., and Galili, G. (2011). Coordinated gene networks regulating arabidopsis plant metabolism in response to various stresses and nutritional Cues[W]. *Plant Cell* 23, 1264–1271. doi: 10.1105/tpc.110.082867

Malacarne, G., Vrhovsek, U., Zulini, L., Cestaro, A., Stefanini, M., Mattivi, F., et al. (2011). Resistance to *Plasmopara viticola* in a grapevine segregating population is associated with stilbenoid accumulation and with specific host transcriptional responses. *BMC Plant Biol.* 11:114. doi: 10.1186/1471-2229-11-114

Martínez, J. A. (2012). *Natural Fungicides Obtained from Plants*. Rijeka: InTech, doi: 10.5772/26336

Matarese, F., Cuzzola, A., Scalabrelli, G., and D'Onofrio, C. (2014). Expression of terpene synthase genes associated with the formation of volatiles in different organs of *Vitis vinifera*. *Phytochemistry* 105, 12–24. doi: 10.1016/j.phytochem.2014.06.007

Mulas, G., Galaffu, M. G., Pretti, L., Nieddu, G., Mercenaro, L., Tonelli, R., et al. (2011). NMR analysis of seven selections of vermentino grape berry: metabolites composition and development. *J. Agric. Food Chem.* 59, 793–802. doi: 10.1021/jf103285f

Negatu, B., Kromhout, H., Mekonnen, Y., and Vermeulen, R. (2016). O04-2 Occupational pesticide exposure and respiratory health of famers and farm workers: a study in three commercial farming systems in ethiopia. *Occup. Environ. Med.* 73, A7–A8. doi: 10.1136/oemed-2016-103951.19

Pedersen, T. L. (2017). *ggraph: An Implementation of Grammar of Graphics for Graphs and Networks version 1.0.0 from CRAN*. Available at: https://CRAN.R-project.org/package=ggraph [accessed June 21, 2017].

Pezet, R., Gindro, K., Viret, O., and Spring, J.-L. (2004). Glycosylation and oxidative dimerization of resveratrol are respectively associated to sensitivity and resistance of grapevine cultivars to downy mildew. *Physiol. Mol. Plant Pathol.* 65, 297–303. doi: 10.1016/j.pmpp.2005.03.002

Polesani, M., Bortesi, L., Ferrarini, A., Zamboni, A., Fasoli, M., Zadra, C., et al. (2010). General and species-specific transcriptional responses to downy mildew infection in a susceptible (*Vitis vinifera*) and a resistant (*V. riparia*) grapevine species. *BMC Genom.* 11:117. doi: 10.1186/1471-2164-11-117

R Core Team (2017). *R: A Language and Environment for Statistical Computing*. Available at: http://www.gbif.org/resource/81287 [accessed June 21, 2017].

Ribnicky, D. M., Shulaev, V., and Raskin, I. (1998). Intermediates of salicylic acid biosynthesis in tobacco. *Plant Physiol.* 118, 565–572. doi: 10.1104/pp.118.2.565

Rigano, F., Albergamo, A., Sciarrone, D., Beccaria, M., Purcaro, G., and Mondello, L. (2016). Nano liquid chromatography directly coupled to electron ionization mass spectrometry for free fatty acid elucidation in mussel. *Anal. Chem.* 88, 4021–4028. doi: 10.1021/acs.analchem.6b00328

Rojas, C. M., Senthil-Kumar, M., Tzin, V., and Mysore, K. S. (2014). Regulation of primary plant metabolism during plant-pathogen interactions and its contribution to plant defense. *Front. Plant Sci.* 5:17. doi: 10.3389/fpls.2014.00017

Rortais, A., Arnold, G., Dorne, J.-L., More, S. J., Sperandio, G., Streissl, F., et al. (2017). Risk assessment of pesticides and other stressors in bees: principles, data gaps and perspectives from the European Food Safety Authority. *Sci. Total Environ.* 58, 524–537. doi: 10.1016/j.scitotenv.2016.09.127

Salvagnin, U., Carlin, S., Angeli, S., Vrhovsek, U., Anfora, G., Malnoy, M., et al. (2016). Homologous and heterologous expression of grapevine E-(β)-caryophyllene synthase (VvGwECar2). *Phytochemistry* 131, 76–83. doi: 10.1016/j.phytochem.2016.08.002

Slaughter, A. R., Hamiduzzaman, M. M., Gindro, K., Neuhaus, J.-M., and Mauch-Mani, B. (2008). Beta-aminobutyric acid-induced resistance in grapevine against downy mildew: involvement of pterostilbene. *Eur. J. Plant Pathol.* 122, 185–195. doi: 10.1007/s10658-008-9285-2

Sparvoli, F., Martin, C., Scienza, A., Gavazzi, G., and Tonelli, C. (1994). Cloning and molecular analysis of structural genes involved in flavonoid and stilbene biosynthesis in grape (*Vitis vinifera* L.). *Plant Mol. Biol.* 24, 743–755. doi: 10.1007/BF00029856

Sumner, L. W., Amberg, A., Barrett, D., Beale, M. H., Beger, R., Daykin, C. A., et al. (2007). Proposed minimum reporting standards for chemical analysis Chemical Analysis Working Group (CAWG) Metabolomics Standards Initiative (MSI). *Metabolomics* 3, 211–221. doi: 10.1007/s11306-007-0082-2

Teixeira, A., Martins, V., Noronha, H., Eiras-Dias, J., and Gerós, H. (2014). The first insight into the metabolite profiling of grapes from three *Vitis vinifera* L. cultivars of two controlled appellation (DOC) regions. *Int. J. Mol. Sci.* 15, 4237–4254. doi: 10.3390/ijms15034237

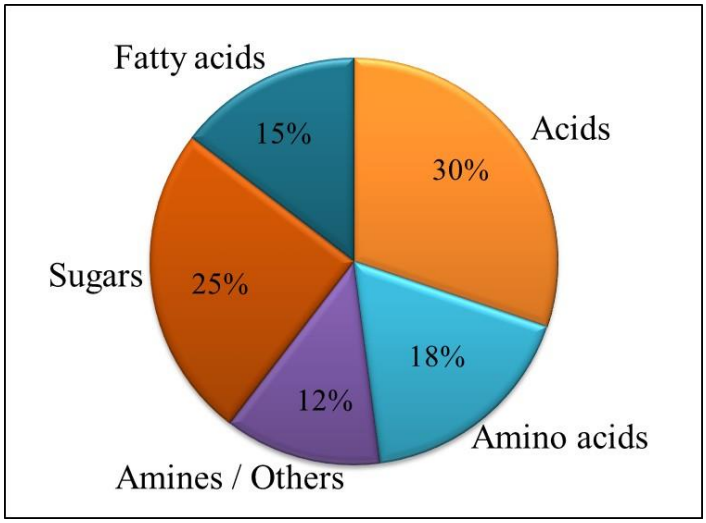
Topolewska, A., Czarnowska, K., Haliński, Ł.P., and Stepnowski, P. (2015). Evaluation of four derivatization methods for the analysis of fatty acids from green leafy vegetables by gas chromatography. *J. Chromatogr. B* 990, 150–157. doi: 10.1016/j.jchromb.2015.03.020

US Department of Health and Human Services (2001). *Guidance for Industry, Bioanalytical Method Validation*. Rockville, MD: US Department of Health and Human Services.

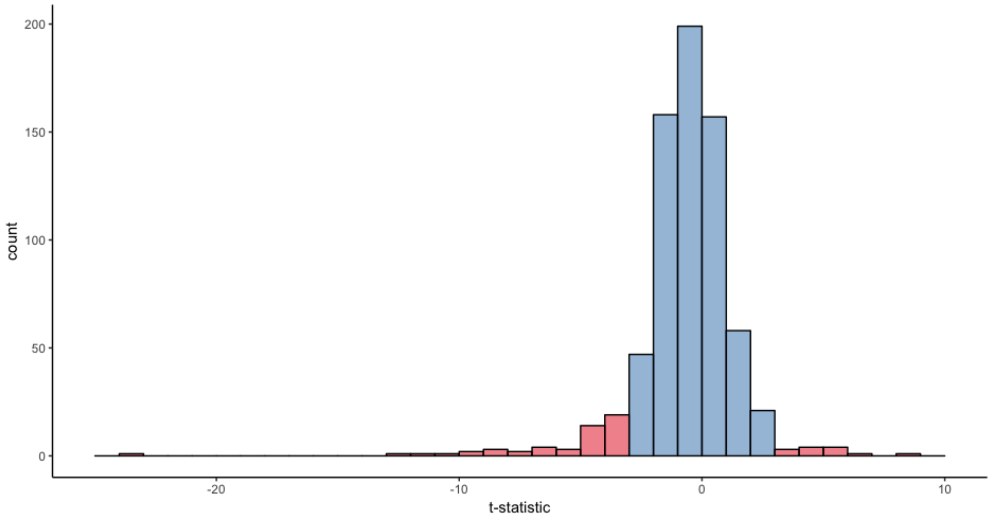
van den Berg, R. A., Hoefsloot, H. C. J., Westerhuis, J. A., Smilde, A. K., and van der Werf, M. J. (2006). Centering, scaling, and transformations: improving the biological information content of metabolomics data. *BMC Genomics* 7:142. doi: 10.1186/1471-2164-7-142

Vrhovsek, U., Masuero, D., Gasperotti, M., Franceschi, P., Caputi, L., Viola, R., et al. (2012). A versatile targeted metabolomics method for the rapid quantification of multiple classes of phenolics in fruits and beverages. *J. Agric. Food Chem.* 60, 8831–8840. doi: 10.1021/jf2051569.ref

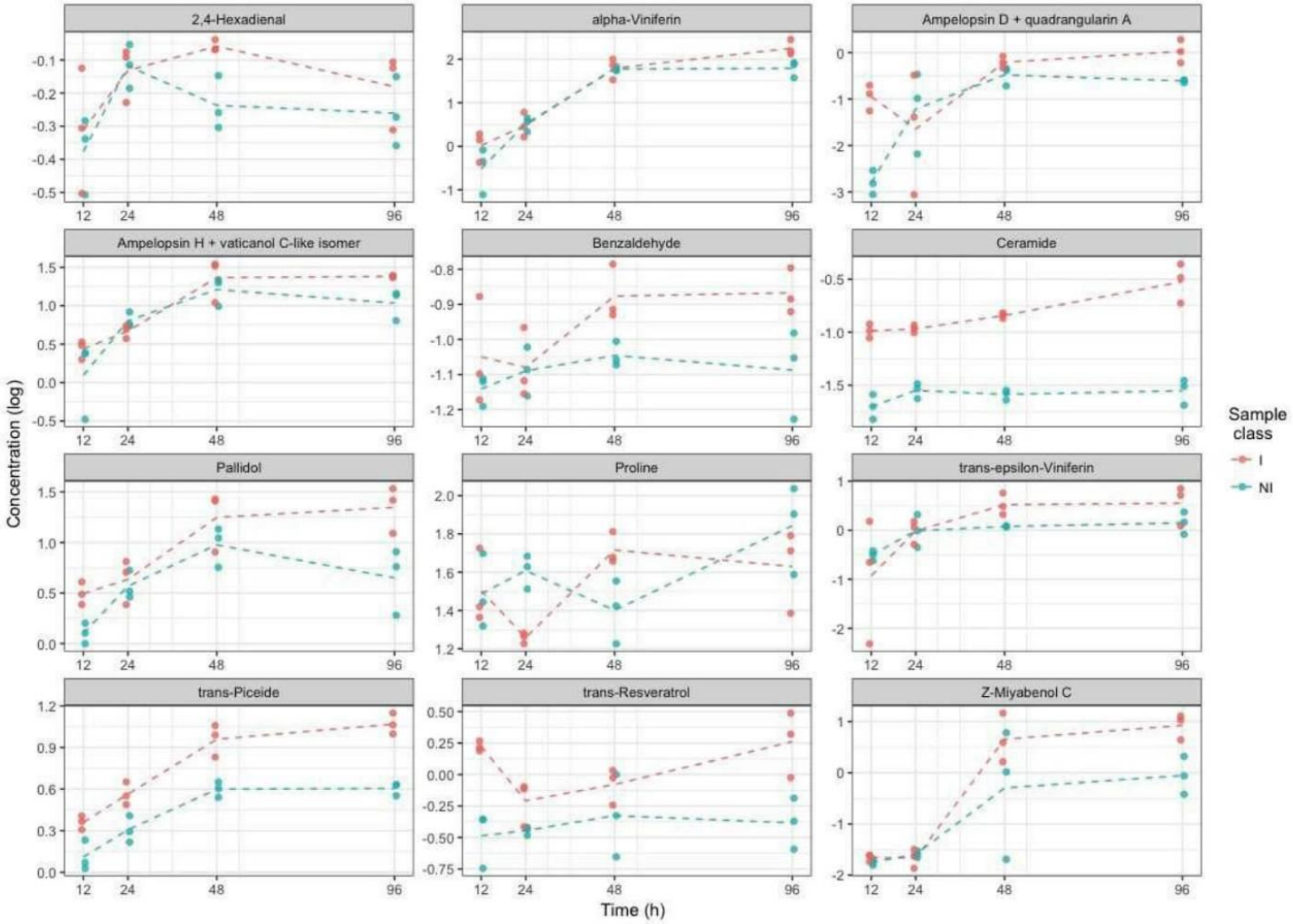
Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.



***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.*

***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.*

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
	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

	γ-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
	***myo*-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 acids											
	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	**Compound**	NI	I	NI	I	NI	I	NI	I
Acids									
	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 ± 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 ± 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 ± 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
	abscisic acid	98.705 ± 0.737	89.089 ± 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 acids									
	valine	9.837 ± 1.102	9.816 ± 3.246	25.372 ± 1.651	45.968 ± 12.718	63.996 ± 5.958	66.239 ± 4.968	108.143 ± 16.837	141.978 ± 42.695
	alanine	127.625 ± 12.826	101.268 ± 9.997	130.278 ± 18.012	88.493 ± 12.984	72.887 ± 24.993	124.058 ± 12.080	124.714 ± 13.440	106.200 ± 36.350
	leucine	8.337 ± 1.002	9.233 ± 1.451	16.116 ± 1.390	21.662 ± 5.037	25.370 ± 3.504	31.684 ± 2.113	35.029 ± 4.011	50.000 ± 13.189
	isoleucine	13.042 ± 1.142	11.542 ± 1.582	19.593 ± 1.065	27.187 ± 5.517	32.171 ± 2.967	36.922 ± 2.978	44.551 ± 5.471	64.249 ± 18.333
	serine	35.384 ± 5.509	27.621 ± 3.786	45.685 ± 7.902	25.877 ± 3.311	45.201 ± 18.032	70.468 ± 8.060	76.914 ± 9.035	123.097 ± 26.088
	threonine	30.646 ± 1.386	29.490 ± 3.028	35.150 ± 3.924	16.914 ± 1.471	28.726 ± 9.582	51.364 ± 4.803	57.485 ± 13.158	66.878 ± 10.059
	proline	32.835 ± 8.747	34.200 ± 9.541	41.131 ± 4.591	18.129 ± 0.661	26.385 ± 5.479	52.655 ± 6.143	75.863 ± 20.355	45.830 ± 11.180
	glycine	2.266 ± 0.400	1.183 ± 0.134	1.211 ± 0.159	0.941 ± 0.124	0.957 ± 0.188	1.519 ± 0.122	1.142 ± 0.243	1.243 ± 0.099
	aspartic acid	120.687 ± 10.410	121.664 ± 3.200	127.224 ± 5.676	97.007 ± 12.825	116.674 ± 19.500	150.049 ± 3.078	118.449 ± 9.328	170.223 ± 11.105
	β-alanine	1.183 ± 0.108	1.180 ± 0.138	1.659 ± 0.079	1.421 ± 0.103	1.264 ± 0.206	1.750 ± 0.085	2.646 ± 0.149	3.173 ± 0.177
	γ-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 ± 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 ± 0.135	1.172 ± 0.166	1.290 ± 0.140	1.249 ± 0.066	1.205 ± 0.286	1.369 ± 0.091	1.238 ± 0.139	1.434 ± 0.070
xylose	7.035 ± 0.368	6.598 ± 0.306	8.129 ± 1.098	7.847 ± 0.482	10.071 ± 0.578	10.869 ± 0.513	9.878 ± 0.554	12.904 ± 1.338
ribose	6.020 ± 0.464	5.286 ± 0.382	6.220 ± 1.019	4.667 ± 0.160	4.601 ± 0.319	6.271 ± 0.363	5.604 ± 0.548	6.990 ± 0.580
α-rhamnose	8.145 ± 0.459	7.657 ± 0.557	8.914 ± 1.004	7.021 ± 0.391	7.992 ± 0.405	9.575 ± 0.329	9.167 ± 0.585	9.874 ± 0.421
arabitol	0.614 ± 0.140	0.580 ± 0.077	0.946 ± 0.004	0.591 ± 0.034	0.701 ± 0.077	0.937 ± 0.113	0.910 ± 0.100	0.961 ± 0.044
adonitol	5.190 ± 0.073	5.042 ± 0.115	5.584 ± 0.241	5.040 ± 0.260	4.963 ± 0.451	6.799 ± 0.614	5.231 ± 0.373	5.956 ± 0.593
fucose	18.641 ± 1.094	19.196 ± 0.948	22.257 ± 2.409	20.307 ± 0.246	20.578 ± 0.963	24.685 ± 0.922	20.297 ± 0.966	23.918 ± 0.769
fructose	566.374 ± 93.893	602.380 ± 60.235	627.578 ± 90.834	527.922 ± 67.471	414.562 ± 59.014	583.836 ± 62.728	418.740 ± 81.852	340.157 ± 74.007
glucose	533.139 ± 81.063	567.669 ± 46.136	588.911 ± 75.589	892.109 ± 132.949	550.403 ± 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	1.689 ± 0.248	1.408 ± 0.064	1.550 ± 0.061	1.449 ± 0.050
myo-inositol	2086.746 ± 87.214	1973.240 ± 118.251	2276.653 ± 382.800	2268.049 ± 235.955	1939.385 ± 90.294	2392.529 ± 52.402	1772.351 ± 83.732	1620.083 ± 241.747
sucrose	2306.535 ± 319.332	2230.674 ± 332.372	2279.989 ± 491.795	1372.737 ± 415.686	1476.273 ± 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	102.346 ± 1.673	113.069 ± 5.166	91.019 ± 1.044	97.372 ± 13.899

***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.*

		12 hpi		24 hpi		48 hpi		96 hpi	
Class	**Compound**	NI	I	NI	I	NI	I	NI	I
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 ± 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	3.2607 ± 1.1109	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	1.2406 ± 0.1223	1.3403 ± 0.0594	1.4877 ± 0.0980	1.2647 ± 0.1761	1.3383 ± 0.1287	1.4553 ± 0.0926	1.5876 ± 0.3102	1.5707 ± 0.1526
	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+*cis*-vaccenic acid	1.1266 ± 0.1068	1.5006 ± 0.4263	1.7163 ± 0.4498	0.3776 ± 0.0437	0.4639 ± 0.4327	0.3956 ± 0.2535	0.2602 ± 0.2602	0.1809 ± 0.1809
	palmitic acid	10.6169 ± 1.7498	12.2662 ± 1.2439	11.9569 ± 2.8327	11.1579 ± 2.1381	8.0167 ± 0.8927	8.3329 ± 2.7452	6.0506 ± 0.4424	10.0884 ± 1.3027
	palmitoleic acid	1.0415 ± 0.1806	1.0724 ± 0.1818	0.9470 ± 0.1366	0.4981 ± 0.0808	0.5795 ± 0.1207	0.5302 ± 0.0802	0.3289 ± 0.0822	0.4934 ± 0.0881
	stearic acid	9.9880 ± 1.1293	9.6090 ± 0.9488	10.4877 ± 0.9624	10.7254 ± 2.4001	11.4102 ± 0.6748	10.8848 ± 0.9477	13.1147 ± 1.8376	15.7781 ± 2.0969
Glycerolipids									
	1,2,3-tripentadecanoylglycerol	0.0160 ± 0.0074	0.0143 ± 0.0030	0.0069 ± 0.0015	0.0138 ± 0.0042	<LOQ	<LOQ	<LOQ	<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-glycerol)sodium 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**: 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 ± error standard, expressed as µg/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.*

						12 hpi		24 hpi		48 hpi		96 hpi	
Class	**Compound**	RT	Ri Sper.	Ri Theor.	ident.	NI	I	NI	I	NI	I	NI	I
Acids													
	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
	nonanoic-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
	octanoic	37.82	2079	2073	A,B,C	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
Alcohols													
	cyclobutanemethanol	19.29	1341		C	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
	1-hexanol	20.48	1371	1380	A,B,C	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
	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	173.290 ± 10.063	167.780 ± 26.285	220.359 ± 32.143
	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
	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	155.885 ± 13.968	160.270 ± 14.746	181.987 ± 36.140	192.293 ± 30.649
	3-nonen-1-ol	29.82	1698	1696	B,C	121.044 ± 17.405	135.379 ± 15.187	167.887 ± 0.943	162.439 ± 15.562	195.647 ± 13.321	224.391 ± 5.378	203.201 ± 25.064	246.893 ± 15.278
	1-octanol	26.71	1572	1563	A,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 ± 8.720
Aldehydes													
	2,4-heptadienal	25.09	1513	1503	B,C	138.441 ± 13.542	153.274 ± 10.861	157.877 ± 10.188	135.462 ± 11.982	161.276 ± 6.259	210.994 ± 39.470	156.023 ± 18.286	219.590 ± 11.771
	2-heptenal	19.29	1341	1330	B,C	53.207 ± 3.518	56.077 ± 4.332	63.835 ± 4.860	54.905 ± 4.697	63.423 ± 3.192	80.194 ± 16.664	53.604 ± 7.801	62.934 ± 4.386
	2,4-hexadienal	22.32	1422	1409	B,C	429.970 ± 61.955	519.578 ± 126.424	767.780 ± 115.477	746.795 ± 78.308	586.665 ± 64.827	874.412 ± 20.499	559.579 ± 79.047	673.904 ± 93.316
	hexanal	7.45	1103	1089	A,B,C	4022.485 ± 216.150	4520.207 ± 544.959	5031.063 ± 843.644	3673.208 ± 378.407	3097.018 ± 617.795	5259.611 ± 659.729	4121.559 ± 584.621	4549.167 ± 560.278
	trans-3-hexenal	9.87	1156	1146	A,B,C	135.352 ± 19.993	154.852 ± 28.860	170.986 ± 55.129	160.260 ± 12.136	120.402 ± 13.843	182.956 ± 21.239	158.967 ± 30.243	223.798 ± 14.887
	cis-3-hexenal	10.14	1161	1176	A,B,C	490.799 ± 155.639	528.964 ± 143.416	482.302 ± 232.701	515.478 ± 19.908	265.526 ± 76.414	338.516 ± 107.677	265.264 ± 24.979	191.409 ± 71.412
	trans-2-hexenal	13.13	1221	1230	A,B,C	680.295 ± 55.609	750.105 ± 114.749	841.578 ± 151.778	769.178 ± 46.808	730.967 ± 98.993	1041.885 ± 94.303	1000.652 ± 165.377	1398.056 ± 140.788
	pentanal	4.36	998	984	B,C	54.016 ± 5.699	52.372 ± 1.993	53.378 ± 3.340	49.910 ± 3.221	48.537 ± 4.595	71.669 ± 13.078	62.263 ± 8.450	68.483 ± 11.449
	2-pentenal	9.6	1150	1130	B,C	113.175 ± 11.266	135.288 ± 6.882	120.009 ± 16.685	91.814 ± 8.546	83.413 ± 12.964	133.914 ± 32.105	87.532 ± 11.461	98.113 ± 15.079
Benzenoids													
	benzaldehyde	25.92	1543	1545	A,B,C	72.497 ± 4.051	93.118 ± 20.002	82.036 ± 12.979	84.778 ± 11.832	90.199 ± 4.326	134.401 ± 14.819	84.011 ± 13.205	136.762 ± 11.962
	benzylalchool	34.15	1894	1896	A,B,C	254.358 ± 46.436	274.410 ± 74.218	259.695 ± 39.665	358.599 ± 66.802	333.634 ± 42.838	370.417 ± 32.104	360.058 ± 81.172	483.104 ± 75.501
	eugenol	39.88	2185	2198	A,B,C	3.189 ± 0.671	3.183 ± 0.818	5.226 ± 0.653	6.745 ± 1.370	6.199 ± 1.037	7.983 ± 1.173	8.116 ± 0.975	9.263 ± 1.713
	phenethy-alcohol	34.86	1929	1906	A,B,C	179.431 ± 29.111	230.938 ± 46.579	284.099 ± 4.901	318.067 ± 38.225	378.055 ± 21.867	456.135 ± 44.471	443.241 ± 50.262	547.257 ± 53.705
Ketones													
	2-cyclopentene-1-one-2-pentenyl	31.29	1762		C	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-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
	penten-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-trimetylciclohexanone	18.51	1322	1306	B,C	16.807 ± 1.124	19.320 ± 1.611	23.479 ± 3.766	20.812 ± 2.411	23.269 ± 1.162	24.376 ± 0.664	21.299 ± 2.138	24.036 ± 3.319
Terpenoids													
	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-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-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
	citral	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

	dihydroactinidolide	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
	farnesene	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
	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
	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
	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
	isogeraniol	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
	linalool	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
	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		C	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-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-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-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
	cis-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
Unknowns:																													
	unknown-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
	unknown-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
	unknown-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
	unknown-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
	unknown-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 hpi		24 hpi		48 hpi		96 hpi	
Class	**Compound**	NI	I	NI	I	NI	I	NI	I
Benzoic Acid 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
	p-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
Phenylpropanoids									
	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 ± 113.381	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
Dihydrochalcones									
	phlorizin	2.450 ± 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-ols									
	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 ± 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 ± 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 ± 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 ± 0.007	0.169 ± 0.018	0.205 ± 0.103	0.245 ± 0.060	0.165 ± 0.014	0.278 ± 0.023
	quercetin-3-glucoside+quercetin-3-galactoside (as que-3-glc)	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
	quercetin-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-glucuronide	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	1487.359 ± 16.072
	rutin	4.726 ± 0.271	5.149 ± 0.816	5.219 ± 1.000	4.648 ± 0.450	8.184 ± 0.335	10.059 ± 3.474	4.162 ± 0.478	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*-*ω*-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-*ε*-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 + quadrangularin A	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	1.188 ± 0.383
	ampelopsin H + vaticanol C-like isomer	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
	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
	E-*cis*-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 condensation product	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

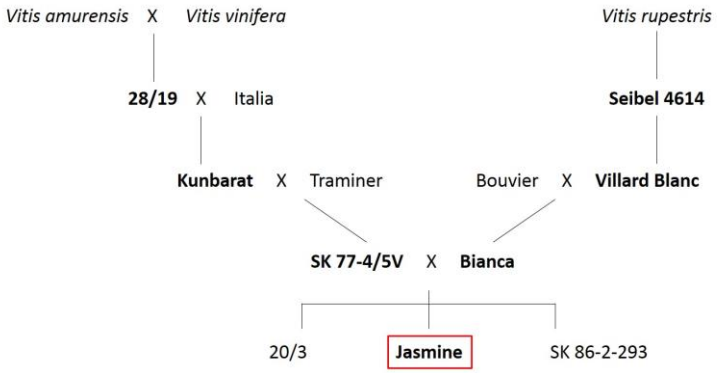
***Supplementary Table 3**: t statistic results of Bianca.*

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
cis-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
myo-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
Proline	24	6.6
Quercetin-3-glucoside+quercetin-3-galactoside (as que-3-glc)	96	-6.6
Quercetin-3-O-glucoside-arabinoside	96	-4.2
Quercetin-3-O-glucuronide	96	-8.6
Ribose	48	-3.5
Rutin	96	-4.1
Sinapic acid	96	-11.9
Succinic acid	24	4.0
Taxifolin	96	-3.0
Threonine	24	5.3
trans-Coutaric acid	96	-7.0
trans-epsilon-Viniferin	48	-3.5
trans-Piceid	12	-3.7
trans-Piceid	24	-3.5
trans-Piceid	48	-4.8
trans-Piceide	96	-9.2
trans-Resveratrol	12	-5.4
trans-Resveratrol	96	-3.4
Unknown-2	96	-4.3
Unknown-3	12	-4.9
Unknown-4	12	4.4
Unknown-5	12	-4.1
Z-Miyabenol C	96	-3.8

Preface to Chapter V

"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 defence-related 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 µL 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 µm) 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 XCALIBUR™ 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 µm). 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 XCALIBUR™ 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 µg/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. 2012; Naithani 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 µg/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.

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

	CLASS	#		CLASS	#
Primary compounds	acids	17	Lipid compounds	carnitines	1
	amino acids	13		sterols	3
	amines	3		fatty acids	13
	sugars	14		glycero lipids	4
				glycero phospholipids	4
				sphingolipids	1
				prenols	1

	CLASS	#		CLASS	#
Phenol compounds	benzoic acid derivateds	4	Volatile compounds	acids	3
	coumarins	2		alchohols	7
	phenylpropanoids	6		aldehydes	9
	dihydrochalcones	1		benzenoids	4
	flavones	1		ketones	4
	flavan-3-ols	9		terpenoids	14
	flavonols	11		other	4
	stilbenes+stilbenoids	14		unknown	6
	other phenolic	2			

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.

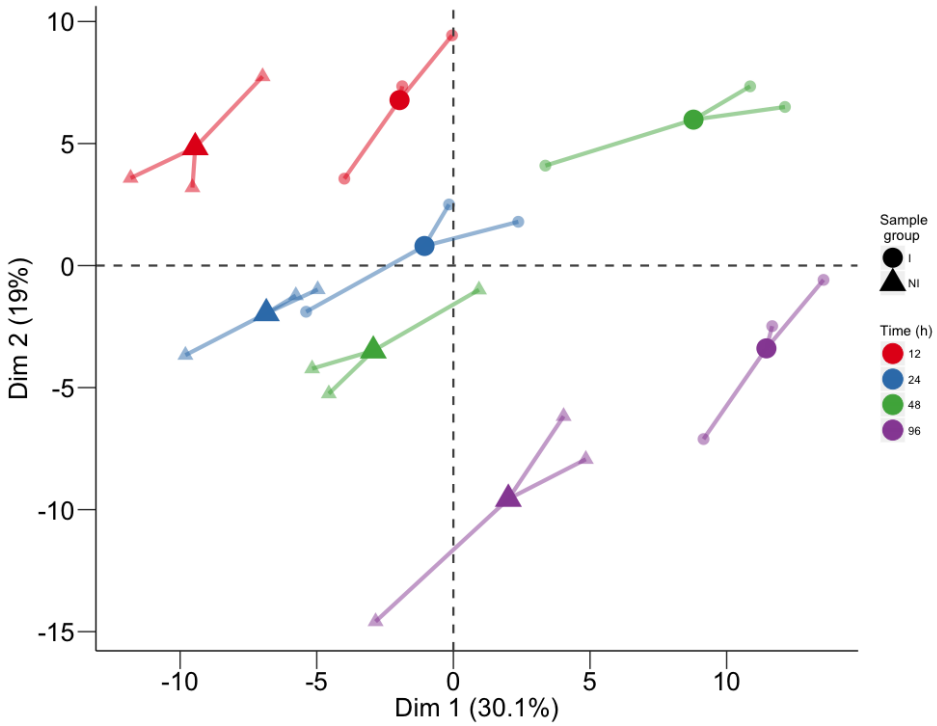


Fig. V. 1. *Principal component analysis performed on the log 10-transformed metabolite concentration of all analysed compounds. For each time point, three biological replicates (smaller dots) are represented for each condition (circle: inoculated samples; triangle: not inoculated) and linked with their 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.

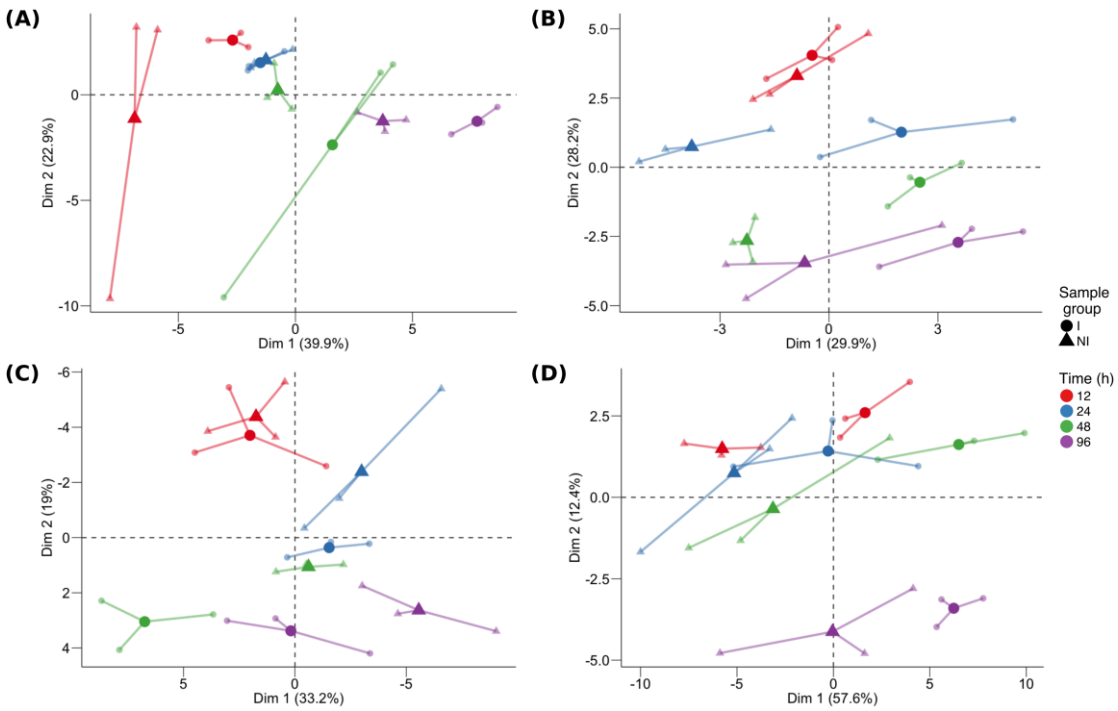


***Fig. V. 2**. Principal component analysis of the log 10-transformed metabolite concentration of individual classes: A) primary compounds B) lipids C) phenol compounds D) volatile compounds. For each time point, three biological replicates (smaller dots) are represented for each condition (circle: 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 NADPH-oxidase 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 stress-related 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).

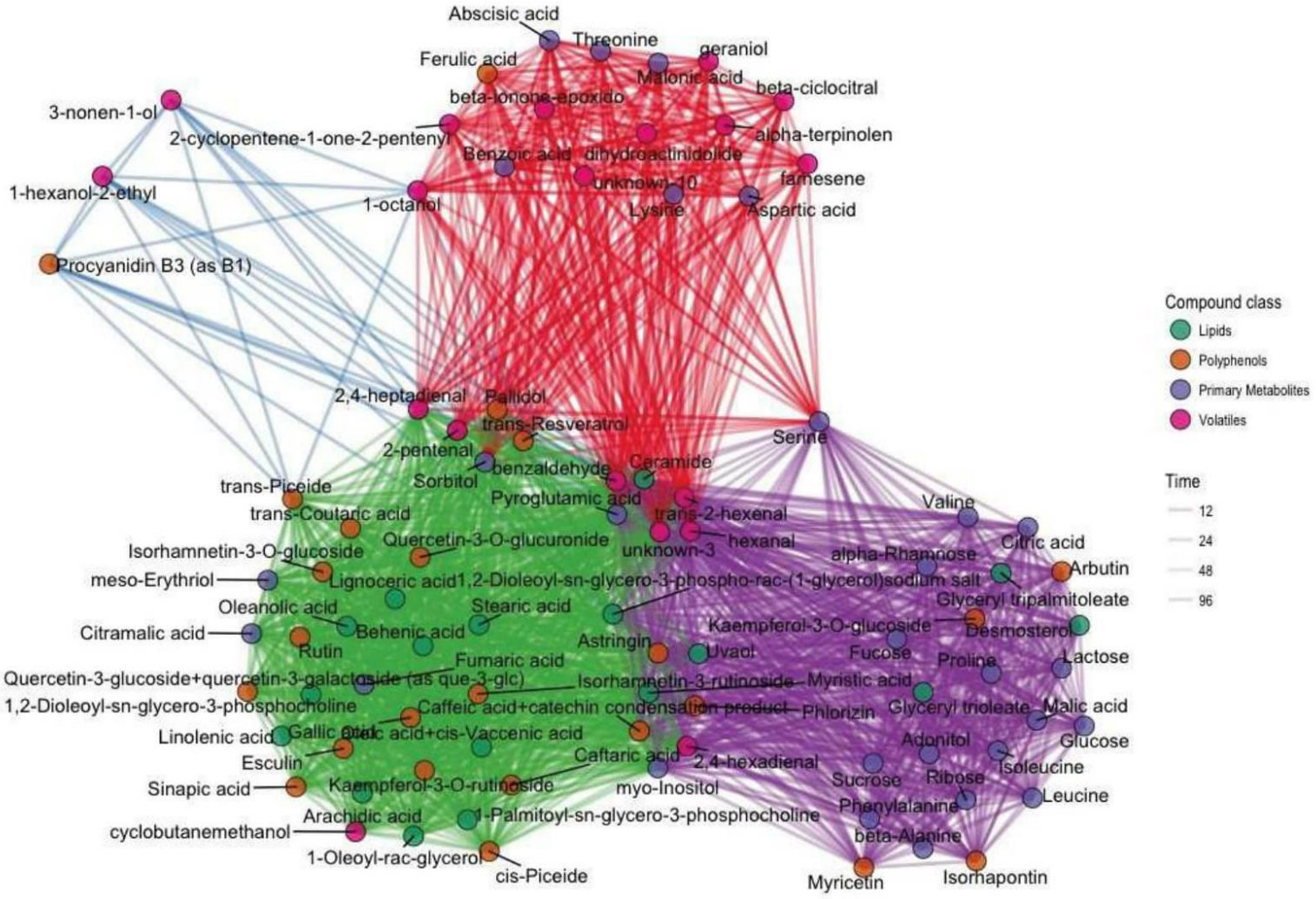


Fig. V. 3*: Network plot representing the 88 most different metabolites in inoculated and not inoculated samples, corresponding to compounds having an absolute value of the t-statistic greater than 3 (|t| >3). Each of the 88 compounds is represented with a dot; dots belonging to different classes are 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).

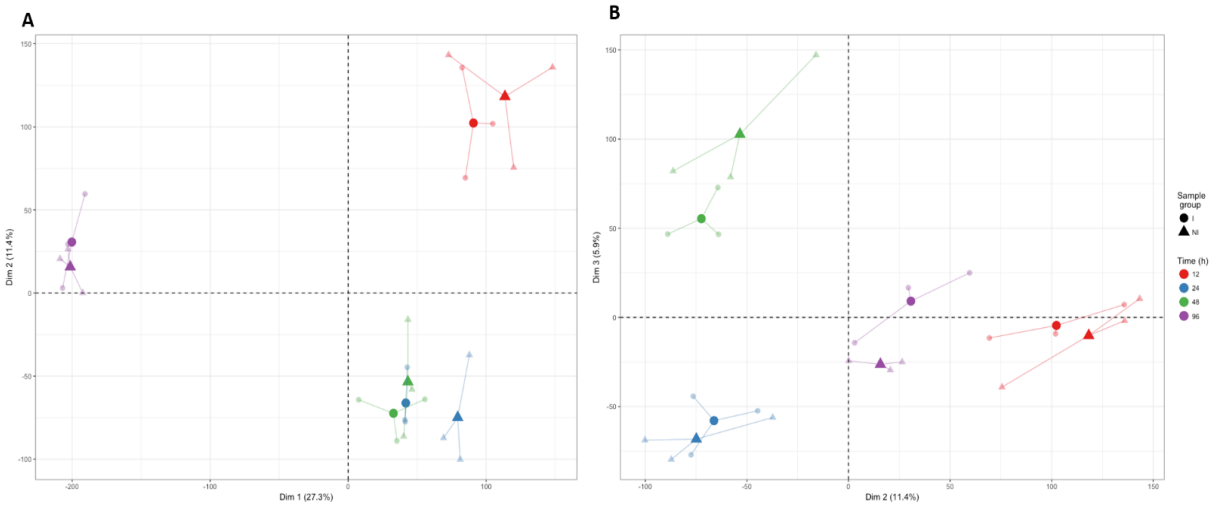


Fig. V. 4*: Principal component analysis performed on the entire transcriptome. A: dimension 1 (27.3%) dimension 2 (11.4%); B: dimension 2 (11.4%), dimension 3 (5.9%) For each time point, three biological replicates (smaller dots) are represented for each condition (circle: inoculated samples; triangle: 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).

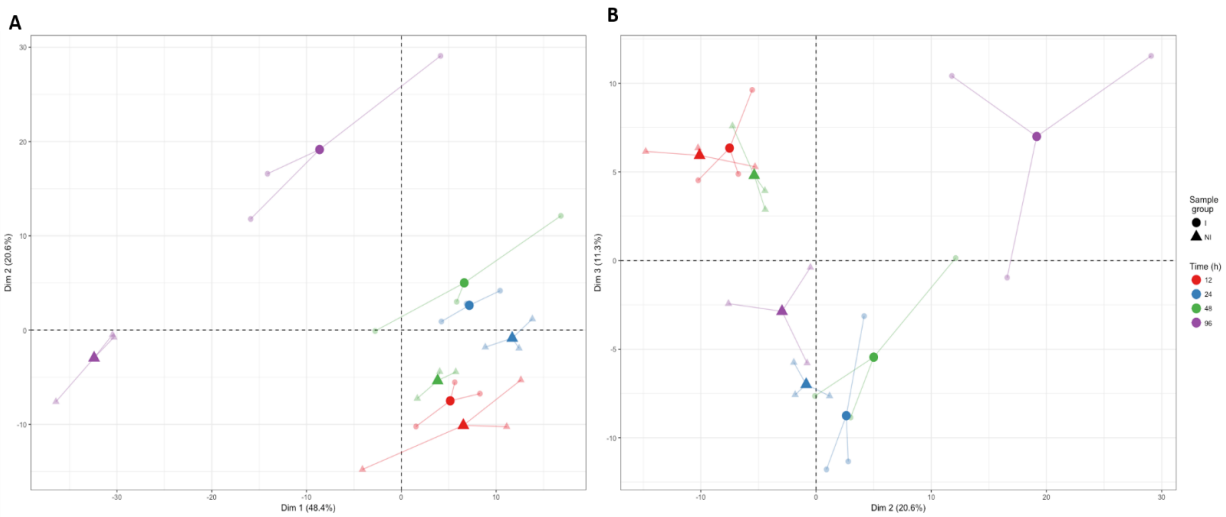


Fig. V. 5*: Principal component analysis performed on the DEGs. A dimension 1 (48.4%) dimension 2 (20.6%); B dimension 2 (20.6%), dimension 3 (11.3%) For each time point, three biological replicates (smaller dots) are represented for each condition (circle: inoculated samples; triangle: not inoculated) 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).

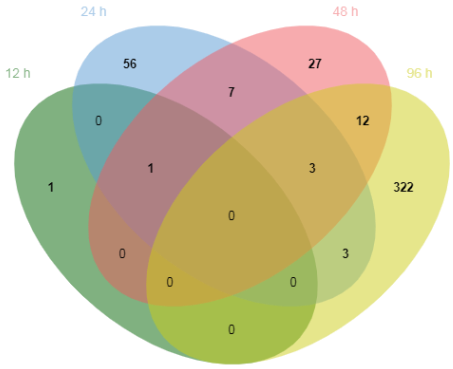


Fig. V. 6*: Common and unique DEGs at 12, 24, 48 and 96 hpi are represented in the Venn diagram.*

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.

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

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

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).

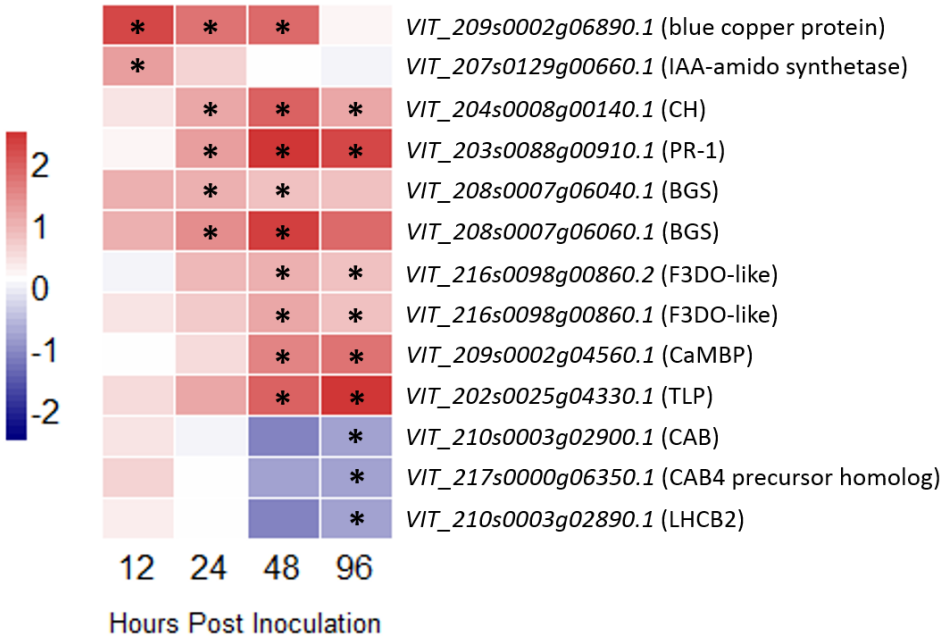


Fig. V. 7*: Heatmap representation of log2 fold change (inoculated/not inoculated) interesting genes. Blue and red boxes represent down and up regulation of the gene under pathogen attack. Asterisks identify significant differences (p<0.05) between inoculated and control. For VIT_216s0098g00860 we reported 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-β-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 up-regulated, 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 up-regulated 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.

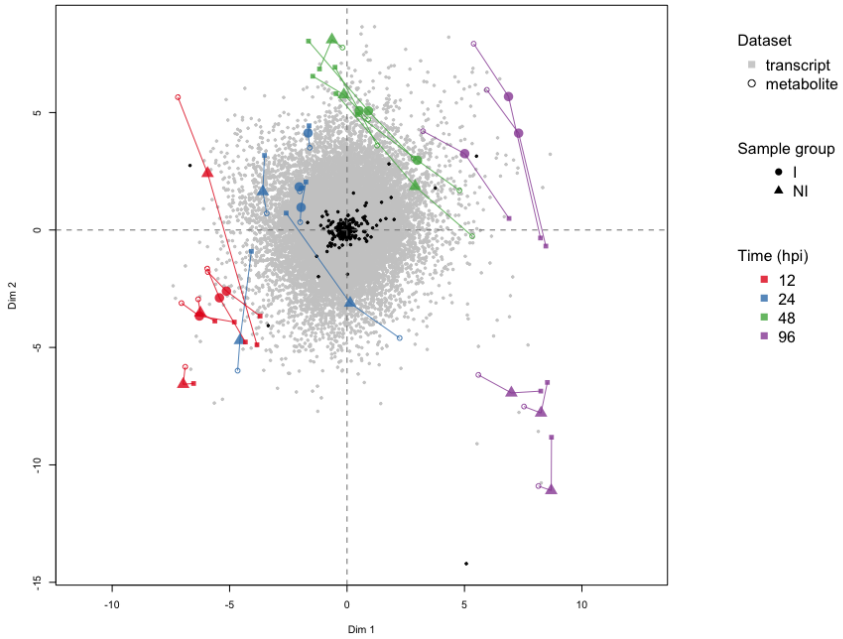


Fig. V. 8*: MCIA analysis of two –omics datasets. Each transcript is represented with a grey filled square and each metabolite with a black filled dot. For each time point, three biological replicates are represented for each condition (circle: inoculated samples; triangle: not inoculated). Each time 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.

Bibliography

Agudelo-Romero P, Erban A, Rego C, et al (2015) Transcriptome and metabolome reprogramming in Vitis vinifera cv. Trincadeira berries upon infection with Botrytis cinerea. J Exp Bot eru517. doi: 10.1093/jxb/eru517

Ali K, Maltese F, Figueiredo A, et al (2012) Alterations in grapevine leaf metabolism upon inoculation with Plasmopara viticola in different time-points. Plant Sci 191–192:100–107. doi: 10.1016/j.plantsci.2012.04.014

Berkey R, Bendigeri D, Xiao S (2012) Sphingolipids and Plant Defense/Disease: The "Death" Connection and Beyond. Front Plant Sci. doi: 10.3389/fpls.2012.00068

Chitarrini G, Soini E, Riccadonna S, et al (2017a) Identification of biomarkers for defence response to Plasmopora viticola in a resistant grape variety. Front Plant Sci. doi: 10.3389/fpls.2017.01524

Chitarrini G, Zulini L, Masuero D, Vrhovsek U (2017b) Lipid, phenol and carotenoid changes in "Bianca" grapevine leaves after mechanical wounding: a case study. Protoplasma 1–12. doi: 10.1007/s00709-017-1100-5

Della Corte A, Chitarrini G, Di Gangi IM, et al (2015) A rapid LC–MS/MS method for quantitative profiling of fatty acids, sterols, glycerolipids, glycerophospholipids and sphingolipids in grapes. Talanta 140:52–61. doi: 10.1016/j.talanta.2015.03.003

Dixon RA, Achnine L, Kota P, et al (2002) The phenylpropanoid pathway and plant defence-a genomics perspective. Mol Plant Pathol 3:371–390. doi: 10.1046/j.1364-3703.2002.00131.x

Enfissi EMA, Fraser PD, Lois L-M, et al (2005) Metabolic engineering of the mevalonate and non-mevalonate isopentenyl diphosphate-forming pathways for the production of health-promoting isoprenoids in tomato. Plant Biotechnol J 3:17–27. doi: 10.1111/j.1467-7652.2004.00091.x

Ferri M, Righetti L, Tassoni A (2011) Increasing sucrose concentrations promote phenylpropanoid biosynthesis in grapevine cell cultures. J Plant Physiol 168:189–195. doi: 10.1016/j.jplph.2010.06.027

Gershenzon J, Dudareva N (2007) The function of terpene natural products in the natural world. Nat Chem Biol 3:408–414. doi: 10.1038/nchembio.2007.5

Grimplet J, Van Hemert J, Carbonell-Bejerano P, et al (2012) Comparative analysis of grapevine whole-genome gene predictions, functional annotation, categorization and integration of the predicted gene sequences. BMC Res Notes 5:213. doi: 10.1186/1756-0500-5-213

Guan L, Dai Z, Wu B-H, et al (2016) Anthocyanin biosynthesis is differentially regulated by light in the skin and flesh of white-fleshed and teinturier grape berries. Planta 243:23–41. doi: 10.1007/s00425-015-2391-4

Jaillon O, Aury J-M, Noel B, et al (2007) The grapevine genome sequence suggests ancestral hexaploidization in major angiosperm phyla. Nature 449:463–467. doi: 10.1038/nature06148

Jang JC, León P, Zhou L, Sheen J (1997) Hexokinase as a sugar sensor in higher plants. Plant Cell 9:5–19. doi: 10.1105/tpc.9.1.5

Kachroo A, Kachroo P (2009) Fatty Acid–Derived Signals in Plant Defense. Annu Rev Phytopathol 47:153–176. doi: 10.1146/annurev-phyto-080508-081820

Kassambara A., Mundt F. (2017) factoextra : Extract and Visualize the Results of Multivariate Data Analyses.

Langmead B (2010) Aligning short sequencing reads with Bowtie. Curr Protoc Bioinforma Ed Board Andreas Baxevanis Al CHAPTER:Unit-11.7. doi: 10.1002/0471250953.bi1107s32

Lawton MA, Lamb CJ (1987) Transcriptional activation of plant defense genes by fungal elicitor, wounding, and infection. Mol Cell Biol 7:335–341.

Lê S., Josse J., Husson F. (2008) FactoMineR: An R Package for Multivariate Analysis. Journal of Statistical Software. J. Stat. Softw. 1–18.

Liu D, Xin M, Zhou X, et al (2017) Expression and functional analysis of the transcription factor-encoding Gene CsERF004 in cucumber during Pseudoperonospora cubensis and Corynespora cassiicola infection. BMC Plant Biol 17:96. doi: 10.1186/s12870-017-1049-8

Love MI, Huber W, Anders S (2014) Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. Genome Biol 15:550. doi: 10.1186/s13059-014-0550-8

Matarese F, Cuzzola A, Scalabrelli G, D'Onofrio C (2014) Expression of terpene synthase genes associated with the formation of volatiles in different organs of Vitis vinifera. Phytochemistry 105:12–24. doi: 10.1016/j.phytochem.2014.06.007

Meng C, Kuster B, Culhane AC, Gholami AM (2014) A multivariate approach to the integration of multi-omics datasets. BMC Bioinformatics 15:162. doi: 10.1186/1471-2105-15-162

Naithani S, Raja R, Waddell EN, et al (2014) VitisCyc: a metabolic pathway knowledgebase for grapevine (Vitis vinifera). Front Plant Sci. doi: 10.3389/fpls.2014.00644

Osbourn A, Goss RJM, Field RA (2011) The saponins: polar isoprenoids with important and diverse biological activities. Nat Prod Rep 28:1261–1268. doi: 10.1039/c1np00015b

Pedersen T. L. (2017) ggraph: An Implementation of Grammar of Graphics for Graphs and Networks version 1.0.0 from CRAN.

R Core Team (2017) R: a language and environment for statistical computing.

Ranty B, Aldon D, Galaud J-P (2006) Plant Calmodulins and Calmodulin-Related Proteins. Plant Signal Behav 1:96–104.

Rojas CM, Senthil-Kumar M, Tzin V, Mysore KS (2014) Regulation of primary plant metabolism during plant-pathogen interactions and its contribution to plant defense. Front Plant Sci. doi: 10.3389/fpls.2014.00017

Salvagnin U, Carlin S, Angeli S, et al (2016) Homologous and heterologous expression of grapevine E-(β)-caryophyllene synthase (VvGwECar2). Phytochemistry 131:76–83. doi: 10.1016/j.phytochem.2016.08.002

Savoi S, Wong DCJ, Arapitsas P, et al (2016) Transcriptome and metabolite profiling reveals that prolonged drought modulates the phenylpropanoid and terpenoid pathway in white grapes (Vitis vinifera L.). BMC Plant Biol 16:67. doi: 10.1186/s12870-016-0760-1

Shiratake K, Suzuki M (2016) Omics studies of citrus, grape and rosaceae fruit trees. Breed Sci 66:122–138. doi: 10.1270/jsbbs.66.122

Tena G, Asai T, Chiu W-L, Sheen J (2001) Plant mitogen-activated protein kinase signaling cascades. Curr Opin Plant Biol 4:392–400. doi: 10.1016/S1369-5266(00)00191-6

van den Berg RA, Hoefsloot HCJ, Westerhuis JA, et al (2006) Centering, scaling, and transformations: improving the biological information content of metabolomics data. BMC Genomics 7:142. doi: 10.1186/1471-2164-7-142

van Loon LC, Rep M, Pieterse CMJ (2006) Significance of inducible defense-related proteins in infected plants. Annu Rev Phytopathol 44:135–162. doi: 10.1146/annurev.phyto.44.070505.143425

Vrhovsek U, Masuero D, Gasperotti M, et al (2012) A Versatile Targeted Metabolomics Method for the Rapid Quantification of Multiple Classes of Phenolics in Fruits and Beverages. J Agric Food Chem 60:8831–8840. doi: 10.1021/jf2051569

Wang M, Vannozzi A, Wang G, et al (2015) A comprehensive survey of the grapevine VQ gene family and its transcriptional correlation with WRKY proteins. Front Plant Sci. doi: 10.3389/fpls.2015.00417

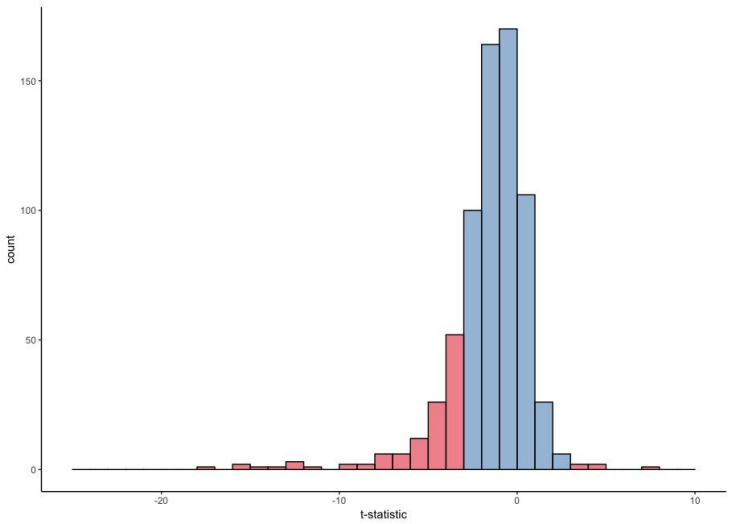
Werner S, Steiner U, Becher R, et al (2002) Chitin synthesis during in planta growth and asexual propagation of the cellulosic oomycete and obligate biotrophic grapevine pathogen Plasmopara viticola. FEMS Microbiol Lett 208:169–173.

Yaeno T, Matsuda O, Iba K (2004) Role of chloroplast trienoic fatty acids in plant disease defense responses. Plant J 40:931–941. doi: 10.1111/j.1365-313X.2004.02260.x

Zamboni A, Carli MD, Guzzo F, et al (2010) Identification of Putative Stage-Specific Grapevine Berry Biomarkers and Omics Data Integration into Networks. Plant Physiol 154:1439–1459. doi: 10.1104/pp.110.160275

Zhang W, Li F, Nie L (2010) Integrating multiple “omics” analysis for microbial biology: application and methodologies. Microbiology 156:287–301. doi: 10.1099/mic.0.034793-0

Zurbriggen MD, Carrillo N, Hajirezaei M-R (2010) ROS signaling in the hypersensitive response. Plant Signal Behav 5:393–396.



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

***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.*

		12 hpi		24 hpi		48 hpi		96 hpi	
Class	**Compound**	NI	I	NI	I	NI	I	NI	I
Acids									
	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	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
	pyroglutamic 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	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
	5-ketogluconic 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									
	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	57.694 ± 5.108	104.950 ± 8.889
	alanine	18.537 ± 4.681	37.457 ± 6.630	41.718 ± 7.204	37.868 ± 4.621	54.240 ± 5.024	56.325 ± 22.975	105.154 ± 9.097	134.509 ± 7.493
	leucine	1.910 ± 0.350	2.663 ± 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
	isoleucine	6.452 ± 0.737	6.912 ± 0.093	9.149 ± 1.176	10.142 ± 1.137	15.409 ± 1.970	22.908 ± 0.246	33.729 ± 2.934	65.245 ± 5.857
	serine	11.632 ± 1.323	18.952 ± 0.864	18.899 ± 0.147	19.559 ± 2.330	25.550 ± 3.256	31.069 ± 9.151	53.272 ± 1.964	80.724 ± 8.411
	threonine	9.916 ± 1.232	16.285 ± 0.529	16.303 ± 0.341	15.481 ± 1.478	18.445 ± 1.055	22.130 ± 6.372	39.353 ± 2.355	59.819 ± 7.856
	proline	6.680 ± 1.165	9.955 ± 0.960	13.788 ± 0.677	14.615 ± 0.407	18.947 ± 1.646	18.186 ± 5.388	36.854 ± 1.323	52.991 ± 4.539
	glycine	0.884 ± 0.070	1.051 ± 0.058	1.393 ± 0.465	1.319 ± 0.050	1.070 ± 0.107	1.066 ± 0.203	1.768 ± 0.467	1.426 ± 0.015
	aspartic acid	58.891 ± 1.670	106.256 ± 6.815	88.490 ± 7.026	74.369 ± 5.888	70.658 ± 0.958	98.985 ± 11.789	119.494 ± 7.781	139.581 ± 16.112
	β-alanine	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
	γ-aminobutyric acid	11.840 ± 0.977	15.254 ± 0.447	19.732 ± 0.615	17.633 ± 1.595	26.025 ± 2.181	25.546 ± 6.658	26.894 ± 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	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
Sugars									
	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
	α-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

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 ± 0.133	1.293 ± 0.068
adonitol	5.913 ± 0.557	6.975 ± 0.262	6.698 ± 0.576	6.993 ± 0.286	6.356 ± 0.483	7.539 ± 0.642	6.504 ± 0.509	8.444 ± 0.195
fucose	10.732 ± 2.795	9.054 ± 0.206	9.270 ± 0.820	9.623 ± 0.338	9.208 ± 0.636	13.773 ± 1.591	9.020 ± 0.405	12.877 ± 0.310
fructose	289.451 ± 110.549	405.362 ± 5.746	398.711 ± 40.610	405.322 ± 11.005	342.667 ± 28.232	425.019 ± 116.760	337.797 ± 34.184	469.591 ± 32.038
glucose	904.094 ± 359.954	504.495 ± 26.259	502.028 ± 82.238	495.693 ± 38.249	424.360 ± 22.719	977.183 ± 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 ± 0.127	1.934 ± 0.100	2.513 ± 0.166	2.478 ± 0.716	2.547 ± 0.182
myo-inositol	2346.249 ± 492.114	2205.945 ± 54.240	2138.925 ± 262.663	2167.144 ± 168.634	1872.523 ± 82.951	3001.778 ± 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 ± 147.489	1491.428 ± 40.826	1731.516 ± 651.124	1027.878 ± 106.681	1657.326 ± 20.040
lactose	79.804 ± 12.515	74.604 ± 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

***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.*

		12 hpi		24 hpi		48 hpi		96 hpi	
Class	**Compound**	NI	I	NI	I	NI	I	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 ± 0.026	0.364 ± 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 ± 0.028	0.388 ± 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 ± 0.009	0.213 ± 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 ± 0.002	0.039 ± 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-glycerol)sodium salt	0.228 ± 0.030	0.215 ± 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
	1-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 ± 0.070	0.621 ± 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 ± 0.003	0.198 ± 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. 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.*

Class	Compound	12 hpi		24 hpi		48 hpi		96 hpi	
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
	p-hydroxybenzoic acid	0.247 ± 0.053	0.381 ± 0.070	0.242 ± 0.032	0.239 ± 0.051	0.278 ± 0.038	0.228 ± 0.025	0.136 ± 0.015	0.216 ± 0.096
	vanillin	0.186 ± 0.007	0.186 ± 0.013	0.153 ± 0.021	0.153 ± 0.017	0.198 ± 0.031	0.241 ± 0.037	0.135 ± 0.010	0.141 ± 0.020
	vanillic acid	0.010 ± 0.005	0.024 ± 0.012	0.029 ± 0.006	0.018 ± 0.005	0.024 ± 0.006	0.014 ± 0.008	0.030 ± 0.004	0.021 ± 0.003
Coumarins									
	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	0.020 ± 0.003	0.027 ± 0.010	0.024 ± 0.004	0.013 ± 0.003	0.015 ± 0.008	0.032 ± 0.012	0.032 ± 0.012	0.016 ± 0.003
Phenylpropanoids									
	trans-coutaric acid	84.769 ± 8.149	86.920 ± 9.332	66.963 ± 1.807	67.014 ± 3.378	73.072 ± 2.404	99.652 ± 2.527	55.977 ± 6.309	77.525 ± 10.951
	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	1108.53 ± 100.63	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	16.806 ± 0.707	15.767 ± 0.861	13.267 ± 1.013	13.869 ± 1.246	14.640 ± 1.788	21.058 ± 1.383	12.199 ± 0.407	15.787 ± 1.766
	ferulic acid	0.054 ± 0.008	0.008 ± 0.004	0.019 ± 0.011	0.022 ± 0.006	0.015 ± 0.004	0.028 ± 0.013	0.016 ± 0.002	0.013 ± 0.003
	sinapic acid	0.175 ± 0.012	0.110 ± 0.017	0.136 ± 0.007	0.142 ± 0.006	0.135 ± 0.003	0.182 ± 0.005	0.107 ± 0.008	0.124 ± 0.012
Dihydrochalcones									
	phlorizin	0.612 ± 0.067	0.688 ± 0.077	0.649 ± 0.135	0.568 ± 0.065	0.696 ± 0.039	0.967 ± 0.077	0.436 ± 0.022	0.662 ± 0.026
Flavones									
	luteolin-7-O-glucoside	0.053 ± 0.005	0.069 ± 0.018	0.058 ± 0.011	0.058 ± 0.019	0.047 ± 0.005	0.065 ± 0.009	0.055 ± 0.012	0.044 ± 0.001
Flavan-3-ols									
	catechin	13.279 ± 1.022	11.921 ± 1.092	9.081 ± 0.769	8.643 ± 0.860	9.211 ± 0.905	10.243 ± 1.490	6.996 ± 0.170	6.879 ± 0.989
	epicatechin	1.876 ± 0.288	1.292 ± 0.386	1.038 ± 0.287	1.071 ± 0.175	1.984 ± 0.632	1.759 ± 0.691	1.210 ± 0.357	1.390 ± 0.314
	epicatechin gallate	0.337 ± 0.173	0.481 ± 0.030	0.429 ± 0.079	0.336 ± 0.084	0.327 ± 0.175	0.273 ± 0.148	0.234 ± 0.023	0.315 ± 0.107
	epigallocatechin	1.420 ± 0.462	1.523 ± 0.518	0.810 ± 0.113	0.818 ± 0.231	0.869 ± 0.295	1.164 ± 0.347	0.528 ± 0.099	0.876 ± 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 B1	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 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
	cis-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

cis + *trans*-ω-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-ε-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 ± error standard, expressed as µg/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
Alcohols										
	cyclobutanemethanol	C	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
Aldehydes										
	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
Benzenoids										
	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
Ketones										
	2-cyclopentene-1-one-2-pentenyl	C	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
Terpenoids										
	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	±	0.0050	0.0273	±	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	±	0.0039	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	±	0.0058	0.0783	±	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	±	0.0015	0.0202	±	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	±	0.0007	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	±	0.0086	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	±	0.0020	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	±	0.0064	0.0857	±	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	±	0.0049	0.0628	±	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	±	0.0078	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
Unknowns:																										
	unknown-1		0.0067	±	0.0003	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	±	0.0007	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	±	0.0023	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	±	0.0016	0.0184	±	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	±	0.0007	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	±	0.0005	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

Supplementary Table V. 5*: List of metabolites with a t statistic absolute value greater than 3 and time point specification.*

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
cis-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
Lysine	12	-6.02651
Malic	96	-3.60199
Malonic	12	-3.59885
meso-Erythriol	48	-4.18336
myo-Inositol	48	-3.3675
myo-Inositol	96	-5.13669
Myricetin	96	-7.3156
Myristic	48	-4.88568
Myristic	96	-4.83239
Oleanolic	48	-7.508
Oleic acid+*cis*-Vaccenic	48	-3.1132
Pallidol	12	-3.14652
Pallidol	48	-3.12785
Phenylalanine	96	-4.37555
Phlorizin	48	-3.34079
Phlorizin	96	-6.62349

Procyanidin B3 as B1	24	4.215774
Proline	96	-3.73868
Pyroglutamic	12	-4.54743
Pyroglutamic	24	-3.06419
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

***Supplementary Table V. 6**: Differentially expressed genes from RNA-Seq analysis.*

DEGs 12 hpi

VIT_209s0002g06890.1
VIT_207s0129g00660.1

DEGs 24 hpi

VIT_200s0187g00010.1
VIT_200s0270g00120.1
VIT_200s0301g00080.1
VIT_200s0346g00110.1
VIT_200s0346g00110.2
VIT_200s0615g00010.1
VIT_200s0615g00010.3
VIT_201s0146g00480.1
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_203s0088g00910.1
VIT_204s0008g00140.1
VIT_204s0008g07150.1
VIT_205s0020g03190.3
VIT_205s0020g03190.5
VIT_205s0020g03190.6
VIT_205s0020g04780.1
VIT_205s0020g04780.3
VIT_207s0031g00530.1
VIT_207s0129g00390.1
VIT_207s0129g00760.1
VIT_207s0129g00800.1
VIT_208s0007g00580.1
VIT_208s0007g01930.1
VIT_208s0007g01940.1
VIT_208s0007g01940.2
VIT_208s0007g04800.4
VIT_208s0007g06040.1
VIT_208s0007g06060.1
VIT_208s0007g07990.1
VIT_209s0002g00890.3
VIT_209s0002g00890.4
VIT_209s0002g01320.1
VIT_209s0002g02990.1
VIT_209s0002g03020.1
VIT_209s0002g05400.1
VIT_209s0002g06890.1
VIT_210s0042g00840.1
VIT_210s0042g00860.1
VIT_210s0042g00870.1
VIT_210s0042g00880.1
VIT_210s0042g00890.1
VIT_210s0042g00920.1
VIT_211s0016g03250.1
VIT_212s0034g01930.1
VIT_214s0060g02170.1
VIT_214s0108g00630.1
VIT_214s0108g00690.1
VIT_218s0001g06090.1
VIT_218s0001g06120.1
VIT_218s0001g10200.1
VIT_218s0041g02010.1
VIT_218s0041g02270.1
VIT_218s0122g00620.1
VIT_218s0166g00050.1
VIT_219s0015g02680.1
VIT_219s0015g02730.1
VIT_219s0015g02890.1
VIT_219s0093g00110.1
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_202s0025g04330.1
VIT_203s0088g00700.1
VIT_203s0088g00710.1
VIT_203s0088g00810.1
VIT_203s0088g00910.1
VIT_204s0008g00140.1
VIT_204s0008g01070.1
VIT_204s0008g02510.1
VIT_204s0008g07150.1
VIT_205s0049g00570.1
VIT_207s0031g01070.1
VIT_207s0031g01710.1
VIT_207s0197g00130.1
VIT_208s0007g06060.1
VIT_208s0040g01130.1
VIT_209s0002g01310.1
VIT_209s0002g04560.1
VIT_209s0002g06890.1
VIT_211s0016g03250.1
VIT_212s0057g00140.1
VIT_212s0057g01020.1
VIT_213s0019g04380.2
VIT_214s0030g00680.1
VIT_214s0066g01810.1
VIT_214s0066g01840.1
VIT_214s0066g01970.1
VIT_214s0108g01000.1
VIT_215s0024g00410.1
VIT_215s0046g01140.1
VIT_215s0046g02410.3
VIT_216s0013g00070.1
VIT_216s0022g00890.1
VIT_216s0050g01890.1
VIT_216s0098g00510.1
VIT_216s0098g00860.1
VIT_216s0100g00940.1
VIT_216s0100g01000.1
VIT_217s0000g03370.1
VIT_217s0000g03370.2
VIT_217s0000g03370.4
VIT_217s0000g04880.1
VIT_218s0001g06170.1
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
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Concluding remarks

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 plant-pathogen 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 Plant-microbe 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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I thank all the members, present and past, of the Metabolomic Unit for the time shared. In particular my friends, *Marynka*, *Domenico*, *Evelyn*, *Stefania* and *Luca* for their support and encouragement. Thanks to you the last four years have been full of love and joy.

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