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"Metabolomica e Lipidomica: approfondimenti sul sistema di difesa delle piante resistenti della vite contro Peronospora e Oidio"

*in co-tutela con Fondazione Edmund Mach **

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AIM of the Ph.D. project

Downy mildew (DM), produced by the oomycete *Plasmopara viticola*, and Powdery mildew (PD), caused by the ascomycete *Erysiphe necator*, are the two most common and commercially important diseases of grapevine. *Vitis vinifera*, a Eurasian species famed for its flavor, is the source of the bulk of cultivated grapevines. However, this species is very vulnerable to *P. viticola* and *E. necator*, implying that grape production is heavily reliant on the usage of fungicides.

A crucial part of managing a vineyard is undoubtedly protecting the vine from infections, notably fungal ones, especially in the context of minimizing the use of chemical pesticides. Getting healthy grapes is crucial for increasing the product's qualitative attributes as well as for the environment, the well-being of agricultural workers, and the control of production costs. In this sense, the selection of vines is crucial since they must simultaneously have the necessary technological, agronomic, and qualitative traits.

In this regard, the most common strategy is the use of vines with pathogen-specific resistance, in other words, mono-locus resistant genotypes carrying one locus associated with *P. viticola* resistance (*Rpv*), respectively with *Erysiphe necator* resistance (*Run*/*Ren*). However, this resistance genes' protection can sometimes be overcome by virulent strains of the pathogens and thus a longer-lasting disease resistance is required. A more promising strategy is the use of pyramided resistant genotypes carrying more than one *Rpv* gene, respectively *Run*/*Ren* gene.

In order to better comprehend the still poorly understood mechanisms of plant defense against *P. viticola* and *E. necator*, the goal of this thesis was to define the plant-pathogen interaction and their metabolic and lipidomic disruption, as well as better understand the defense mechanisms of resistant vines.

We did this by examining vines with various levels of resistance aiming to:

1. Understand if different sources of resistance are associated with different degrees of resistance and, implicitly, with different responses to *P. viticola*
2. Explore the interaction between grapevine and *E. necator* and extend the insufficiently current knowledge about the perturbations occurring in the plant system after biotic stress.
3. Characterize the disruptive impact of *E. necator* within the plant's lipid profiling

Understanding the plant defense mechanisms behind the different levels of vine resistance to diseases is crucial for breeding programs, as well as for lowering the need for treatments and guaranteeing adequate quality levels, particularly in areas where the climate is favorable for the development of the pathogens.

The thesis is composed of five chapters:

- A general introduction in Chapter I;
- An original published paper entitled "Mono-Locus and Pyramided Resistant Grapevine Cultivars Reveal Early Putative Biomarkers upon Artificial Inoculation with *Plasmopara viticola*" in Chapter II;
- An original published paper entitled "Secondary and primary metabolites reveal putative resistance-associated biomarkers against *Erysiphe necator* in resistant grapevine genotypes" in Chapter III

- An original published paper entitled “Semi-targeted profiling of the lipidome changes induced by *Erysiphe necator* in disease-resistant and *Vitis vinifera* L. varieties” in Chapter IV
- Conclusion and future perspectives in Chapter V

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PUBLICATIONS

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ABSTRACT of the Ph.D.

In recent years, increased sensitivity to environmental problems, as well as consumer interest in the nutritional and health aspects of wine production have prompted scientists to deepen their research into the relationships between the vine and its pathogens in order to develop operational strategies to better protect the agricultural environment and improve product quality. Although *Vitis vinifera* is not resistant to the most common fungal pathogens, different levels of resistance were found in the cultivated varieties.

This thesis investigated mono-locus resistant genotypes carrying one locus associated with *Plasmopara viticola* resistance (*Rpv*), respectively with *Erysiphe necator* (*Run/Ren*) as well as pyramided resistant genotypes carrying more than one resistant gene against two major parasitic diseases of the vine: downy mildew, *P. viticola*, and powdery mildew, *E. necator*.

The choice of vines was done considering their degree of resistance and susceptibility to the pathogens. The study looked into five resistant mono-locus varieties: BC4, 'Bianca', F12P160, 'Kishmish vatkhana', 'Solaris'; five resistant pyramided varieties: F12P127, F13P71, F12P60, F26P92, and NY42; and two susceptible varieties: 'Pinot Noir' and 'Teroldego'. In order to confirm any connections with the various degrees of resistance, the OIV of the infected leaf tissues was also determined.

We have performed metabolomic and lipidomic analyses on completely detached leaves, which gave us a molecular snapshot of the complex and quickly evolving metabolic perturbations taking place inside the leaves as a reaction to the pathogen's infection. The targeted metabolomics approach was used for the analysis of the main classes of plant metabolites (primary compounds, lipids, phenols, and volatile organic compounds), while the semi-targeted lipidomics approach was used for the analysis of lipids only.

These cutting-edge "omics" technologies enabled us to investigate alterations in the most important categories of plant metabolites involved in plant defense. Understanding the interactions between plants and diseases aids in the understanding of plant defense systems as well as the characterization of the plant-pathogen relationship and its metabolic disruption. It may also aid in the discovery of pathogen resistance-related biomarkers, which can provide a thorough interpretation of the antagonistic interactions between *V. vinifera* and the two pathogen infections, as well as useful information for breeders.

The metabolomics response of resistant vines to *P. viticola* during the first 96 hours after pathogen inoculation revealed 22 potential biomarkers of resistance. Metabolite modulation was greatest in mono-locus genotypes at 48 and 96 hpi, compared to pyramided genotypes, where changes began as early as 12 hpi.

The metabolomics changes that occurred inside the *E. necator*-resistant vines provided us with a picture of plant metabolome disturbance, which contributed to the expansion of current understanding about the perturbations that occur in the defense plant system following biotic stress. Several molecules were altered in the pyramided and mono-locus genotypes as compared to the susceptible variety. Among these compounds, ten were highly accumulated after the infection with *E. necator*. Thus, they have been proposed by our study as potential biomarkers of the resistant varieties.

A deeper investigation and a better comprehension of the role of lipids in the plant defense response were necessary in light of the little information currently known about the participation of lipids in the pathosystem of resistant grapevine genotypes—*E. necator*. Our research found that lipidome changes were most obvious at 24 and 48 hours after inoculation. The extra-plastidial lipids (PC, PE), the signaling lipids (PA and PI), the plastid lipids (PG, MGDG, and DGDG), and in lesser amounts: LPC, LPG, LPI, and LPE were among the lipids that were most frequently discovered in the leaves of the grapevine that had been infected with *E. necator*. Furthermore, the down-accumulation of the lipid classes distinguished the resistant genotypes, while the up-accumulation of the lipid classes distinguished the susceptible genotype.

CHAPTER I

INTRODUCTION

Chapter I. INTRODUCTION

1. VITACEAE FAMILY AND ITS ECONOMIC IMPORTANCE

Vitaceae is a Rhamnales order family with two subfamilies: Lecoideae and Ampelideae. Ampelideae is made up of five genera: *Ampelopsis*, *Cissus*, *Parthenocissus*, *Ampelocissus*, and *Vitis*. The first four are utilized as decorative plants, whereas the *Vitis* genus contains species that are widely cultivated and have significant economic value. This last genus is further subdivided into two subgenera: *Muscadinia* and *Euvitis*, which contain approximately 40 Asian and 30 American species (Fregoni, 2005).

The Euvitis subgenus is classified into four groups based on their geographical distribution and optimal climate requirements. There are two American groupings, one Euro-Asian and one Eastern Asian. American grapevines have been classified into two groups: those acclimated to temperate regions and those adapted to tropical ones (Fregoni, 2005). Most American and Asian grapevine species are resistant to various infections, but their wines are not well embraced by customers due to poor quality. The *Vitis vinifera* L. species is the most significant in the Euro-Asiatic group for qualitative characteristics, but unfortunately, all varieties are highly sensitive to various infections; only a few exceptions have been found.

The global surface area planted with vines for wine, table grapes, juice, and raisins reached in 2019 roughly 7.4 million hectares (mha), including immature plants that had not yet begun production. In the same year, worldwide wine production (excluding juices and musts) was predicted to exceed 260 million hectoliters, while the global wine export market has increased in both volume and value since 2018 (Vezzulli et al., 2022).

Vitis vinifera L. is widely regarded as one of the most important crops grown in Europe, with a significant social and economic impact. This continent has the world's largest wine production and vineyard acreage - 3.2 million hectares of land under vines, as well as some of the most important and well-known winemaking regions and wines. These are extremely prevalent in the Mediterranean region, and especially in the world's top wine-producing countries: Italy, France, and Spain account for three-quarters (74.9 %) of the area under vines in the EU and about two-fifths (38.7 %) of vineyard holdings in 2020 (EUROSTAT, 2022; Droulia et al., 2021).

2. GRAPEVINE PATHOGENS

The Eurasian grape species *(V. vinifera* L.) is widely impacted by a large number of diseases that influence production, fruit quality, processing, and exports. Grapevine is known to contain a diverse range of pathogens, including fungi, oomycetes, bacteria, phytoplasmas, viruses, and plant-parasitic nematodes, all of which have distinct infection methods, life cycles, and survival strategies (Figure 1). These organisms attack all sections of the grapevine plant, including the roots, trunk, arms, cordons, canes, shoots, leaves, rachis, and berries (Vezzulli et al., 2022).

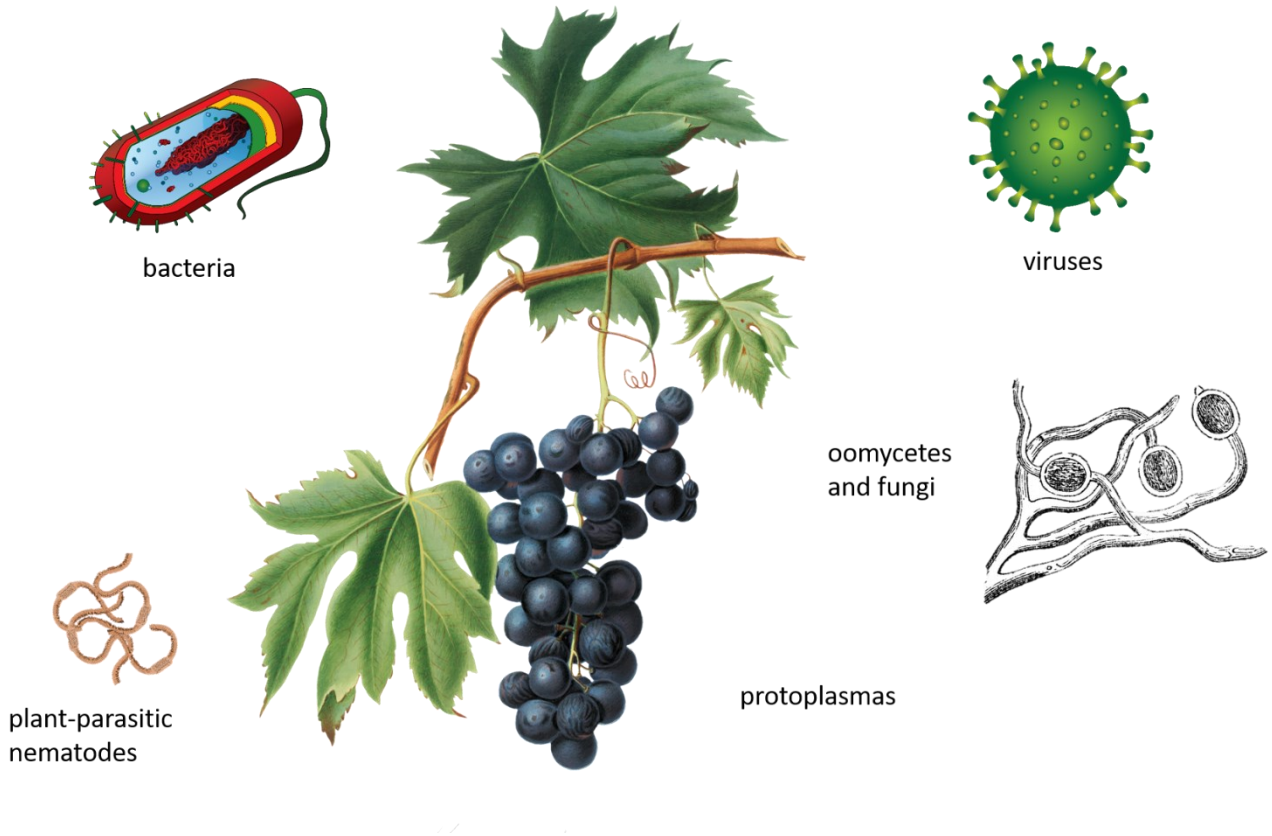

Figure 1. Grapevine plant and its pathogens

Among of the most serious diseases affecting *V. vinifera* our study considered downy mildew (DM) and powdery mildew (PM), caused by *Plasmopara viticola* and *Erysiphe necator*. DM is caused by a fungus-like (oomycete) organism whose reproduction and dissemination mechanisms differ from those of diseases caused by actual fungi, such as PM. However, they are both a disease of the foliage that affects the leaves and fruits, resulting in yield loss and a decline in the quality of must and wine.

2.1 Downy mildew

The oomycete *P. viticola* (Berk. & Curt.) Berl. & de Toni causes DM, one of the most extremely destructive diseases of the grapevine. DM was accidently introduced into Europe from North America in the late 1870s, due to the importation of American rootstocks resistant to *Phylloxera* a root aphid that causes grapevine wilt and death. The disease quickly expanded across grape-growing areas around the world, especially in temperate-humid climates.
This disease affects leaves, shoots, and bunches, causing up to 75% crop damage in one season if no treatments are used (Buonassisi et al., 2017), resulting in significant economic losses.

In favorable weather conditions, the pathogen can have numerous infectious cycles in one season. It overwinters as oospores in dead leaf lesions and shoots and, sometimes, as mycelium in infected twigs. In suitable weather conditions (temperatures above 10 °C and rainy periods), the oospores germinate to produce a sporangium whose zoospores are transported by wind or water to the wet leaves near the ground (Buonassisi et al., 2018). During the infection process, the zoospores of *P. viticola* penetrate the host through the stomata of the lower surface and develop intercellular mycelium in the mesophyll of grapevine leaves, where it feeds through the haustoria. After the

colonization period, the sporangiophores and sporangia that emerge from the stomata can be carried by, wind or rain to nearby healthy plants, germinate quickly, and produce many zoospores that cause secondary infections under climatic conditions similar to those for primary infections. A disease cycle may take from 5 to 18 days, depending on temperature, humidity, and varietal susceptibility (Buonassisi et al., 2018; Gessler et al., 2011).

The pathogen can attack all green plant tissue. The most distinctive signs of infection are the sporangia formation apparent as whitish spots, commonly found on the abaxial surface of the first-formed leaves, which are accompanied by chlorotic spots (known also as oil spots) on the adaxial surface. Sporulation can be seen on the leaf's abaxial side as well as the surfaces of tendrils, inflorescences, and young berries (Buonassisi et al., 2018).

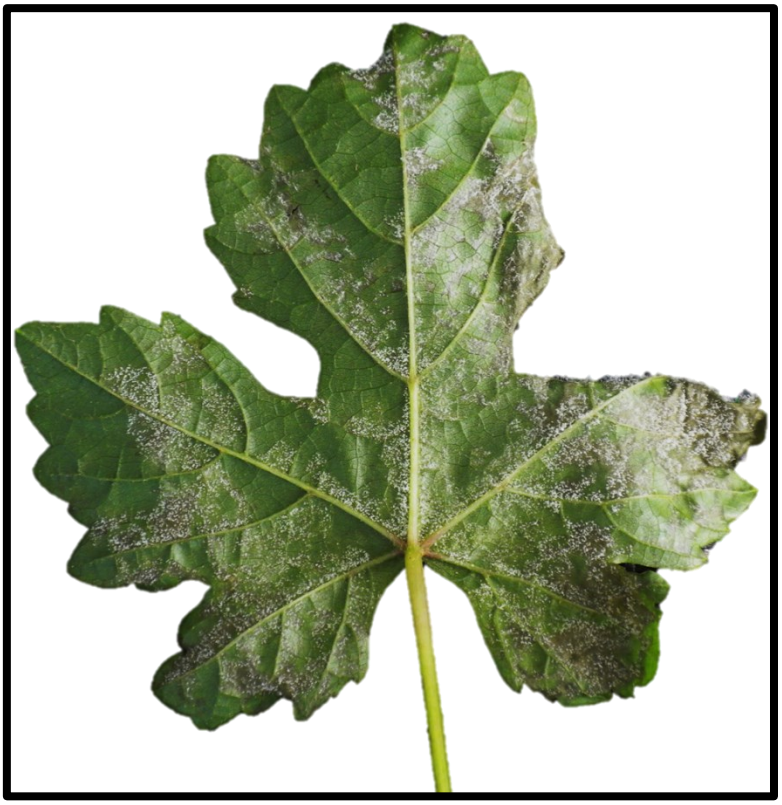

Figure 2. Grapevine leaves with downy mildew infection

2.2 Powdery mildew

Powdery mildew (PM), caused by the obligate biotrophic ascomycete *E. necator* (syn. *Uncinula necator* (Schw.) Burr; anamorph *Oidium tuckeri* Berk), is one of the most common diseases in vineyards.

This pathogen is mostly found in arid and warm climates and it can colonize all green tissues of the cultivated grapevine *V. vinifera*. The ideal development happens at a temperature of 26^0 C and relative humidity of 85% in the spring, but the epidemiologic process can begin as soon as the temperatures start to rise above 15^0C and the relative humidity exceeds 25% (Gadoury et al., 2012; Wilcox, 2003).

It's a polycyclic disease with two stages. Primary infections are caused by sexual spores (ascospores), while secondary infections are caused by asexual spores (conidia) on all green tissues of grapevines,

primarily leaves and berries, eventually leading to bunch rotting (Gadoury and Pearson, 1988; Gadoury et al., 2001; Caffi et al., 2011; Gadoury, 2012;Wilcox, 2015).

E. necator's growth and development are entirely dependent on its host. The conidium does this by attaching to plant tissue cells, permitting the creation of a primary germ tube that matures into a specialized infectious structure (i.e., appressorium). In order to infiltrate and invade the host cells, it generates mechanical pressure (Armijo et al., 2016). The successful invasion results in the creation of the haustorium, via which the fungus absorbs nutrients required to complete its lifespan (Qiu et al., 2015). Once this structure is developed, secondary hyphae spread throughout the infected tissue, allowing asexual reproductive bodies (conidiophores and conidia) to arise. When environmental or nutritional conditions become unfavorable, *E. necator* generates cleistothecia, sexual reproduction structures that contain four to six asci at maturity, each containing four ascospores (Agrios, 1997; Armijo et al., 2016). However, physiological maturity may take several months, especially in colder climates. Ascospores, like conidia, germinate with a single germ tube that ends in appressorium development (Gadoury et al., 2012).

All green grapevine organs above ground are affected by *E. necator*. Symptoms include white-greyish powdery or dusty spots of fungus development on the upper side of the leaves and other green parts of the vines. One of the most distinctive signs of infection is the ascospore colonies. They are most commonly found on the lower surface of the first-formed leaves. Young colonies appear whitish unless they did not sporulate and that is when they have a metallic brightness. On the opposite, the senescent colonies are greyish. Required conditions for sporulation are humidity > 93% and temperatures of 18–20 °C. Another distinctive sign of the infection is the so-called flag shoots. These are shoots that arise from these buds where the mycelium is overwintered. They may be heavily coated with fungal growth, and white in color, which makes them look like white flags in the vine (Buonassisi et al., 2018; Gadoury et al., 2012). Berries in infected clusters become hard, brown, smaller than those in uninfected clusters, and may crack open (Gomès and Thévenot, 2009) affecting wine quality significantly (Gadoury et al., 2001; Calonnec et al., 2004).

Figure 3. Grapevine leaves with powdery mildew infection

2.3 Control of downy and powdery mildew

Early treatments are the most commonly used method for providing efficient plant protection for highly PM and DM susceptible *V. vinifera* cultivars. To prevent an outbreak of the pathogens, viticulturists, including organic wine producers, apply fungicides such as sulphur- and copper-compounds or other synthetic protectants up to 12 times during the growing season, depending on weather conditions and geographic location (Chen, 2020). As a result, viticulture ranks as one of the most significant agricultural users of fungicides (Zendler, 2020).

This aspect raised worries about the negative influence of pesticides on the environment and human health, leading to restrictions on fungicide usage, such as EU rules (e.g., Directive 1107/2009/EU). The European Commission currently enforces national pesticide reduction action plans, supporting the use of monitoring networks (Directive 128/2009/EC), forecasting models, and dissemination mechanisms to exchange this information among growers and technicians. As a result, a dependable monitoring and forecasting system is required for developing prediction indices to support long-term protective efforts (Volpi, 2021).

3. PLANT-PATHOGEN INTERACTION

Plants have two main ways to defend themselves against pathogens in nature: through constitutive and induced defenses. A constitutive defense is one that is always present in the plant, whereas an induced defense is a temporary defense that is targeted to defend against an area of the plant where it has been attacked or injured.

The mechanical barriers, which are part of the constitutive defense, include morph-anatomical properties of grapevine organs such as spines, trichomes, thick cuticles, and hard, sticky, or smooth surfaces that inhibit pathogens from penetrating or laying eggs. In the induced defense, the most essential antimicrobial compounds are the phytoanticipins. These compounds are present in plants even before the attack of a pathogen or an infection (Tiku, 2020) and they include a variety of chemicals that are poisonous, repulsive or make plant tissues indigestible to attackers (Dearing, 2005).

Plants can have either separate mechanical and chemical defenses or a mix of the two (Dussord, 1991). Nevertheless, many pathogens manage to successfully breach this pre-invasive layer of protection. To inhibit future pathogen invasion, a wide range of inducible plant defenses can be activated. Thus, several efficient methods to perceive the attack of their adversary and translate this perception into an effective immune response were developed for a post-invasive line of defense (Pieterse, 2009).

3.1 Plant immunity system

The primary immune response identifies microbial pathogen characteristics such as flagellin, chitin, glycoproteins, and lipopolysaccharides. The name given to these microbial determinants is pathogen-associated molecular patterns (PAMPs). PAMPs engage pattern-recognition receptors (PRRs), which in turn initiate a variety of downstream signalling cascades that eventually culminate in the activation

of PAMP-triggered immunity (Figure 4A). In turn, pathogens acquire effector molecules that are carried into the host cell to decrease PTI and boost pathogen pathogenicity, leading to an effector-triggered susceptibility (Figure 4B). Plants then developed resistance (R) proteins that detect these attacker-specific effectors, resulting in effector-triggered immunity, a secondary immune response (Figure 4C). Therefore, the balance between the pathogen's ability to inhibit the plant's immune system and the plant's ability to recognize the pathogen and activate effective defences determines the final success (Pieterse, 2009).

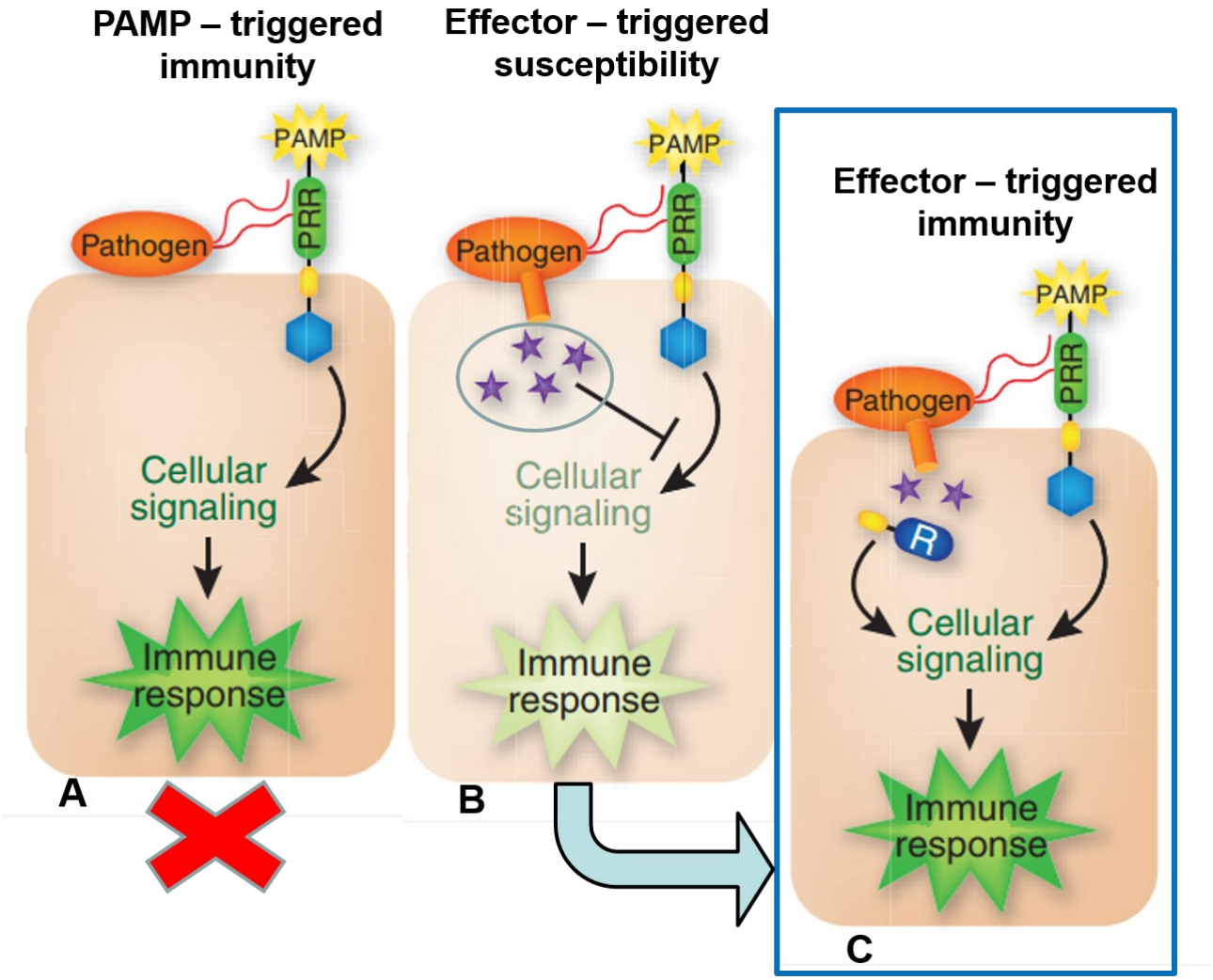

Figure 4. Simplified schematic representation of the plant immune system (adapted from Pieterse et all, 2009)

3.2 Defence specialized metabolites in grapevine

Plant metabolites are low molecular weight compounds classified in primary and secondary compounds. Through our research, we examined both primary and secondary metabolism following *P. viticola* and *E. necator* infection in order to address the most significant groups of plant metabolites with a defense role.

Although primary metabolites are principally involved in physiological tasks such as vegetative growth and reproduction, it has been discovered that they are also involved in plant defense. Various molecules such as carbohydrates, organic acids, amines, amino acids, and lipids act not only as a source of energy but also as a source of signaling molecules to trigger direct or indirect defense responses. Phytoalexins, which are secondary metabolites, are produced in response to biotic and abiotic stressors and they actively participate in the complex defense system between invading pathogens and plants. One of the most common phytoalexins found in the grapevine *Vitis vinifera* belongs to the class of molecules known as stilbenes (Wang et al., 2010).

According to current research, the wide class of stilbenoids has been proven to be crucial for disease resistance at various time points post-pathogen inoculation (Vezzulli, 2022). Stilbenes were among the initial choices as biomarkers for disease resistance (Gindro et al., 2012; Viret et al., 2018; Billet et al., 2020). Stilbenic phytoalexins are considered to be active compounds with antifungal activity, therefore they are key defense molecules implicated in the resistance of grapevine cultivars to the two major fungal pathogens, *P. viticola* (downy mildew) and *E. necator* (powdery mildew) (Viret, 2018).

Aside from these phenolic chemicals, several lipids have been linked to DM resistance (Chitarrini et al., 2017; Cavaco et al., 2018; Negrel et al., 2018).

A few suggestions about potential resistance biomarkers (or elicitors) as VOCs were found in the metabolite profiles of resistant grapevine species compared to those of representatives of the more susceptible cultivars (Elfert et al., 2013). Volatile sesquiterpenes and monoterpenes were also found in grapevine genotypes after *P. viticola* inoculation in vitro (Algarra Alarcon et al., 2015).

4. GRAPEVINE RESISTANCE

To develop new cultivars with strong and enduring field resistance to several diseases and pests, it is crucial to comprehend genetic resistance. Breeding initiatives are being driven by climate change and a desire to reduce plant protection measures.
In the current grapevine breeding, *V. vinifera* has been crossed with resistance traits from wild *Vitis* species, and interspecific hybrids have been found with resistance against *P. viticola* and *E. necator* (Buonassisi et al., 2017; Frobel and Zyprian, 2019; Merdinoglu et al., 2018; Vezzulli et al., 2018).
It is important to note that the pathogen still manages to complete its life cycle in these hybrids, although it produces fewer sporangia than on sensitive cultivars (Bellin et al., 2009).
More virulent strains of the pathogen can overcome the protection provided by these resistance genes (R genes), particularly in genotypes bearing one *Rpv* gene (Peressotti et al., 2010; Merdinoglu et al., 2018; Fröbel et al., 2019). Therefore, longer-lasting disease resistance is essential to avoid such resistance breakdowns (Merdinoglu et al., 2018; Stam and McDonald, 2018).
Pyramiding resistance is a proposed approach that involves accumulating numerous resistance (R)-loci from diverse genetic sources in the same variety to prevent resistance breakdown by a specific pathogen. Over the last several decades, extensive research to find R-loci against *E. necator* (previously *U. necator*) and *P. viticola* has resulted in a significant number of R-loci adopting a stacking ("pyramiding") strategy in breeding (Töpfer et al., 2011; Dry et al., 2019).
At present, 31 grapevine genomic areas have been linked to downy mildew resistance (*Rpv* loci) (Ruiz –Garcia, 2021) and 14 to powdery mildew resistance (*Run* and *Ren* loci); a descriptive list of them is available online (www.vivc.de/loci) (Possami et al., 2021).

The availability of markers that identify the presence of these loci may enable marker-assisted selection (MAS) of potentially resistant genotypes (Eibach et al., 2007; Kozma et al., 2009; Vezzulli et al., 2019; Zini et al., 2019).

5. "OMICS" APPROACHES TO STUDY THE GRAPEVINE – DOWNY AND POWDERY MILDEW INTERACTION

The new emerging technologies such as next-generation sequencing/genomics, QTLomics, transcriptomics, proteomics, and metabolomics, in association with comparative studies, are revealing insights on the early host responses to DM and PM attack as well as the complex plant defensive mechanisms. Multi-omics or integrated omics are the terms used to describe the recent combining and integration of various omics on a single sample or material. The development of analytical tools like the mass spectrometer (MS) and next-generation DNA sequencing (NGS) has helped the advancements in omics studies.

These comprehensive studies have been applied to model plant research in recent decades and they refer to large-scale molecular analyses of several genes, gene products, or gene regions in case of genomics; they study the full set of RNAs in transcriptomics and proteins derived from the genome in proteomics. The study of the metabolome, which is the collection of tiny molecules produced by a cell, tissue, or organism under specific conditions is analysed trough metabolomics, as well as the full lipid profile found in a cell, tissue, organism, or ecosystem, which is a subset of the "metabolome".

The use of metabolomics is the best way for exploring the interaction between the grapevine-*P. viticola* and *E. necator* and expanding current understanding about the perturbations of a wide range of molecules during biotic stress. Understanding how resistant and susceptible grapevine types react to the two infections may lead to the discovery of pathogen resistance-associated biomarkers that can provide a holistic explanation of the incompatible interactions and provide significant information for breeding.

Biomarkers are physiologically relevant chemicals that are created or released during pathogen-host interactions. Upon pathogen infection, such chemicals may be found first among all of the key classes of metabolites in grapevine leaves—phenolics, organic acids, terpenoids, and lipids (Vezzulli, 2021). There is a large amount of research on the pathosystem grapevine-*P. viticola*, including our studies. On the contrary, the pathosystem grapevine-*E. necator* is still little understood, and our research contributed to putting some further light on it.

The focus of the metabolomics research done to comprehend the mechanism of grapevine defense was DM. There have been studies that have concentrated on numerous elements, including the differences in berry composition between grapevine varieties in certain cases (Mulas et al., 2011; Degu et al., 2014; Teixeira et al., 2014; Bavaresco et al., 2016), and the discovery of metabolite alterations in infected leaves in others (Ali et al., 2012). Some studies focused on the metabolomic analysis of DM-infected grapevine tissues (Figueiredo et al., 2008; Ali et al., 2009; Buonassisi et al., 2017) and metabolite changes caused by the mono-locus resistance mechanism (Chitarrini et al., 2017, 2020). There is little metabolomics data available to help understand grapevine resistance to PM. There has recently been research underlying the synergy between metabolomics and various omics techniques (Maia et al., 2020; Pimentel et al., 2021; Yin et al., 2022), metabolic changes in berry and leaf composition in numerous grapevine cultivars (Atak et al., 2021; Rienth et al., 2021), and disease control (Gur et al., 2022). In general, there is a greater need for studies that use metabolomics to contribute to and better understand plant defense mechanisms against *E. necator*. To date, our research has contributed to a better understanding of the downy and powdery mildew-induced metabolic alterations in grapevine genotypes with one or more resistance loci.

CHAPTER II

MONO-LOCUS AND PYRAMIDED RESISTANT GRAPEVINE CULTIVARS REVEAL EARLY PUTATIVE BIOMARKERS UPON ARTIFICIAL INOCULATION WITH *PLASMOPARA VITICOLA*

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Mono-Locus and Pyramided Resistant Grapevine Cultivars Reveal Early Putative Biomarkers Upon Artificial Inoculation With *Plasmopara viticola*

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One of the most economically important grapevine diseases is Downy mildew (DM) caused by the oomycete *Plasmopara viticola*. A strategy to reduce the use of fungicides to compensate for the high susceptibility of *V. vinifera* is the selection of grapevine varieties showing pathogen-specific resistance. We applied a metabolomics approach to evaluate the metabolic modulation in mono-locus resistant genotypes carrying one locus associated with *P. viticola* resistance (*Rpv*) (BC4- *Rpv1*, Bianca- *Rpv3-1*, F12P160- *Rpv12*, Solaris- *Rpv10*), as well as in pyramided resistant genotypes carrying more than one *Rpv* (F12P60- *Rpv3-1; Rpv12* and F12P127- *Rpv3-1, Rpv3-3; Rpv10*) taking as a reference the susceptible genotype Pinot Noir. In order to understand if different sources of resistance are associated with different degrees of resistance and, implicitly, with different responses to the pathogen, we considered the most important classes of plant metabolite primary compounds, lipids, phenols and volatile organic compounds at 0, 12, 48, and 96 h post-artificial inoculation (hpi). We identified 264 modulated compounds; among these, 22 metabolites were found accumulated in significant quantities in the resistant cultivars compared to Pinot Noir. In mono-locus genotypes, the highest modulation of the metabolites was noticed at 48 and 96 hpi, except for Solaris, that showed a behavior similar to the pyramided genotypes in which the changes started to occur as early as 12 hpi. Bianca, Solaris and F12P60 showed the highest number of interesting compounds accumulated after the artificial infection and with a putative effect against the pathogen. In contrast, Pinot Noir showed a less effective defense response in containing DM growth.

Keywords: downy mildew, metabolomics, mono-locus, pyramided, resistance

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INTRODUCTION

Grapevine was among the first fruit species to be domesticated and today represents one of the most important crops in the world, with an essential role in the economy of many countries. Unfortunately, viticulture is threatened by numerous pathogens causing severe harvest losses. One of the most destructive diseases affecting grapevine is Downy mildew (DM), caused by the biotrophic pathogen *Plasmopara viticola*. DM affects the members of the family *Vitaceae* and in particular the cultivated species *Vitis vinifera* and it can attack all green parts of the vine (leaves, fruits, and shoots in particular) (Buonassisi et al., 2018; Vezzulli et al., 2018). DM infection leads to significant crop losses due to defoliation and to the production of low-quality, deformed or entirely damaged grapes (Yildirim et al., 2019; Nogueira Júnior et al., 2020). The most distinctive signs of infection are the sporangia formation apparent as whitish spots, commonly found on the abaxial surface of the first-formed leaves, which are accompanied by chlorotic spots (known also as oil spots) on the adaxial surface. The sporulation requires humidity > 93% and temperatures of 18–20°C and it can be observed on the abaxial side of the leaf and the surface of tendrils, inflorescence, and young berries (Buonassisi et al., 2018).

The application of large amounts of fungicides is the most diffused strategy to control DM, this practice, however, is not only expensive and in conflict with the requirements for sustainable and environment-friendly agriculture, but also promotes the emergence of fungicide-resistant strains (Buonassisi et al., 2018; Merdinoglu et al., 2018; Fröbel and Zyprian, 2019; Yildirim et al., 2019). A possible alternative to the use of fungicides is the valorization of the interspecific hybrids of *V. vinifera* with resistant genotypes from *Muscadinia*, several wild North American and Asian *Vitis* species which have been found resistant against *P. viticola* (Buonassisi et al., 2017; Merdinoglu et al., 2018; Vezzulli et al., 2018; Fröbel and Zyprian, 2019).

The resistance response to *P. viticola* is given by quantitative trait loci (QTLs) named *Rpv* (i.e., resistance to *P. viticola*). To date, 27 quantitative trait loci (QTL) have been identified in wild *Vitis* species and a descriptive list of them is available online (www.vivc.de/data on breeding and genetics/ Table of loci for Traits in Grapevine) (Bellin et al., 2009; Bove et al., 2019; Eisenmann et al., 2019; Vezzulli et al., 2019; Maul et al., 2020; Nogueira Júnior et al., 2020). The protection offered by these resistance genes (*R* genes) can be overcome by virulent strains of the pathogen, particularly in the genotypes carrying one *Rpv* gene (Peressotti et al., 2010; Merdinoglu et al., 2018; Fröbel et al., 2019). To avoid such resistance breakdowns a longer-lasting disease resistance is required. A possible strategy is pyramiding resistance, by accumulating several resistant genes in the same variety to create a durable disease resistance (Merdinoglu et al., 2018; Stam and McDonald, 2018). The study of varieties with different resistance genes can help us explore the mechanisms of resistance in *P. viticola*-grapevine interactions. Thus, this study initially screened four cultivars with mono-locus resistance (BC4, Bianca, F12P160 and Solaris) and subsequently two cultivars with pyramided resistance (F12P60 and F12P127).

Grapevine cultivar Bianca is a Bouvier and Villard Blanc hybrid, created in 1963 at the Kölyuktetö viticulture research facility in Hungary. Its resistance is given by the *Rpv3-1* locus located in chromosome 18 (Bellin et al., 2009). The cultivar Solaris was obtained by crossing the variety Merzling (*Rpv3-3*) with Gm6493 (*Rpv10*). It was created at the Geisenheim grape-breeding Institute (Germany) and is a carrier of resistance locus *Rpv10* that maps to chromosome 9 (Schwander et al., 2012). Both varieties are officially registered for use in wine production (http://www.vivc.de/). The resistance of the F12P160 genotype is explained by the *Rpv12* locus, located in chromosome 14 (Venuti et al., 2013). The cultivar BC4 was created in 2017 at INRA (France) as a cross between *Muscadinia* (*Rpv1*) X Regent (*Rpv3-1*). The *Rpv1* locus is responsible for its resistance and it maps to chromosome 12 (Merdinoglu et al., 2003). None of the two hybrids are officially registered for use in wine production. The latest cultivars, F12P60 and F12P127, are two pyramided hybrids created at Fondazione Edmund Mach (Italy). *Rpv3-1* and *Rpv12* are responsible for the resistance in cultivar F12P60 and they map to chromosomes 18 and 14, respectively (Bellin et al., 2009; Venuti et al., 2013). The resistance loci *Rpv3-1*, *Rpv3-3*, and *Rpv10* map to the chromosomes 18 and 9 and are engaged in the resistance of the cultivar F12P127 (Bellin et al., 2009; Di Gaspero et al., 2012; Schwander et al., 2012). Both varieties are not yet registered for cultivation.

Information about the different behavior of resistant and susceptible varieties coming from several cultivars is useful to understand the protection mechanisms involved in resistance to *P. viticola*. The plasticity of the plants in response to the pathogen is probably associated with the modulation of several classes of primary and secondary metabolites. For this reason, metabolomics is the most suitable approach in exploring the interaction between the grapevine and *P. viticola* and in extending the current knowledge about the perturbations of a wide range of molecules after biotic stress. To date, metabolomics studies have focused on several aspects: the differences between grapevine cultivars in berry composition in some cases (Mulas et al., 2011; Degu et al., 2014; Teixeira et al., 2014; Bavaresco et al., 2016), and the identification of metabolite changes in infected leaves in others (Ali et al., 2012). Some works focused on the metabolomic profiling of grapevine tissues infected with DM (Figueiredo et al., 2008; Ali et al., 2009; Buonassisi et al., 2017) and on metabolite changes due to the mono-locus resistance mechanism (Chitarrini et al., 2017, 2020). However, there is not yet a full description of which metabolites play a key role in resistance in the pyramiding resistance cultivars. This suggests the need to investigate further to identify the biomarkers of the defense response in resistant varieties.

In this study, we chose to examine first the reaction of primary and secondary metabolism of genotypes with mono-locus resistance against DM, and then we extended our investigation to the analysis of pyramided resistance genotypes. Among the hundreds of compounds identified, we decided to focus on those metabolites (not stilbenes and stilbenoids) that showed significant accumulation in resistant vs. susceptible genotypes over the course of the infection, and that can therefore be identified as putative markers of resistance. Within the class

of stilbenes and stilbenoids we decided to investigate not only the putative markers of resistance but also the markers of infection. The aim was to find previously unreported biomarkers of resistance, which are expected to pave the way for a better understanding of the different resistance mechanisms that underlie the hybrids-pathogen interaction affecting the *Vitis* species. All genotypes in the study were observed over 2 consecutive years and examined with a metabolomics approach for primary and secondary metabolism at 0, 12, 48, and 96 h post-inoculation. The assessment of the resistance level after artificial inoculation on leaves was carried out using the OIV-452 method (**Supplementary Table 1**).

MATERIALS AND METHODS

Plant Material and Artificial Inoculation

Grapevine plants with genotypes having different degrees of resistance to DM and one with a susceptible genotype were used in this study. The mono-locus resistance genotypes consisted of the varieties BC4, Bianca, F12P160 and Solaris whereas the pyramided resistance genotypes were F12P60 and F12P127 (**Table 1**). All the grapevine plants were grown in pots in controlled conditions in the Fondazione Edmund Mach grape germplasm collection located in San Michele all'Adige (Trento), Italy (46^0 12′ 0″ N, 11^0 8′ 0″ E). The mono-locus resistance experiment was conducted in the 2 consecutive years 2016 and 2017; while the pyramided resistance experiment was conducted in the 2 consecutive years 2017 and 2018. For each experiment, the susceptible variety Pinot Noir was used as control genotype (**Table 1**).

During the experiment, the healthy plants ($n = 18$ per variety) were divided into two homogeneous groups (control and inoculated); the plants in the same group were further divided into three groups, each one representing one biological replicate (**Figure 1**). Plants were artificially infected with spores of the pathogen in the greenhouse. The inoculum was collected each year in late spring/early summer from naturally infected plants of the same untreated vineyard (grape cultivar: Pinot Noir) and was characterized by a mix of strains. Grapevine plants were inoculated by spraying the sporangial suspension at the rate of $1x10^6$ sporangia/mL on the lower surface of all leaves of plants, whereas the control plants were sprayed using milliQ water. Plants were kept in the greenhouse at a controlled temperature of 21°C and over 80% of relative humidity until the sampling. Leaves were sampled at four time points following a randomization scheme at 0, 12, 48, and 96 h post-inoculation/mock (**Figure 1**). Three biological replicates were sampled at each time point. Each sample was ground under liquid nitrogen and stored at −80°C until the extractions. The OIV-452 score was evaluated at 7 days post-inoculation on the first six fully expanded leaves (**Supplementary Table 1**) to assign a resistance score to *P. viticola* (leaves): 1 = very low 3 = low 5 = medium 7 = high 9 = very high or total. At the same time the Hypersensitive Response (HR) identified by the necrosis spots was evaluated.

Extraction Procedures and Analysis of Compounds

Primary Compounds

Primary compounds were extracted from 100 mg of fresh leaves and then subjected to derivatization using methoxamine hydrochloride in pyridine to inhibit the cyclization of reducing sugars and then with N-methyl-N-trimethylsilyl-trifluoroacetamide with 1% trimethylchlorosilane for trimethylsilylation following the Chitarrini et al. (2017) procedure. The derivatized extract was then injected for GC/MS analysis using a Trace GC Ultra with a fused silica RXI-5-Sil MS w/Integra Guard (30 m × 0.25 mm × 0.25 μm) column, combined with mass spectrometer TSQ Quantum GC (Thermo Electron Corporation) following the Chitarrini et al. (2017) parameters.

Volatile Compounds

Volatile compounds were measured using a solid phase micro-extraction starting from 100 mg of fresh leaves and following the method of Chitarrini et al. (2017). Gas chromatography separation was done using a Trace GC Ultra gas chromatograph with a fused silica Stabilwax-DA column (30 m × 0.25 mm × 0.25 μm) (Restek Corporation) coupled to a Quantum XLS mass spectrometer (Thermo Electron Corporation) following the parameters of Matarese et al. (2014).

TABLE 1 | The genotypes used in this study, their source of resistance and their associated resistance-related loci (*Rpv*) with their references.

Genotypes		Resistance related loci (*Rpv*)			References
		Downy mildew	Preliminary leaf resistance level	Source of resistance	
Mono-locus resistance	BC4	*Rpv1*	Resistant	*M. rotundifolia*	Merdinoglu et al., 2003
	Bianca	*Rpv3-1*	Resistant	*V. rupestris*	Bellin et al., 2009
	F12P160	*Rpv12*	Resistant	*V. amurensis*	Venuti et al., 2013
	Solaris	*Rpv10*	Resistant	*V. amurensis*	Schwander et al., 2012
Pyramided resistance	F12P60	*Rpv3-1; Rpv12*	Resistant	*V. rupestris* *V. amurensis*	Bellin et al., 2009; Venuti et al., 2013
	F12P127	*Rpv3-1, Rpv3-3; Rpv10*	Resistant	*V. rupestris* *V. amurensis*	Bellin et al., 2009; Di Gaspero et al., 2012; Schwander et al., 2012
Control	Pinot Noir	–	Susceptible	–	

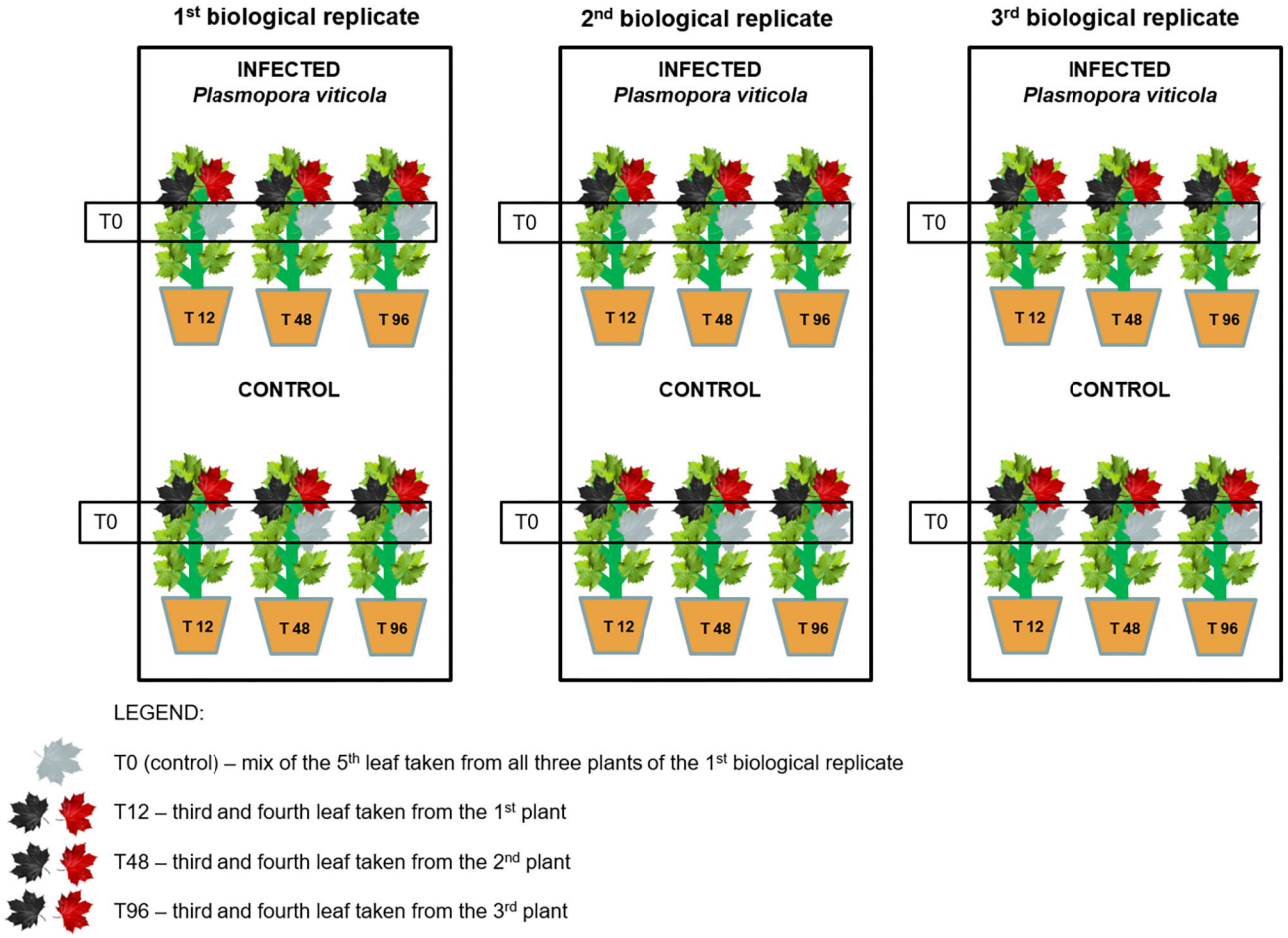

FIGURE 1 | Experimental design and randomization scheme.

Lipidic Compounds

Lipid compounds analysis was done according to Della Corte et al. (2015) following the sample preparation described by Chitarrini et al. (2017). One hundred mg of fresh leaves were extracted using 0.3 mL of methanol; 0.6 mL of chloroform containing butylated hydroxyl toluene (500 mg/L); 0.25 mL water and then with 0.4 mL of chloroform containing butylated hydroxyl toluene (500 mg/L)/methanol/water 86:14:1 v/v/v; the combined lower lipid-rich layer was evaporated to dryness under N_2 and the samples were re-suspended in 300 µl of acetonitrile/isopropanol/water (65:30:5 v/v/v/). Samples were injected into a UHPLC Dionex 3000 (Thermo Fisher Scientific) with RP Ascentis column (15 cm × 2.1 mm; 2.7 µm C18) following a 30 min multi-step gradient coupled with an API 5500 triple-quadrupole mass spectrometer (Applied Biosystems/MDS Sciex) (Della Corte et al., 2015).

Phenolic Compounds

The phenolic compounds were extracted from 100 mg of fresh leaves using 0.4 mL of chloroform and 0.6 mL of methanol:water (2:1); the extraction was repeated by adding 0.6 mL of methanol and water (2:1 v/v) and 0.2 mL of chloroform according to Vrhovsek et al. (2012) with some modifications, previously applied by Chitarrini et al. (2017). The aqueous-methanol phase of two extractions was collected, combined, and evaporated to dryness under N_2. Samples were re-suspended in 500 µl of methanol: water (1:1 v/v) and injected in a Waters Acquity UPLC system (Milford) with a Waters Acquity HSS T3 column (10 mm × 2.1 mm; 1.8 µm) coupled with a Xevo triple-quadrupole spectrometer (Waters) following Vrhovsek et al. (2012).

Data Processing and Statistical Analysis

Data processing of primary and volatile compounds was performed using the software "Xcalibur" (version 4.0), whereas "Analyst" (version 1.7) and "MassLynx" (version 4.1) were used for processing lipids and phenols, respectively.

Lipid, phenols and primary compounds were identified using reference standards, retention time, quantifier and qualifier ion, and quantified using their standard calibration curves as mg/kg of fresh leaves. Volatile organic compounds were identified in the mass spectral database NIST MS Search 2.3 and results were semi

quantified as the equivalent of the internal standard (1-heptanol) and expressed as μg/kg of fresh leaves.

Statistical analysis and visualization were performed with R (R Core Team, 2020) relying on the following packages: tydiverse (Grolemund et al., 2019; Wickham et al., 2019) and egg (Baptiste, 2019) for data handling, manipulation and visualization; emmeans packages (Russell, 2020) for marginal means estimations; effsize for the effect size calculation (Sawilowsky, 2009; Torchiano, 2020). Logarithmic transformation was used to correct for the expected non-normality of metabolomics data. The average effect of each year was subtracted for each metabolite/genotype, to compensate for the expected year-to-year variability in the overall metabolic response. A linear modeling approach was used for each metabolite/genotype to assess the effects of time and artificial inoculationt (inoculated and non-inoculated). Cohen's d was used to estimate the size of the metabolic modulation induced by the pathogen inoculation for each time point. A metabolite was considered significantly perturbed if its concentration in the inoculated samples was significantly different from the control plants at least at one time point (uncorrected $p < 0.05$).

RESULTS

Dynamics of Metabolic Perturbations in Plant Defense Mechanism

In the 2 years considered, 264 compounds were identified in leaf samples under investigation. Among these, we quantified 175 compounds belonging to several classes: organic acids (29), amino acids (17), amines and others (12), sugars (25), benzoic acids derivatives (6), coumarins (3), dihydrochalcones (1), flavan-3-ols (11), flavanones (2), flavones (4), flavonols (15), phenylpropanoids (5), stilbenes and stilbenoids (13), fatty acids (15), glycerolipids (4), glycerophospholipids (2), prenols (1), sphingolipids (1), sterols (2) and other phenols (7). We semi-quantified 89 volatile organic compounds: volatile acids (5), alcohols (14), aldehydes (13), benzenoids (6), esters (3), hydrocarbons (1), other volatiles (6), fatty acids (2), benzofurans (1), terpenoids (10), terpenes (10), ketones (4) and unknown volatiles (14). In **Supplementary Table 2** the concentrations of VOCs (sheet 1) lipids (sheet 2) and polyphenols (sheet 3) identified as putative markers of resistance following the criteria described in section Putative Biomarkers of Resistance to *Plasmopara viticola* and The Effect of Pathogen Inoculation have been reported together with stilbenes and stilbenoids involved in the response to the infection and fight against the pathogen (sheet 3; see section Putative Biomarkers of Resistance to *Plasmopara viticola* and Stilbenes and Stilbenoids as Markers) for each genotype and for each year (**Supplementary Table 2**).

Putative Biomarkers of Resistance to *Plasmopara viticola*

A global view of the metabolites that showed a significant effect after inoculation ($p < 0.05$ in at least one time point) is presented for the mono-locus resistant genotypes (BC4, Bianca, F12P160, Solaris) in **Figure 2** and for the pyramided resistant genotypes (F12P60, F12P127) in **Figure 3**.

In the plots, the dots indicate in which genotype(s) each metabolite was showing a significant difference in the inoculated vs. non-inoculated samples at least at one time point. This global visualization highlights that the resistant varieties Bianca, Solaris and F12P60 are showing a higher number of modulated metabolites. On the other hand, BC4 and F12P127 seem to show a more limited response to inoculation. The OIV-452 score was evaluated in the experiments (**Supplementary Table 1**) showing a very high degree of resistance for Bianca, F12P160, F12P60 and F12P127 (OIV-452 = 9); a high level for Solaris (OIV-452 = 7), medium for BC4 (OIV-452 = 5) and very low for Pinot Noir (OIV-452 = 1). At the same time, the hypersensitive response (HR) taking into account the necrosis was evaluated following the OIV-452 score. HR response was absent in Pinot Noir, medium in F12P160 and Bianca and high in BC4 and Solaris. For the pyramided genotypes, F12P127 was characterized by an high level of HR response, whereas the HR response was absent in F12P60 (**Supplementary Table 1**).

Figures 2, **3** clearly show that the interaction with the pathogen profoundly alters the plant metabolism, and some of the metabolites appear modulated after the artificial inoculation in both resistant genotypes and the susceptible Pinot Noir.

In order to pinpoint the most promising compounds, we defined the following for potential resistance biomarkers:

for metabolites excluding stilbenes and stilbenoids:

1. the metabolite was showing a significant modulation only in the resistant genotypes and, in addition, it was showing a large positive modulation (effect size d > 1) at the last two time points.

for stilbenes and stilbenoids:

1. the metabolite was showing a significant modulation only in the resistant genotypes and, in addition, it was showing a large positive modulation (effect size d > 1) at the last two time points (see section Stilbenes and Stilbenoids as Markers);
2. if modulated also in Pinot Noir, the metabolite was showing an effect size with a delta d > 1 compared with Pinot Noir (see section Stilbenes and Stilbenoids as Markers).

In the case of non-stilbenoids, we acknowledge that the magnitude and the timing of the accumulation of a compound could be important in characterizing the response of the plant to the pathogen attack (Pezet et al., 2004; Chitarrini et al., 2017), but the presence of a significant modulation also in Pinot Noir suggests that this metabolite is actually associated with infection. The second part of the first criterion (d > 1 in the last two time points), instead, stemmed from the hypothesis that the presence of the pathogen in the inoculated leaves was the main cause for the accumulation of the metabolites over time. In the case of stilbenoids, a more liberal criterion was applied since this class of compounds is known to hold a prominent role in the response of *V. vinifera* to pathogen infection; for these reasons we considered also those compounds with an effect size in the inoculated conditions with a delta d > 1 compared with Pinot Noir.

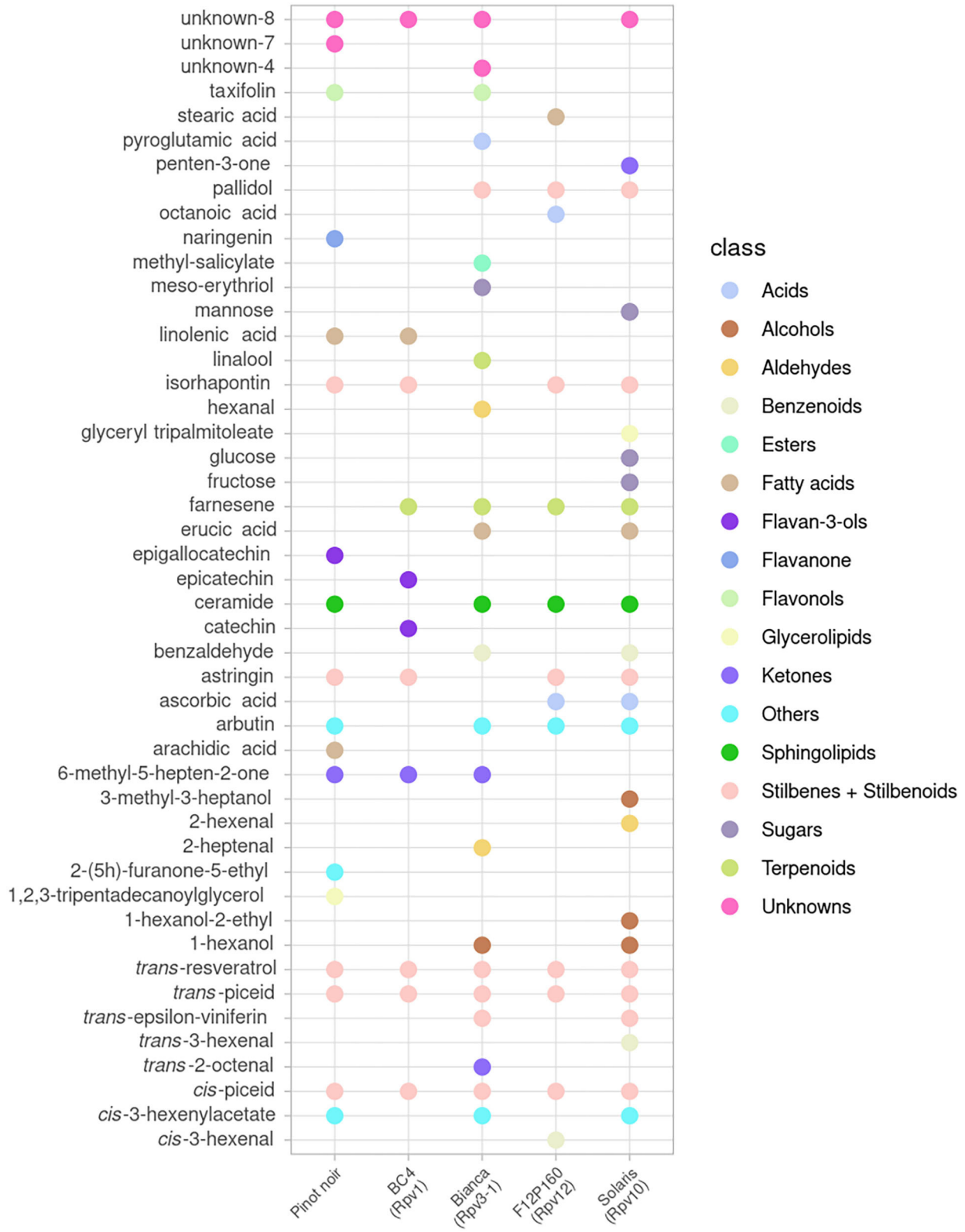

FIGURE 2 | Metabolites significantly modulated by the infection in at least one-time point for mono-locus resistant genotypes (BC4, Bianca, F12P160, Solaris) and for the susceptible Pinot Noir. All time points were considered in the 2 years of data analysis (2016–2017) and the color of each metabolite identifies the different chemical classes.

FIGURE 3 | Metabolites significantly modulated by the infection in at least one time point for the pyramided resistant genotypes (F12P60, F12P127) and for the susceptible Pinot Noir. All time points were considered in the 2 years of data analysis (2017–2018) and the color of each metabolite identifies the different chemical classes.

The Effect of Pathogen Inoculation

The previous criteria led to the identification of 20 compounds, excluding the stilbenes and stilbenoids class (discussed in section Stilbenes and Stilbenoids as Markers), as putative biomarkers of resistance belonging to the plant primary metabolism: fatty acids (4) and secondary metabolism: flavan-3-ols (1), alcohols (4), aldehydes (2), benzenoids (1), benzoic acid esters (1), terpenoids (4), esters (1), and unknown volatiles (2) (**Table 2**). The concentrations of these compounds of interest are reported in **Supplementary Table 2**.

In order to discuss the strength of the modulation induced by the pathogen, the effect size (Cohen d) was calculated for each putative biomarker and for each time point (0, 12, 48, 96 hpi). According to the study of Sawilowsky (2009), the "d" values are associated with an effect size which can vary from a very small ($d = 0.01$) to a huge effect ($d = 2.0$). The "d" values of the identified putative biomarkers and their associated effect size are being presented in the **Supplementary Table 3**.

BC4

In the resistant genotype BC4 we identified two compounds as putative biomarkers; one phenol, epicatechin, and one volatile, farnesene. Catechin and epicatechin have been recently identified as discriminatory factors, with a significantly higher amount in resistant/partial resistant plants (Maia et al., 2020). In our experiment, the effect size of epicatechin strongly grew at 48 and 96 hpi (1.99 and 1.64). Farnesene, instead, showed a higher and rapid accumulation after 12 hpi with high d values at 48 and 96 hpi (5.15 and 2.78) (**Figure 4**).

Bianca

In the resistant genotype Bianca, six VOCs have been identified as potential biomarkers: 1-hexanol, erucic acid, benzaldehyde, farnesene, linalool, methyl-salicylate (**Figure 5**). In five of them we found an accumulation with a positive effect at both 48 and 96 hpi. The effect size of inoculation for 1-hexanol increased at 48 and 96 hpi, where it reached a positive effect (1.77 and 1.53, respectively). Linalool started increasing at 48 hpi (1.60), reaching a positive effect (3.05) at 96 hpi. Farnesene increased showing an effect size of 1.56 at 48 hpi and reaching a huge effect size of 4.23 at 96 hpi. The last two significant compounds of this resistant genotype, benzaldehyde, and methyl salicylate kept a positive effect immediately after the inoculation reaching an effect size at 48 and 96 hpi (benzaldehyde 1.66 and 3.16 at 48 and 96 hpi; methyl salicylate 2.96 and 2.61 at 48 and 96 hpi). Benzaldehyde was present also in F12P60 and Pinot Noir for 2017–2018, whereas methyl salicylate was detected in Pinot Noir for 2017–2018. Since in these cases the effect of the inoculation was much smaller, they remain putative biomarkers of resistance as initially assumed. Erucic acid reaches a peak with positive effect at 96 hpi (3.32).

TABLE 2 | Potential biomarkers among all metabolite classes except stilbenes and stilbenoids as identified by the selection criterion—modulation only in the resistant genotypes ($d > 1$).

Class of the compounds	Compounds	GENOTYPES					
		Mono-locus resistance				Pyramided resistance	
		BC4 (*Rpv1*)	Bianca (*Rpv3-1*)	F12P160 (*Rpv12*)	Solaris (*Rpv10*)	F12P60 (*Rpv3-1; Rpv12*)	F12P127 (*Rpv3-1, Rpv3-3; Rpv10*)
Fatty acids	erucic acid		•				
	oleic acid + *cis*-vaccenic acid					•	
	palmitic acid					•	
	stearic acid			•			
Flavan-3-ols	Epicatechin	•					
Alcohols	1-hexanol		•		•	•	
	1-hexanol-2 ethyl				•		
	(*E*)-2 hexenol					•	
	1-octen-3-ol					•	
Aldehydes	2-hexenal				•		
	nonanal						•
Benzenoids	benzaldehyde		•		•	•	
Benzoic acid esters	methyl salicylate		•				
Terpenoids	farnesene	•	•	•			•
	linalool		•				
	(*E*)-nerolidol					•	
	neral					•	
Esters	*cis*-3-hexenyl benzoate					•	
Unknowns VOCs	unknown 4					•	
	unknown 13					•	

F12P160

For the resistant genotype F12P160, we identified farnesene and stearic acid in our inclusion criteria list. **Figure 6** highlights the interesting accumulation of farnesene with an increase of the effect size at 48 and 96 hpi (2.12 and 2.03, respectively).

Solaris

In the resistant genotype Solaris, we identified four compounds: 1 hexanol, 1-hexanol-2-ethyl, 2-hexenal and benzaldehyde (**Figure 7**). All the four metabolites are accumulated at 48 hpi with a peak at 96 hpi and an effect size of 2.33, 1.61, 1.97 and 3.65.

F12P127

The pyramided genotype F12P127 revealed two compounds in the inclusion criteria list, farnesene and nonanal (**Figure 8**). Farnesene was accumulated at 48 and 96 hpi with an effect size of 3.28 and 2.88, while nonanal showed an unclear trend among the time with an effect size of 1.48 and 1.10 at 48 and 96 hpi.

F12P60

In F12P60 pyramided genotype, we identified eleven potential biomarkers (**Table 2**) in total. Benzaldehyde, as for F12P127 and Bianca genotypes, increased reaching an effect size of 4.92 and 4.76 at 48 and 96 hpi. Similar trends were found for (*E*)-2-hexenol (2.97 at 96 hpi) and 1-hexanol (2.74 at 96 hpi). The two terpenoids (*E*)-nerolidol and neral are accumulated after 24 hpi, with a peak at 48 hpi for (*E*)-nerolidol (2.17) and at 96 hpi for neral (2.27), respectively. Finally, we found a lipid compounds accumulation: oleic acid+*cis*-vaccenic and palmitic acid have an accumulation trend over time with an effect size of 7.01 at 48 hpi for palmitic aid and 4.44 and 4.5 for oleic acid+*cis*-vaccenic at 48 and 96 hpi (**Figure 9**).

Stilbenes and Stilbenoids as Markers

Following the described criteria (see section Putative Biomarkers of Resistance to *Plasmopara viticola*), we found six significant compounds (**Table 3**); among them, pallidol and *trans*-epsilon-viniferin were the only compounds not modulated in Pinot Noir (for the concentrations seen **Supplementary Table 2** sheet 3). Pallidol reached the first criteria for stilbenes and stilbenoids in Bianca (effect size of 3.45 at 96 hpi), F12P160 (1.30 at 48 hpi and 3.07 at 96 hpi), and Solaris (2.02 at 48 hpi and 6.47 at 96 hpi); looking at the trend figures we found a comparable reaction in BC4 without a significant effect size (**Supplementary Figure 1**). The same situation is reported for *trans*-epsilon-viniferin, that reached the selected criteria in Bianca (3.17 at 96 hpi) and Solaris (1.19 at 48 hpi and 6.61 at 96 hpi) and reacted with a similar trend in F12P160 and BC4 but not with a significant effect size (**Supplementary Figure 1**).

The monomer *trans*-resveratrol was identified as significant in mono-locus resistant genotypes and in Pinot Noir comparing inoculated vs. not inoculated samples (**Supplementary Table 2**); anyhow, in mono-locusresistant genotypes the effect size was higher with a delta d > 1 compared with Pinot Noir. In the mono-locus genotypes the effect size had d > 1 already at 48 hpi with a peak at 96 hpi (Bianca 2.82; F12P160 3.41; Solaris 4.07; BC4 2.02); instead, in Pinot Noir we found an effect size of 1.5 at 96 hpi. As previously reported, *trans*-resveratrol has been identified as a monomer and precursor of active compounds in biotic stress plant defense (Langcake and Pryce, 1977; Jeandet et al., 2002). *trans*-Piceid, and *cis*-piceid were

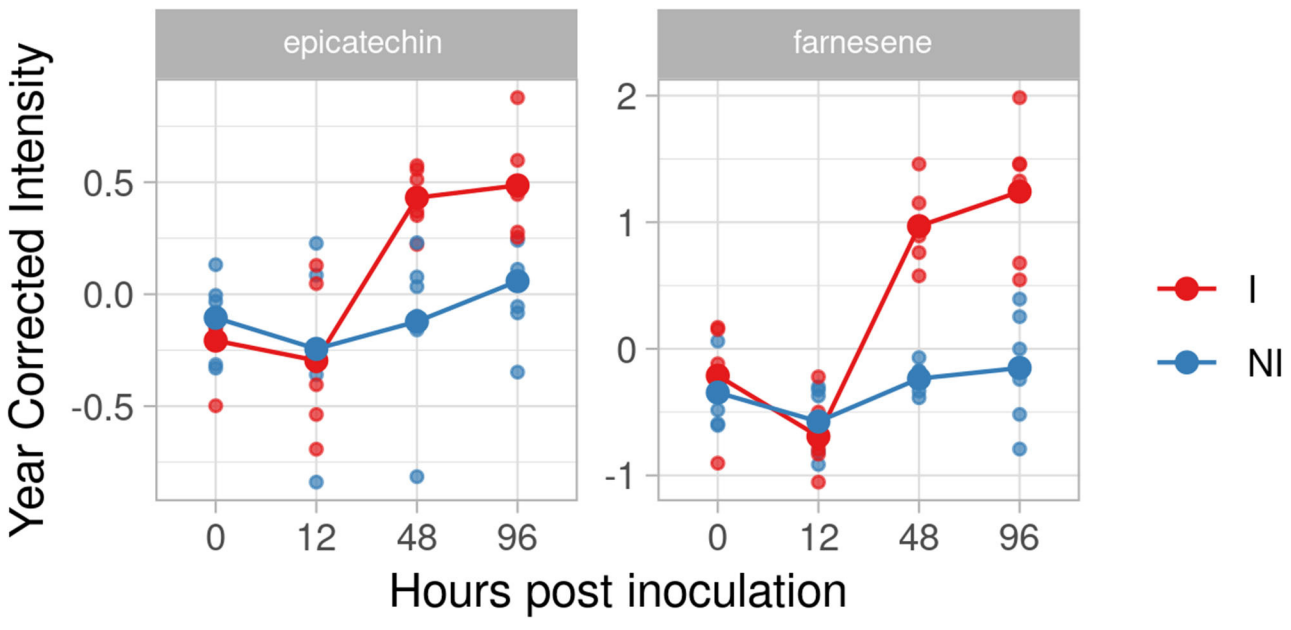

FIGURE 4 | Graphs for specific putative biomarkers of resistance to *Plasmopara viticola* in inoculated (Red) and not inoculated (Blue) BC4 genotype. Values of the 2 years are reported after subtracting the year's effect. I, inoculated; NI, not inoculated.

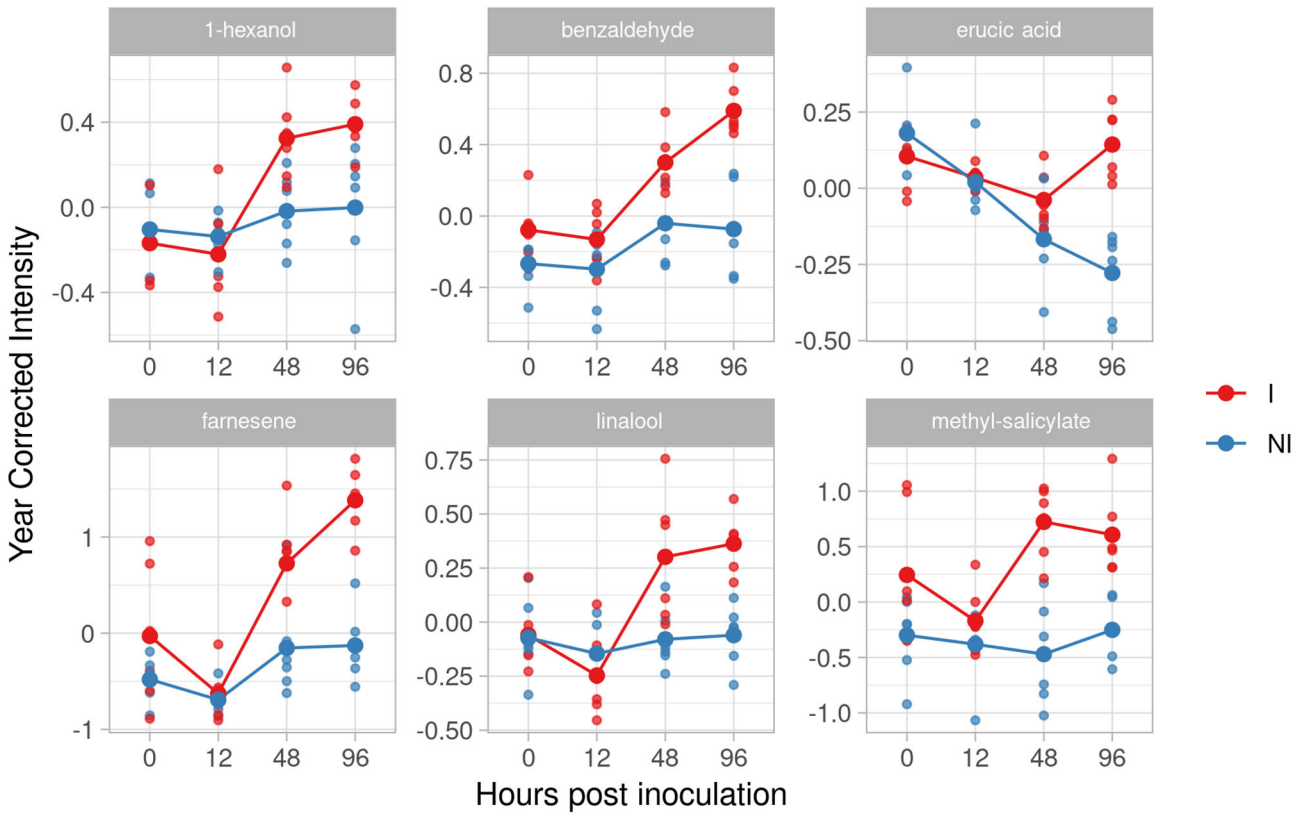

FIGURE 5 | Graphs for specific putative biomarkers of resistance to *Plasmopara viticola* in inoculated (Red) and not inoculated (Blue) Bianca genotype. Values of the 2 years are reported subtracting the year's effect. I, inoculated; NI, not inoculated.

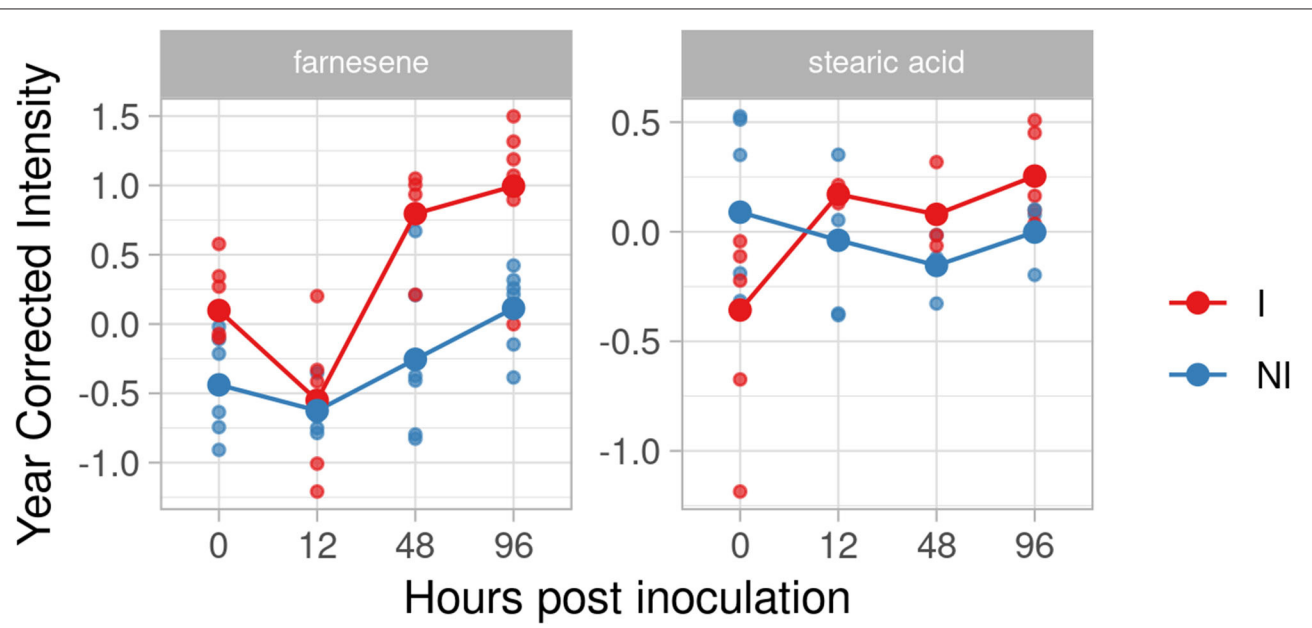

FIGURE 6 | Trend graph over time of putative biomarkers of resistance to *Plasmopara viticola* in F12P160 genotype inoculated (Red) and not inoculated (Blue). Values of the 2 years are reported subtracting the year's effect.

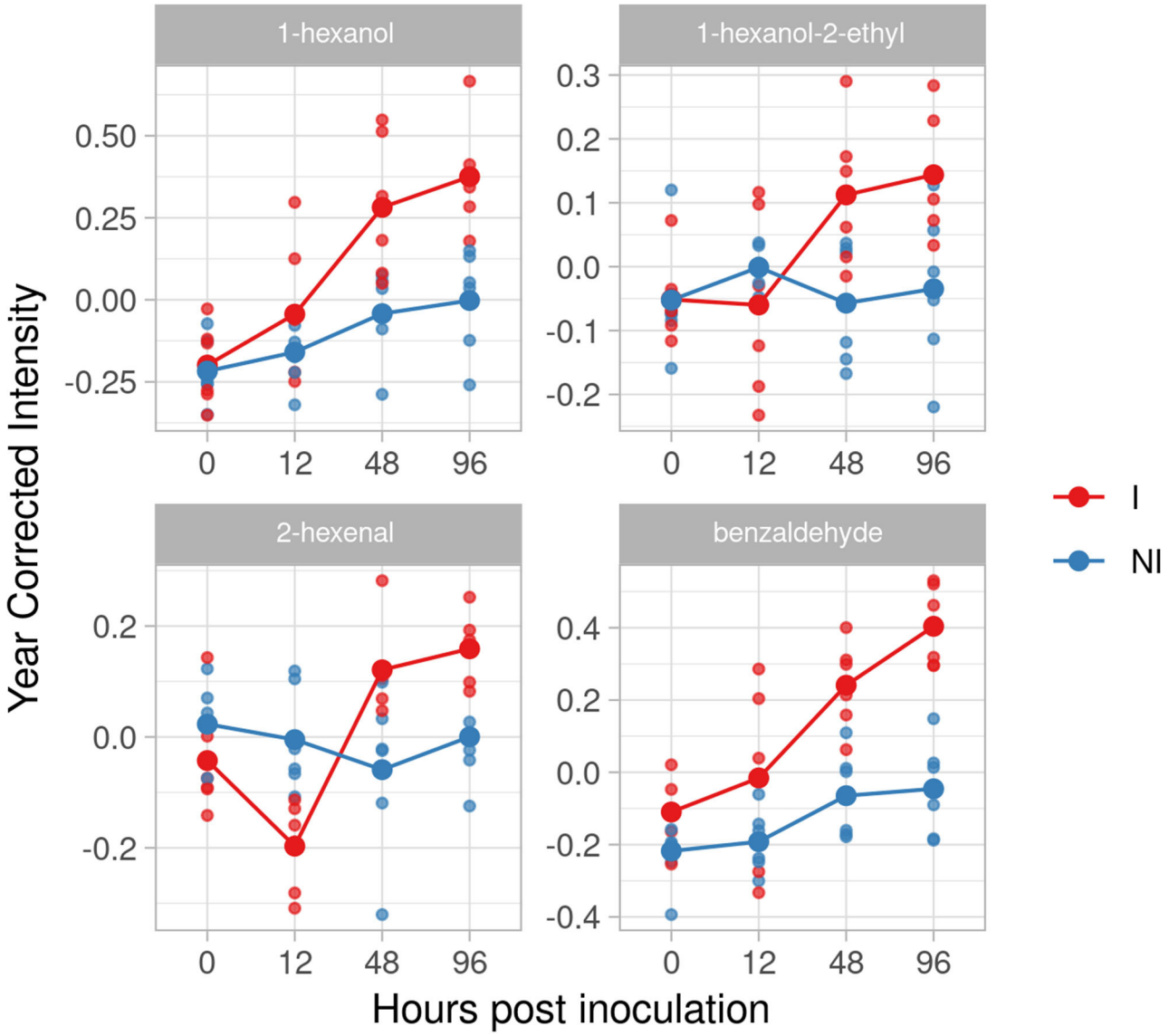

FIGURE 7 | Graphs for specific putative biomarkers of resistance to *Plasmopara viticola* in inoculated (Red) and not inoculated (Blue) Solaris genotype. Values of the 2 years are reported subtracting the year's effect.

identified as highly significant both in the mono-locus resistant varieties and in Pinot Noir and in the pyramided genotype F12P127 (**Figures 2**, **3**; **Supplementary Figures 1**, **2**). The effect size values showed an accumulation ($d > 1$) of these two compounds at 48 hpi and 96 hpi in the resistant genotypes while in Pinot Noir the accumulation has appeared only at 96 hpi (**Supplementary Table 3**). Astringin was significantly modulated in F12P160 (1.95 at 48 hpi and 1.72 at 96 hpi) and Solaris (1.50 at 48 hpi and 2.85 at 96 hpi) genotypes together with Pinot Noir (1.68 at 96 hpi). The trend of these compounds suggests a role in the response to biotic stress, supported by an early accumulation in the resistant genotypes compared to the susceptible one, but they are probably not directly involved in the defense against the pathogen. We are hypothesizing their modulation confirms that the artificial inoculation of the pathogen was successful. All the identified compounds increased with time after pathogen inoculation and their peak concentration was measured at 48 and 96 hpi (**Supplementary Figures 1**, **2**). The different behavior noticed for the phytoalexins agrees with the reports of Ali et al. (2010, 2012) who found that grapevine-specific phytoalexins can also be produced by the susceptible cultivars upon infection if we consider that at the beginning of the inoculation process the metabolic differences might be acting as the first inducible line of defense. Interesting accumulation was found for pallidol, and *trans*-epsilon-viniferin in all mono-locus genotype, with a significant effect size of these active compounds especially in Solaris at 48 and 96 hpi; these results confirm the importance of dimers biosynthesis and their accumulation in resistance

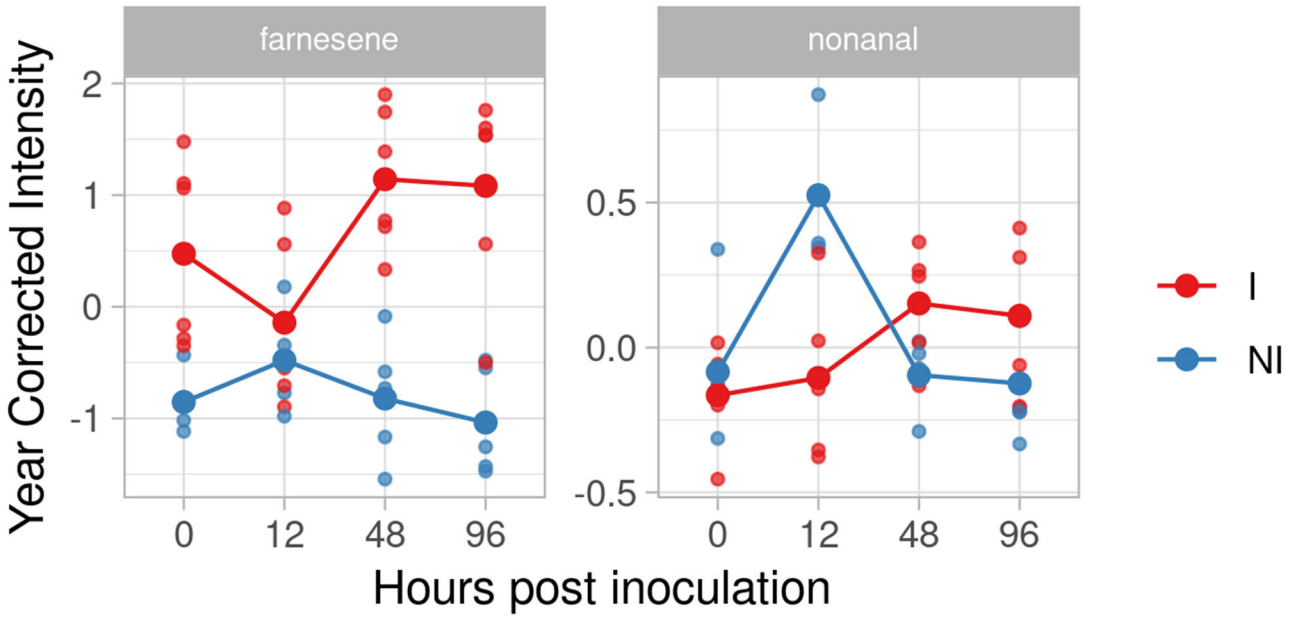

FIGURE 8 | Graphs for specific putative biomarkers of resistance to *Plasmopara viticola* in inoculated (Red) and not inoculated (Blue) F12P127 genotype. Values of the 2 years are reported subtracting the year's effect.

process (Malacarne et al., 2011; Bavaresco et al., 2012; Fröbel et al., 2019).

DISCUSSION

In time, plants have developed different mechanisms of defense against abiotic and biotic stress. Among these mechanisms, the one between grapevine and *P. viticola* still raises questions concerning the interaction between the pathogen and the metabolism of the plant. It is already known that secondary metabolism has a defensive role against predators, parasites and diseases (Ali et al., 2010), but we shouldn't overlook the role of primary metabolism which, besides controlling the growth, development, and reproduction of plant species, also contributes to the plant defense. It can act as a source of energy, and it can signal molecules to directly or indirectly trigger defense response. This study showed findings of putative biomarkers in primary and secondary metabolism within resistant genotypes, as a defense response to *P. viticola.*

In the present 2-year study, we were able to use four analytical methods to identify and quantify or semi-quantify a large number of metabolites covering the most important compound classes. Among the extensive amount of obtained data, we arbitrarily choose to focus our investigation on the metabolites showing the most significant differences between inoculated vs. not inoculated samples, considering the time points with the criterion described in section Putative Biomarkers of Resistance to *Plasmopara viticola*.

An interesting aspect was observed in the alterations of the metabolism of most of the varieties, but mainly in mono-locus resistant genotypes. Several compounds identified as resistance putative biomarkers had their concentration reduced until 12 h after inoculation, followed by an increase at later time points. A similar reaction to the inoculation with DM was described by Ali et al. (2012) for quercetin-3-O-glucoside, glutamic acid and succinic acid in the resistant genotype Regent (*Rpv3-1*). Although we do not have substantial evidence to explain this behavior, we hypothesize that the pathogen might use these compounds to leak the necessary nutrients from the host cells, right before the activation of the plant defense.

During the infection, the pathogen disturbed the plant metabolism to different degrees. In F12P160 and Solaris, a decrease of the sugars was noticed at 12 h after inoculation, possibly because the pathogen was using them as a source of energy for its proliferation. Although sugars are mainly known in plants as a primary substrate to provide energy during the defense responses, they may also act as signal molecules interacting with the hormonal signaling network to regulate the plant immune system. In their role as plant resistance enhancers, sugars also stimulate the synthesis of flavonoids known as defense-related metabolites (Morkunas and Ratajczak, 2014).

In mono-locus resistant genotypes, the modulation of the metabolites was mainly noticed at 48 hpi and 96 hpi; this clearly indicates that 48 h after inoculation the plant defense mechanisms were active, just like Chitarrini et al. (2017) had noticed in a previous study. Solaris was an exception among the mono-locus resistant genotypes, as it reacted like the pyramided F12P60 genotype, where the modulation of 1-hexanol and benzaldehyde started earlier, between 0 and 12 hpi and reached its peak at 48 or 96 hpi. At the time of the experiments, one *Rpv* resistance gene was described in Solaris (**Table 1**); the latest report of Possamai et al. (2020) and Vezzulli et al.

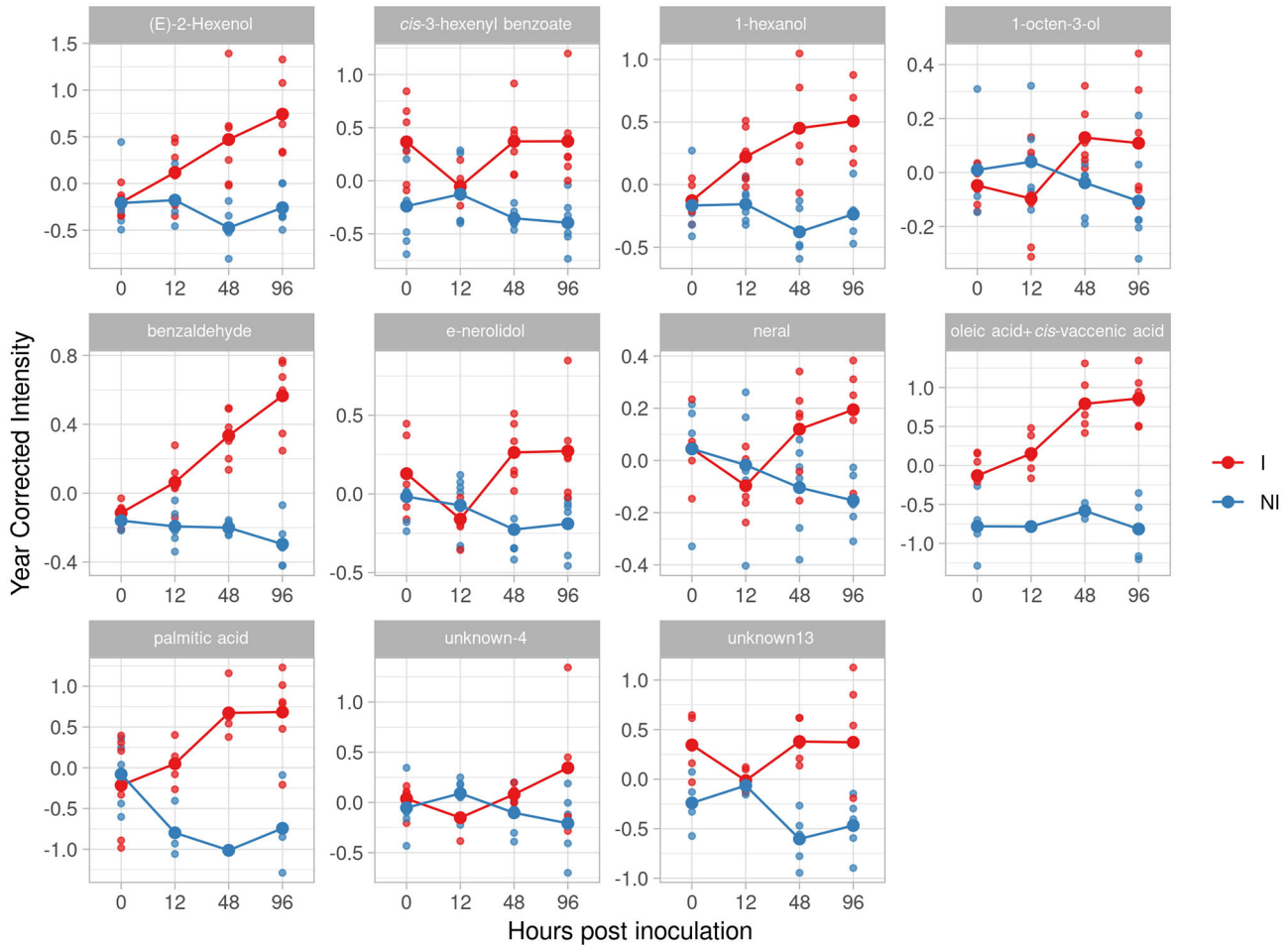

FIGURE 9 | Graphs for specific putative biomarkers of resistance to *Plasmopara viticola* in inoculated (Red) and not inoculated (Blue) F12P60 genotype. Values of the 2 years are reported subtracting the year's effect.

TABLE 3 | Potential biomarkers among stilbenes and stilbenoids as identified by the selection criterion.

Compounds	Genotypes						
	Susceptible	Mono-locus resistance				Pyramided resistance	
	Pinot Noir	BC4 (*Rpv1*)	Bianca (*Rpv3-1*)	F12P160 (*Rpv12*)	Solaris (*Rpv10*)	F12P60 (*Rpv3-1; Rpv12*)	F12P127 (*Rpv3-1, Rpv3-3; Rpv10*)
cis-piceid	•	•	•	•	•		•
trans-piceid	•	•	•	•	•		•
trans-resveratrol	•	•	•	•	•		
pallidol			•	•	•		
trans-epsilon-viniferin			•		•		
astringin	•			•	•		

(2019) reveal the presence of two resistance sources in Solaris (*Rpv3-3* and *Rpv10*), explaining our results and supporting our conclusions. However, additional considerations at the genetic and metabolomics level should be made to fully support that the metabolic changes in Solaris are due to both *Rpv3-3+Rpv10*. The earlier activation of the defense response in the pyramided genotypes could be linked to the fact that the pathogen might take around 12 h to germinate and penetrate the leaf, inducing the first metabolic changes due to its colonization (Chitarrini et al., 2017). Another assumption is the presence of two or more resistance

sources for *P. viticola* within these genotypes. Besides ensuring a higher degree of resistance and a more stable and durable trait (Merdinoglu et al., 2018; Possamai et al., 2020) it could possibly also trigger a faster reaction against the pathogen.

In plants, lipids are energy storage and signaling compounds. In the defense against environmental factors and pathogens, they function as the structural components of cell membranes, which serve as permeable barriers to the external environment of cells. The accumulation of fatty acids (i.e., stearic acid, erucic acid, palmitic acid, oleic acid+*cis*-vaccenic acids) in plant metabolome after pathogen inoculation indicates their action in the adjustment of membrane fluidity mediated by desaturases and in the intracellular signaling processes (Nishida and Murata, 1996; Laureano et al., 2018) and their profile can be also involved in the protection of photosynthetic machinery in the early stages after the inoculation (Laureano et al., 2018). Thus, due to their role in activating the plant defense response, they are proposed as putative biomarkers. In plants, fatty acids have already been reported as important signaling molecules influencing genes involved in plant-microbe and plant-insect interaction (Savchenko et al., 2010; Walley et al., 2013). In previous experiments we found a decrease in oleic acid+*cis*-vaccenic acid together with other unsaturated fatty acids (16:1, 18:2, and 18:3) at the stage of 24 hpi in *Rpv3* and *Rpv12*-mediated resistance genotypes (Chitarrini et al., 2020). Previous studies report that the deactivation of the desaturase which converts stearic acid to oleic acids leads to an upregulation of salicylic acid (SA)-mediated responses and PR genes, with an inhibition of jasmonic acid (JA)-inducible defenses (Kachroo et al., 2008; Mandal et al., 2012). In our experiment we found an increase of palmitic acid and oleic acid+*cis*-vaccenic acid in F12P60; this situation, which is the opposite of what occurs with the mono-locus genotypes Bianca and F12P160, can be related to a different resistance response of the pyramided genotype.

A large variety of volatile compounds was emitted by the plants after the physiological stress induced by the *P. viticola* (green leaf volatiles, benzenoids, terpenoids, and some unknown compounds). This suggests that the secondary metabolism of the plant was seriously affected to a much higher degree by the pathogen. Green leaf volatiles (GLV) produced by the plant are volatile organic compounds that are released when plants suffer stress at the tissue level. Although the plants release GLVs constantly, they do so to a higher extent under conditions of stress (Hammerbacher and Coutinho, 2019). After pathogen inoculation, we identified two classes of GLVs that were released by plant leaves: alcohols and aldehydes. At physiologically relevant concentrations, a defense role of GLVs is suggested by this study based on their antifungal properties (Fallik et al., 1998). Plants are known to release *trans*-3-hexenal within minutes after they experience pathogen stress, and that such release can last for hours, after which it decreases in concentration as it undergoes enzymatic conversion to 2-hexenal (accumulated in our experiment in Solaris at 48 and 96 hpi) and unsaturated alcohols and esters (Davis et al., 2007). Chitarrini et al. (2017, 2020) had already suggested benzaldehyde as a putative biomarker of resistance, thanks to his role as a promoter of salicylic acid (SA)-mediated defense and its significant accumulation in the plant metabolome at 48 and 96 hpi, with an earlier accumulation in *Rpv12*-mediated resistance compared to the *Rpv3*-mediated one. This confirms our findings, and supports benzaldehyde being a biomarker also in the genotypes Solaris and F12P60, where it was found in significantly increased concentrations. Salicylic acid is the phytohormone precursor of the volatile methyl salicylate found in high concentration in the Bianca resistant genotype; in some plants, it is derived directly from the shikimate pathway in the plastids. Methyl salicylate is known for inducing systemic resistance after the attack of biotrophic organisms, like *P. viticola* (Hammerbacher and Coutinho, 2019).

The resistant grapevine genotypes in our study emitted significantly higher concentrations of terpenoids, both monoterpenes (linalool, neral) and sesquiterpenes (farnesene, (*E*)-nerolidol) than the susceptible genotype Pinot Noir. Hammerbacher and Coutinho (2019) found a positive correlation between an increased plant volatile emission and resistance to *P. viticola*. Algarra Alarcon et al. (2015) found a higher emission of sesquiterpenes and monoterpenes in grapevine genotypes resistant to *P. viticola*. Confirming their role in the fight against the pathogen, the antifungal activity of farnesene, and nerolidol together with ocimene and valencene have been recently tested by Ricciardi et al. (2021) showing a positive effect against the pathogen. In our experiment, farnesene was expressed in high concentrations in three mono-locus resistant genotypes (BC4, Bianca, F12P160) and included in the inclusion criteria for F12P127; linalool was significant only in Bianca genotype and (*E*)-nerolidol and neral were significant in the pyramided genotype F12P60.

The molecules of "unknown4" and "unknown13", have emerged in the pyramided genotype F12P60. Unfortunately, we do not have enough information about the chemical structure of these compounds; the likelihood of these molecules having a role in plant response to *P. viticola* infection is mentioned in the study by Lazazzara et al. (2018), who described an increase in the abundance of the unknown compounds in resistant genotypes compared to Pinot Noir. Nevertheless, further studies are required to identify the chemical structure and potential roles of these molecules.

Among the flavonoids, epicatechin has been identified in BC4 and, as per the studies of Ali et al. (2012) and Chitarrini et al. (2017); it plays a role in the resistance against pathogens, likely due to its antimicrobial properties.

The stilbenes and stilbenoids identified in mono-locus genotypes and F12P127 are produced through the phenylalanine/polymalonate pathway, and they can have a direct effect on fungal growth and sporulation by slowing down the growth of the pathogen and increasing plant resistance. Fröbel et al. (2019) found a significant induction of phenylalanine ammonium lyase (PAL) and stilbene synthase (STS) genes in *Rpv10* homozygous genotype stating the importance of the quantitative stilbenes produced to stop the pathogen. A recent study by Eisenmann et al. (2019) found that *Rpv3-1*-mediated resistance induces the production of toxic stilbenes and triggers programmed cell death, reducing, but not suppressing, the pathogen growth and development. The accumulation of

monomers (*trans*-resveratrol and *cis*- and *trans*-piceid) at the infection site is mainly related to the response to the pathogen inoculation, also found in the susceptible Pinot Noir. Instead, dimers biosynthesis and accumulation, significantly found only in resistant genotypes, can be related to the activity of these compounds against the pathogen (*trans*-epsilon viniferin and pallidol). These dimers have already been identified as markers of resistance representing key defense molecules because they are produced in response to biotic stress (Viret et al., 2018). Moreover, several studies (Del Rio et al., 2004; Atak et al., 2017) found a positive correlation between increased host resistance and an expression of a high content of phenolic compounds; indeed, according to Pezet et al. (2004) our observations demonstrate that stilbenes have significant inhibitory effects on the mobility of *P. viticola* zoospores and on subsequent disease development.

Tables 2, **3** give us a clear identification of the founded markers for each locus.

CONCLUSIONS

This study describes different metabolic responses to the inoculation with *Plasmopara viticola* at various time points post-infection depending on the loci for resistance present in the genotypes.

To our knowledge, this work is the first study to investigate biomarkers present in mono-locus and pyramided-resistant cultivars. We first screen the genotypes with one *Rpv* resistant gene, afterwards we look for genotypes with pyramided resistance to find potential biomarkers associated with different types of resistance to *P. viticola*.

We identified several classes of compounds responsible for the diversification of the resistant cultivars from the susceptible one. We found an interesting modulation on stilbenes and stilbenoids, already known as biomarkers of resistance (dimers active compounds) in the *Vitaceae* and we confirmed the implication of benzaldehyde as a valid biomarker. We found an increase of terpenes emitted by the resistant genotypes confirming their role against the pathogen. Our findings suggest the possibility to test the pathogen inhibition by these VOCs compounds on receiving tissues and the future perspective to use it as a formulation. Interesting accumulations of fatty acids and volatile organic compounds were observed in the pyramided genotype F12P60 which is the variety with the greatest accumulation of potentially active compounds. The high accumulation of the remaining identified metabolites in the resistant genotypes, as compared to the susceptible Pinot Noir, suggests their possible involvement as biomarkers of resistance in a successful defense against *P. viticola*. Further experiments are required to test the putative compounds investigating their effect on infected tissues.

Overall, the results indicate that the way the cultivars responded to pathogen attacks can be linked to genotype and/or to resistant gene differences; however, resistance is not exclusively related to the *Rpv* genes. In our experiment we did not find a strict relation between mono-locus and pyramided response genotypes, even if they have the same *Rpv* genes. We found a higher accumulation of potential resistance biomarkers in Bianca Solaris and F12P60 genotypes. As expected, in the resistance genotypes we identified an Hypersensitive Response (HR) with cell death and necrosis. The pyramided F12P60 genotype that showed interesting metabolites modulation, did not provide any phenotypic evidence of the HR response. Finally, this study provides novel insights into the resistance mechanisms underlying the hybrids-pathogen interaction that could be valuable for the genetic improvement of grapevines.

DATA AVAILABILITY STATEMENT

Metabolomics raw data are available from MetaboLights (Study Identifier: MTBLS2876, https://www.ebi.ac.uk/metabolights/MTBLS2876)

AUTHOR CONTRIBUTIONS

RC, GC, LZ, MS, and UV designed the experiment. MS provided the plant material. RC, GC, and LZ performed the experiment. RC, GC, DŠ, and MR did the extractions and analytical analysis. PF, RC, and GC conducted the data treatment and statistical analysis. RC, GC, and UV prepared the manuscript. UV, GC, MO, and PR supervised the project. 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: https://www.frontiersin.org/articles/10.3389/fpls.2021.693887/full#supplementary-material

Supplementary Figure 1 | Stilbenes and Stilbenoids meeting the described criteria in mono-locus genotypes; inoculated (Red) and not inoculated (Blue).

Supplementary Figure 2 | Stilbenes and Stilbenoids meeting the described criteria in pyramided genotypes; inoculated (Red) and not inoculated (Blue).

Supplementary Table 1 | Degree of resistance to *Plasmopara viticola* (OIV-452-leaves) evaluated at 7 days post-inoculation on the first six fully expanded leaves; 1, very low; 3, low; 5, medium; 7, high; 9, very high or total; HR, Hypersensitive Response (necrosis).

Supplementary Table 2 | Concentrations of the 22 compounds identified as putative markers of resistance (see section The Effect of Pathogen Inoculation) (VOCs in sheet 1; Lipids in sheet 2 and Polyphenols in sheet 3) and the four stilbenes and stilbenoids involved in the response to the infection (see section Stilbenes and Stilbenoids as Markers) (sheet 3) reported for each genotype and for each year.

Supplementary Table 3 | The "d" values of the identified putative biomarkers for the mono-locus and pyramided varieties.

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CHAPTER III

SECONDARY AND PRIMARY METABOLITES REVEAL PUTATIVE RESISTANCE-ASSOCIATED BIOMARKERS AGAINST *ERYSIPHE NECATOR* IN RESISTANT GRAPEVINE GENOTYPES

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Secondary and primary metabolites reveal putative resistance-associated biomarkers against *Erysiphe necator* in resistant grapevine genotypes

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Numerous fungicide applications are required to control *Erysiphe necator*, the causative agent of powdery mildew. This increased demand for cultivars with strong and long-lasting field resistance to diseases and pests. In comparison to the susceptible cultivar 'Teroldego', the current study provides information on some promising disease-resistant varieties (mono-locus) carrying one *E. necator*-resistant locus: BC4 and 'Kishmish vatkana', as well as resistant genotypes carrying several *E. necator* resistant loci (pyramided): 'Bianca', F26P92, F13P71, and NY42. A clear picture of the metabolites' alterations in response to the pathogen is shown by profiling the main and secondary metabolism: primary compounds and lipids; volatile organic compounds and phenolic compounds at 0, 12, and 48 hours after pathogen inoculation. We identified several compounds whose metabolic modulation indicated that resistant plants initiate defense upon pathogen inoculation, which, while similar to the susceptible genotype in some cases, did not imply that the plants were not resistant, but rather that their resistance was modulated at different percentages of metabolite accumulation and with different effect sizes. As a result, we discovered ten up-accumulated metabolites that distinguished resistant from susceptible varieties in response to powdery mildew inoculation, three of which have already been proposed as resistance biomarkers due to their role in activating the plant defense response.

KEYWORDS

powdery mildew, metabolomics, GC-MS, LC-MS, resistance, loci, biomarkers

1 Introduction

Vitis is a genus widely dispersed and with diverse taxonomy, yet practically most of the world's commercial grape production is focused on a single species, *Vitis vinifera* L., which is native to Europe and Asia Minor. *Vitis vinifera* is a species highly susceptible to various economically devastating pests and diseases, such as powdery mildew. This disease has several causal agents depending on the plant host. In grapevine, the causal agent of powdery mildew is the ascomycete *E. necator* [(syn. *Uncinula necator* (Schweinf.) Burrill] (Gadoury et al., 2012; Dry and Thomas, 2015).

Originating from northern America, grapevine powdery mildew was recently discovered in extremely diverse climatic conditions, including temperate regions with high rainfall, especially during spring months (Pirrello et al., 2019). The causal pathogen is obligatorily parasitic on the genus *Vitis*, as well as on *Cissus*, *Parthenocissus*, and *Ampelopsis* within the Vitaceae family (Gadoury et al., 2012). *Erysiphe necator* can infect all green tissues of the host and cause significant losses in yield and reduction in berry quality (Pimentel et al., 2021). Due to the devastating effects of the disease, breeding studies have been initiated to develop varieties that are tolerant or resistant to this disease all over the world (Atak and Şen, 2021; Atak, 2022).

During the infection process, *E. necator* produces conidia that germinate and grow epiphytically on the plant tissue forming a germ tube and a lobed appressorium. This breaks the cell wall invading the underlying epidermal cells with haustoria, a feeding structure. Through it, the fungus retrieves nutrients and secretes effectors that suppress the plant's immunity, PAMP (pathogen-associated molecular pattern)-triggered immunity (PTI), allowing the colonization of plant tissue surfaces by the development of secondary hypha. The newly formed conidiophores sporulate to infect other host tissues and start a new infection cycle, which leads to an effector-triggered susceptibility (ETS) within the host (Gadoury et al., 2012). As an answer, the plants react using resistance (R) genes that are related to their evolutionary history (Feechan et al., 2011). These genes encode mainly for NBS-LRR (nucleotide-binding site – leucine-rich repeat) proteins that regularly express an interaction of the effector-triggered immunity (ETI) type in which the NB-LRR proteins act as receptors interacting with the strain-specific effectors of the pathogen released during infection. This is likewise true for the R genes that are transcribed into the *Vitacea*e plant family after *E. necator* infection (Qiu et al., 2015). The interaction generates a signaling cascade that leads to transcriptional re-programming in the host plant. The R genes activate several defense responses, including programmed cell death, the generation of reactive oxygen species, biosynthesis/signaling of plant stress/defense hormones, phytoalexin biosynthesis, and cell wall strengthening (Agurto et al., 2017; Welter et al., 2017).

Powdery mildew threatens many commercially important grapevine species and varieties, and thus, nowadays, the most used and efficient method of control is based on chemical treatments (Dry and Thomas, 2015). The most suitable fungicides against *E. necator* are benzimidazoles, ergosterol biosynthesis inhibitors, the quinone-outside inhibitor (QoI) compounds (strobilurins, quinolones), and the succinate dehydrogenase inhibitor (SDHI) group. Since the majority of these fungicides are site-specific, their repeated use leads to fungicide-resistant isolates (Gadoury et al., 2012). Thus, the introduction of resistant cultivars represents the most promising strategy to reduce the use of fungicides in viticulture, avoiding the appearance of *E. necator* resistance isolates. Although all *V. vinifera* cultivars are highly susceptible to *E. necator*, several *Vitaceae* species belonging to various American and Asian genotypes have developed resistance mechanisms against this pathogen (Gadoury et al., 2012; Agurto et al., 2017; Schneider et al., 2019). The resistance quantitative trait loci (QTLs) in *Vitaceae* are clustered in tandem repeats of genomic areas that have been genetically mapped, revealing many loci that encode R gene sequences conferring resistance on *E. necator* and have been utilized to obtain resistant plants by pseudo-backcrossing (Agurto et al., 2017). The R genes identified in *Vitaceae* are named Ren (i.e. resistance to *E. necator*) and Run (i.e. resistance to *Uncinula necator*).

To date, 17 grapevine powdery mildew resistance loci have been identified and described (Sosa-Zuniga et al., 2022); a descriptive list of them is available online (www.vivc.de/loci). It is important to note, however, that the presence of only one gene or locus, even if it has a large effect, can favor the selection of fungus isolates capable of overcoming resistance (McDonald and Linde, 2002). In other words, if the resistance is based solely on the presence of a gene, the fungus may mutate and evade immune recognition through the emergence of new virulent isolates.

In this context, better and longer-lasting disease resistance would be beneficial (Merdinoglu et al., 2018) and a pyramiding technique that integrates multiple resistance loci in the same genotype has been proposed (Mundt, 2018) as a potential solution. To guarantee the longevity of this type of resistance, it is required that loci with different mechanisms of action, spectrums of target isolates and contributions (minor and major) to the resistance be combined. This approach should bring in a more improved, durable and secure implementation strategy, given that, if any mutation or virulence factor occurs, the pathogen will be still recognized by at least one R gene (Peressotti et al., 2010; Cadle-Davidson et al., 2011; Feechan et al., 2015; Pap et al., 2016; Agurto et al., 2017).

Understanding disease resistance or tolerance mechanisms against *E. necator* in grapevine cultivars with different resistant loci at various time points post-inoculation may provide a holistic interpretation of the incompatible interactions between *Vitis* and *E. necator* and provide valuable information for breeding programs. In this respect, characterizing the metabolic profiles associated with disease resistance and susceptibility represents a key step for the identification of trait-related biomarkers. As we have seen in our previous study (Ciubotaru et al., 2021), metabolomics provided novel insights into the resistance mechanisms underlying the hybrid-pathogen interaction by identifying 22 putative biomarkers of grapevine resistance to *Plasmopara viticola.* Thus, the aim of our study is to provide important metabolomics evidence by monitoring changes in the concentration of a large set of metabolites belonging to four chemical classes in grapevine leaves subjected to artificial infection with *E. necator*. The significance of these findings is important for experiments studying the different behavior of resistant (totally or partially) varieties and susceptible ones in terms of the biochemical mechanisms involved in disease resistance. A

better understanding of resistance biochemistry may lead to an improved selection of resistant plants promoting the reduction of fungicide treatments.

In this sense, metabolomics provides a comprehensive and quantitative investigation of metabolites belonging to both primary and secondary classes, including metabolites that play an important role in fighting pathogens. Moreover, metabolomics studies can help in the identification of key metabolites in plant adaptation to biotic stress. Despite the broad interest in more sustainable agriculture, metabolomics studies performed so far have focused on understanding the mechanism of grapevine defense against downy mildew, while only a limited number of investigations focused on *E. necator* (Pimentel et al., 2021). Recent studies have shown the mechanisms underlying the synergy between metabolomics and various omics approaches (Maia et al., 2020; Pimentel et al., 2021; Sosa-Zuniga et al., 2022; Yin et al., 2022), the metabolic differences in the composition of the berries and leaves in several grapevine cultivars (Atak et al., 2021; Rienth et al., 2021) as well as control of the pathogen (Gur et al., 2022).

In this work, we focused on two mono-locus resistant genotypes ('VRH 3082-1-42'- commonly named BC4 - and 'Kishmish vatkana') and four pyramided resistant genotypes ('Bianca', F29P92, F13P71, and NY42) comparing them with the susceptible cultivar (cv) 'Teroldego'. To date, our current work is the first study that addresses the way *E. necator* induces metabolic changes in grapevine genotypes harboring one or more R loci.

2 Material and methods

2.1 Genetic material

Six different resistant grapevine genotypes and the *V. vinifera* cv 'Teroldego' which is highly susceptible to powdery mildew were used in this study. BC4 and 'Kishmish vatkana' had a mono-locus resistance to powdery mildew, whereas 'Bianca', F26P92, F13P71, and NY42 had a pyramided resistance. The grapevine varieties, their pedigree, and their resistance-related loci are listed in Table 1.

TABLE 1 Grapevine varieties used in this study together with their origin [[1] North American *Vitis*; [2] Asian *Vitis*; [3] Interspecific hybrids of *V. vinifera* with North American *Vitis species*, [4] pure *V. vinifera*], host response [PCD (programmed cell death), ROSs (reactive oxygen species)] and their powdery mildew associated resistance related loci *(Ren/Run)*.

Genotypes		Resistance related powdery mildew loci (Ren/Run)	Resistance mechanism within the hosts			Preliminary leaf resistance level	Source of resistance	References
			PCD	ROS	Callose			
mono-locus resistance	BC4	*Run1*	yes	yes	yes	total resistance	*M. rotundifolia*[1]	Feechan et al., 2013; Agurto et al., 2017;
	'Kishmish vatkana'	*Ren1*	yes	yes	yes	partial resistance	*V. vinifera*[4]	Hoffmann et al., 2008;
pyramided resistance	'Bianca'	*Ren3*	yes	yes	yes	partial resistance	*V. rupestris*[3]	Welter et al., 2007; Zendler et al., 2020;
		Ren9	yes	n.d	n.d	partial resistance	*V. rupestris*[3]	Zendler et al., 2017; Zendler et al., 2020;
	F26P92	*Ren3*	yes	yes	yes	partial resistance	*V. rupestris*[3]	Welter et al., 2007; Zendler et al., 2020;
		Ren9	yes	n.d	n.d	partial resistance	*V. rupestris*[3]	Zendler et al., 2017; Zendler et al., 2020;
	F13P71	*Run1*	yes	yes	yes	total resistance	*M. rotundifolia*[1]	Feechan et al., 2013; Agurto et al., 2017;
		Ren1	yes	yes	yes	partial resistance	*V. vinifera*[2]	Hoffmann et al., 2008;
	NY42	*Run1*	yes	yes	yes	total resistance	*M. rotundifolia*[1]	Feechan et al., 2013; Agurto et al., 2017;
		Ren2	yes	n.d	n.d	partial resistance	*V. cinerea*[2]	Feechan et al., 2015;
		Ren3	yes	yes	yes	partial resistance	*V. rupestris*[3]	Welter et al., 2007; Zendler et al., 2020;
		Ren9	yes	n.d	n.d	partial resistance	*V. rupestris*[3]	Zendler et al., 2017; Zendler et al., 2020;
control	'Teroldego'	-	-	-	-	susceptible	-	

The levels of resistance described in the table: Total = greatly suppressed symptoms or the absence of visible symptoms; Partial = in cases where the symptomatology decreases without disappearing completely (Sosa-Zuniga et al., 2022; Julius Kühn-Institut, 2022).

The so-called BC4 hybrid was created in France and was derived from the intergeneric cross between *Muscadinia rotundifolia* and *V. vinifera* (Volynkin et al., 2021). It is resistant to the pathogen *E. necator* through the locus *Run1*, which is the earliest *E. necator* resistance loci to be identified in grapevine and one of the very few well characterized from the causal gene viewpoint (Agurto et al., 2017). The genotype 'Kishmish vatkana' is a cultivated grape from Central Asia obtained from the cross of 'Vasarga chernaya' with 'Sultanina' and resistant trough *Ren1* locus (Hoffmann et al., 2008).

'Bianca' is a hybrid between 'Bouvier' and 'Villard Blanc' created in 1963 at the Kölyuktetö - viticulture research facility in Hungary. Its resistance is conferred by the *Ren3* locus that was discovered on chromosome 15 of the hybrid 'Regent' (Welter et al., 2007) and the *Ren9* locus.

F29P92 and F13P71 are two pyramided hybrids created at Fondazione Edmund Mach (Italy). F26P92 is a mid-resistant genotype derived from 'Bianca' and 'Nosiola' with two resistance loci, *Ren3* and *Ren9*, while F13P71 is a cross between BC4 and 'Kishmish vatkana' having resistance through *Run1* and *Ren1* loci. The pyramided genotype NY42 is derived from a cross performed at USDA-Geneva (NY-USA) between NY95 and Eger99 and its resistance is given by the loci *Run1*, *Ren2*, *Ren3*, and *Ren9*. All three pyramided genotypes are considered breeding selections as they are still under the evaluation process. As a result, our paper is the first to report them in the literature.

The genotypes were grafted on Kober 5BB rootstock and grown in potted soil in controlled greenhouse conditions at the Fondazione Edmund Mach located in San Michele all'Adige (Trento), Italy (46^0 12′ 0′′ N, 11^0 8′ 0′′ E). Fourteen days prior to the experiment, all plants were treated with sulfur to make sure they were uniformly healthy. The sulfur treatment was repeated at the beginning of the experiment for all non-inoculated plants, which represented the control.

2.2 Pathogen inoculation

The inoculation of *E. necator* onto grapevine-potted plants in the greenhouse was done using conidia from the greenhouse and field; thus, the inoculum actually represented a mixture of *E. necator* strains.

The pathogen requires strict and stable climatic conditions for proper development, which is why in this study we tested two inoculation methods by following three different protocols. Three to four infected leaves were used as a source of inoculum for each round of inoculation depending on the spore quantity present on the leaves.

2.2.1 Dry inoculation

The first inoculation method was a dry dispersion of spores. For this method, we tested a combination of Deliere et al. (2010) modified protocol: the upper surfaces of healthy leaves were inoculated by dispersing spores with an air pump from infected leaves, and a cellophane funnel as per Valdés-Gómez et al. (2011) was placed around the inoculated shoots. Funnels were stapled, to allow air circulation, and were left in place for 24 h instead of 12 h as per the original inoculation method of Deliere et al. (2010) (Figure 1A-left).

2.2.2 Wet inoculation

The second method of pathogen inoculation was based on a conidial suspension. We tested the protocol described by Atak (2017). We collected conidia of *E.necator* by washing three severely infected grapevine leaves in 15 ml of sterile distilled water with one drop of Tween-20 (2µl). The conidial suspension obtained had a concentration of 8.4 at 10^5 conidia mL^{-1}. Leaves were inoculated by spraying the conidia suspension using a spraying bottle of 10 mL, using roughly 0.5 mL of suspension per leaf (4 times spray per leaf). Inoculated leaves were immediately covered with thin plastic for 24 hours to obtain high humidity (Figure 1B-middle). For the same

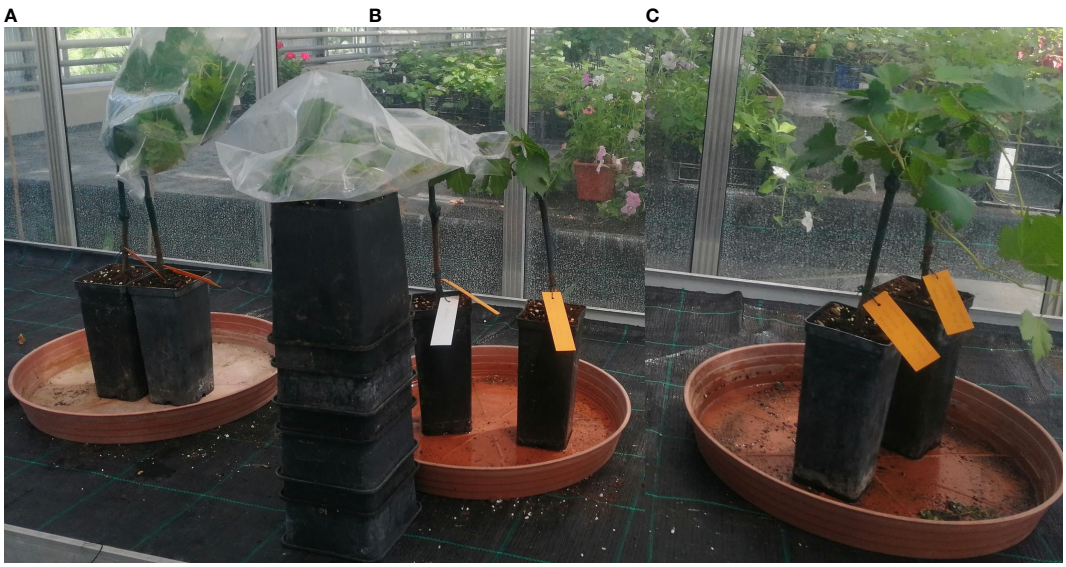

FIGURE 1
The artificial inoculation of *E. necator* conidia onto a susceptible genotype using three different methods: **(A)** - dry dispersion of spores covered by a stapled funnel (left); **(B)** - spray of a conidial suspension covered with plastic (middle); **(C)** - spray of a conidial suspension air-dried (right).

method (conidial suspension), we also tested the protocol described by Miclot et al. (2012) in which the above-prepared suspension was used to spray the upper surface of the leaves. The plants were subsequently air-dried using a ventilator and left uncovered (Figure 1C-right).

We carried out our experiment using the dry inoculation method. For each individual plant, the second, third and fourth fully expanded leaves from below the apex were inoculated by dusting the spores with an air pump for aquariums Newa Wind (Newa Tecno Industria, IT) that had attached a Pasteur glass. The spores were dusted directly into the adaxial surface of the leaves. The climatic conditions in the greenhouse were set at min 20°C – max of 22°C for temperature and 80% for relative humidity (Pertot and Gessler, 2006).

To evaluate the success of the experiments and of the inoculation with *E.necator*, we measured a parameter related to the pathogen performance: the OIV - 455 descriptor at 3, 7, and 11 dpi according to Miclot et al. (2012) (Supplementary Table 1). Briefly, we monitored the disease progression on a daily basis and quantified it based on observations of the plants' reactions.

2.3 Experimental design

Around the twelve-leaf shoot stage, the plants (n=15 plants/genotype) were randomly sorted into two homogenous groups: control and inoculated. The two groups were kept in the same greenhouse (under same conditions) separated by a physical barrier to create two separate compartments in order to prevent any possible transmission of the pathogen. The plant material (three leaves below the shoot apex) was collected at 0, 24, and 48 h post-inoculation (hpi), starting from the morning (8:00 am, which is time zero), and immediately stored at -80°C until use. Three biological replicates were performed per time-point (Figure 2). The experiment was conducted for a 2-year period, in 2019 and 2021.

2.4 Metabolomics analysis

Extraction procedure and analysis of compounds:

Primary compounds were extracted following the method published by Chitarrini et al. (2017a). They were then subjected to derivatization using methoxamine hydrochloride in pyridine and later N-methyl-N-trimethylsilyl-trifluoroacetamide with 1% trimethylchlorosilane for trimethylsilylation. One µL of the derivative extract was then injected for GC/MS analysis using a Trace GC Ultra combined with a TSQ Quantum GC mass spectrometer and a 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 using the software "Xcalibur" (version 4.0) in full scan mode from 50 to 700 m/z. Compounds were identified using their reference standards, retention time, quantifier and qualifier ion, and quantified using their standard calibration curves as mg/kg of fresh leaves.

Lipidic compounds were extracted according to the method of Folch et al. (1957) with some modifications. In the first phase, 0.3 mL of methanol; 0.6 mL of chloroform containing butylated hydroxyl toluene (500 mg/L), and 10 µl of internal standard (stearic acid 100 µg/mL) were used. In a second phase, 0.4 mL of chloroform containing butylated hydroxyl toluene (500 mg/L)/methanol/water 86:14:1 v/v/v was used for the extraction. The combined lower lipid-rich layer of the two extracted phases was finally evaporated to dryness under N_2 and the samples were re-suspended in 300 µl of acetonitrile/isopropanol/water (65:30:5 v/v/v/) containing cholesterol as the internal standard at a concentration of one µm/mL. Samples were injected into 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). The UHPLC system was coupled to an API 5500 triplequadrupole 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 1.7 software.

Volatile compounds were extracted and injected following the method of Chitarrini et al. (2017a) by using a solid phase micro-extraction. A Trace GC Ultra gas chromatograph coupled to a Quantum XLS mass spectrometer (Thermo Scientific, Electron Corporation, Waltham, MA) was used with 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 fiber from Supelco (Bellefonte, PA). Data processing was performed using the software "Xcalibur" (version 4.0). The identification of volatile compounds was done by reference to standards or by comparing retention index and mass spectra using the NIST MS Search 2.3 mass spectral database. Results were semi-quantified as the equivalent of the internal standard (1-heptanol) and expressed as µg/kg of fresh leaves.

Phenolic compounds were extracted according to Vrhovsek et al. (2012) with some modifications made by Chitarrini et al. (2017a). Briefly, the phenolic compounds were extracted from 100 mg of fresh leaves using 0.4 mL of chloroform and 0.6 mL of methanol: water (2:1 v/v); the extraction was repeated by adding 0.6 mL of methanol and water (2:1 v/v) and 0.2 mL of chloroform. The aqueous-methanolic phase of two extractions was collected, combined, and evaporated to dryness under N2. Samples were re-suspended in 500 µl of methanol: water (1:1 v/v) and injected into 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, were quantified using their standard calibration curves and expressed as mg/kg of fresh leaves. Data processing was performed using Waters MassLynx V4.1 software.

2.5 Data analysis

A customized R script was used for statistical analysis and data visualization (R Core Team, 2020). To perform multivariate analysis, the metabolomics dataset's missing values were filled in using median imputation. To account for the anticipated year-to-year fluctuation in the overall metabolic response, the average effect of each year was subtracted for each metabolite/genotype. The base 10 logarithm was

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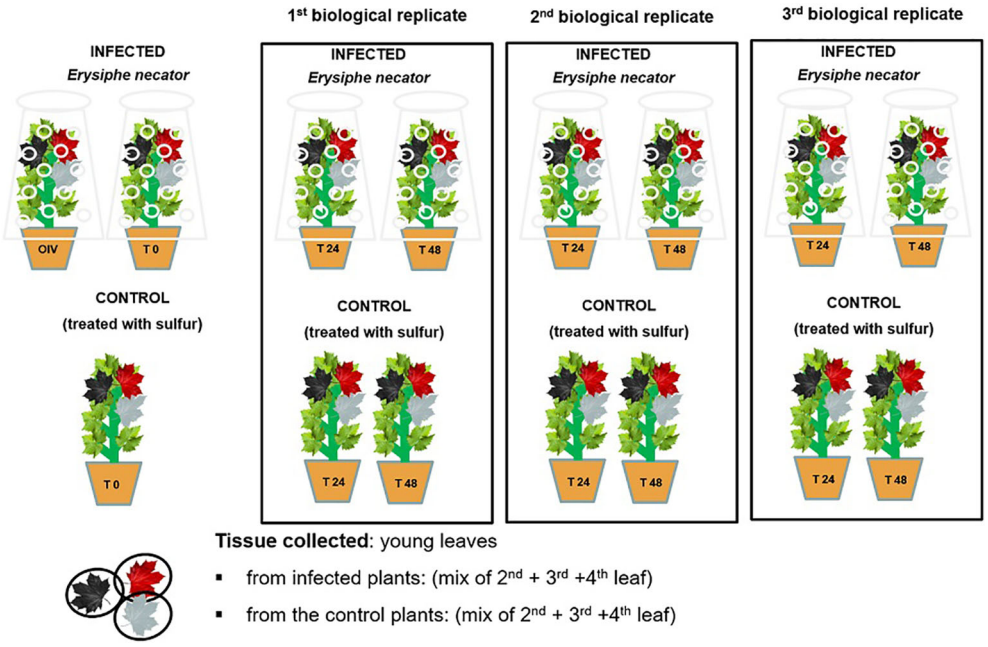

FIGURE 2
A schematic representation of the randomized experimental design of *E. necator's* inoculation.

used to transform the metabolite concentrations in order to compensate for the heteroscedasticity of the data (van den Berg et al., 2006). Thereafter, metabolic principal component analysis (PCA) was carried out on the resulting multidimensional dataset after UV scaling.

The differential response of the individual metabolites at 24 and 48 hpi was characterized by applying a series of univariate non-parametric tests to the data corrected for the effect of the year. To focus on widely present metabolites, only the compounds detected in eight samples were considered for the univariate analysis. Cohen's d-effect size was calculated to identify the metabolites that were strongly altered following infection. Statistical significance and effect size were combined in a set of "volcano plots". Uncorrected $p < 0.05$ and a $d > 1$ were used as arbitrary thresholds to identify strongly responding metabolites. The "d" values can range from a very small effect (d = 0.01) to a huge one (d = 2.0), as per the study of Sawilowsky (2009). Supplementary Table 6 displays the "d" values of the identified up-accumulated metabolites, as well as their related effect size and *p* values. No statistical analysis was conducted on the qualitative assessments of leaf health status.

3 Results

The results of *E. necator's* inoculation were phenotypically observed and the best infections (highest sporulation observed on the leaves) were obtained with the modified dry methods of Deliere et al. (2010) and Valdés-Gómez et al. (2011). The dry inoculation method provided more effective infections than the wet inoculation, most likely due to conidia germination being inhibited or reduced in the presence of water, which was reported to have a detrimental influence on the viability and infectivity of powdery mildew conidia (Miclot et al., 2012). Furthermore, high humidity has been demonstrated to have a severe negative influence on grapevine powdery mildew conidia germination (Carroll and Wilcox, 2003). The reduced efficacy of the wet inoculation is most likely due to residual water remaining in the leaves during or after the drying step. Pictures of the inoculated genotypes taken during the OIV-455 score evaluated at 3, 7 and 11 dpi are available as Supplementary Figures (2- 22).

Over a two-year period, we were able to identify and quantify/semi-quantify 177 metabolites from four chemical classes. These include 60 primary compounds, 56 volatile organic compounds, 43 phenolic compounds and 17 lipids. In the class of primary compounds, we quantified (26) acids, (13) amino acids, (3) amines, one gamma-butyrolactone, and (17) sugars. Within the lipids, we quantified: (2) glycerophospholipids, one sphingolipid, one glycerolipid, one prenol, and (12) fatty acids. We semi-quantified: (4) acids, (9) alcohols, (8) aldehydes, (6) benzenoids, one ester, (2) other volatile organic compounds, (3) fatty acids, (3) fatty acids esters, one fatty alcohol, one benzofuran, (8) terpenoids, (2) terpenes, (3) ketones, one secondary alcohol, and (4) unknowns for the organic volatile compounds. For phenols, we quantified: (3) benzoic acid derivatives, one coumarin, one dihydrochalcone, (12) flavan-3-ols, one flavanone, (12) flavonols, (3) phenylpropanoids, (8) stilbenes and stilbenoids and two other compounds.

The obtained concentrations of all investigated metabolites for each genotype in both years are presented in Supplementary Table 2 for primary compounds, in Supplementary Table 3 for lipids, in Supplementary Table 4 for VOC(s), and in Supplementary Table 5 for phenolic compounds.

3.1 Resistant and susceptible genotypes reveal different kinetics upon pathogen inoculation

After the removal of the effect of the year, PCA was used to depict the global metabolite changes of the 177 identified metabolites in response to pathogen inoculation in all seven genotypes for both years (Figure 3). The six biological replicates of each genotype (three per year) are represented in the plots as small colored dots (the red color corresponds to the inoculated samples and the blue color to the non-inoculated samples). Samples collected at 24 and 48 dpi were analyzed separately to account for possible differences in response among the different genotypes.

The PCA revealed different timescales for the onset of the metabolic response. In fact, in 'Bianca' and 'Teroldego', the separation of infected and non-infected samples began at 24 hpi along the first dimension and became very evident at 48 hpi (Figure 3). Oddly, 'Kishmish vatkana' and F13P71 did not show any separation, neither at 24 hpi nor at 48 hpi (Figure 3). BC4, F26P92, and NY42 showed instead a partial separation through the second dimension at 24 hpi, which was no longer observable by 48 hpi (Figure 3).

3.2 The modulation of classes of compounds upon pathogen inoculation

To determine to which classes of compounds the metabolites that were responsible for the various sorts of separations between genotypes belong, we analyzed the percentages of compounds per class that had a significant effect after infection (Figure 4). The graph represents the percentage of metabolites per each class of compounds that were highly modulated in the plants of each genotype out of the total number of identified and quantified/semi-quantified metabolites per class in both years, as a response to the infection (i.e., 61 primary compounds, 56 volatile organic compounds, 43 phenolic compounds, and 17 lipids).

The class of compounds that were highly modulated due to the infection consisted in lipids. This class showed higher levels compared to the control (non-infected plants), due to the biotic stress in five out of the seven studied genotypes (i.e., BC4, F13P71, F26P92, NY42, and 'Teroldego'). The estimated percentage of lipids affected in BC4 was around 80%; in F13P71 and in 'Teroldego', the percentage of affected lipids decreased to 60% and continued to decrease in F26P92 and NY42 down to 40% reaching 20% in 'Bianca' and less than 20% in 'Kishmish vatkhana'.

Within the class of phenols, the genotype BC4 had the topmost modulated metabolites with a percentage of around 40%. 'Bianca', F26P92, and NY42 reached an approximate value of 20%, whereas the modulation of the metabolites in 'Kishmish vatkana' and 'Teroldego' remained below 20%. An exception was the genotype F13P71, which showed a very low percentage of modulation, not reported in the figure.

The primary compounds exhibited a similar trend of approximately 20% modulated metabolites within the genotypes BC4 and 'Bianca', with a slow decrease in F13P71 and 'Teroldego'. A much lesser percentage was observed in NY42 and F26P92.

The modulation of metabolites in the class of volatile compounds was estimated below 40% for the genotype F26P92, 20% for BC4, 'Kishmish vatkana', and 'Teroldego'; below 20% for NY42, and lower in F13P71.

3.3 Modulated metabolites induced by *Erysiphe necator*

We set out to identify specific metabolites that varied during the infection consistently in both years based on the results of the classes of compounds shown above. As discussed in materials and methods, the most relevant metabolites were identified by combining statistical

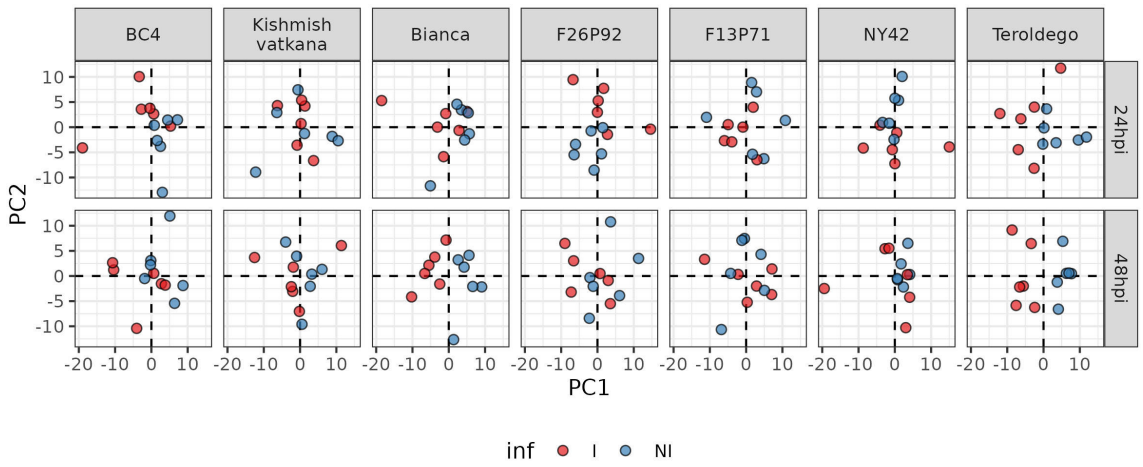

FIGURE 3
Principal component analysis performed on the log 10-transformed metabolite concentration of 24 and 48hpi samples. Each genotype has three biological replicates (small dots) for each year (2019 and 2021). The red color is for inoculated samples (I, inoculated samples) and the blue is for non-inoculated samples (NI, not inoculated samples).

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FIGURE 4
Global visualization of highly modulated metabolites by chemical class (in percentage) in response to *E. necator* inoculation. The size and color intensity of the dots are proportional to the estimated percentage of metabolites modulated in each genotype in both years, based on the total number of identified and quantified/semi-quantified metabolites per class.

significance (assessed by a univariate test) and strength of the effect (estimated by calculating the effect size). We then presented this information in a series of volcano plots (Figure 5 and 6) that highlight the modulation of the distinct classes for each genotype. Positive impact magnitude suggests abundant production (up-accumulation) of the metabolite in infected plants. As a result, a high tail in the volcano's right arm indicates a favorable metabolic response to infection. The lowered (down-accumulation) quantity of metabolites as a reaction to infection, on the other hand, has a negative effect size. It can be seen graphically as the high tail in the volcano's left arm.

Overall, Figure 5 and 6 confirm the trends observed in the initial PCA results, but the plots can be used to get an insight into the classes

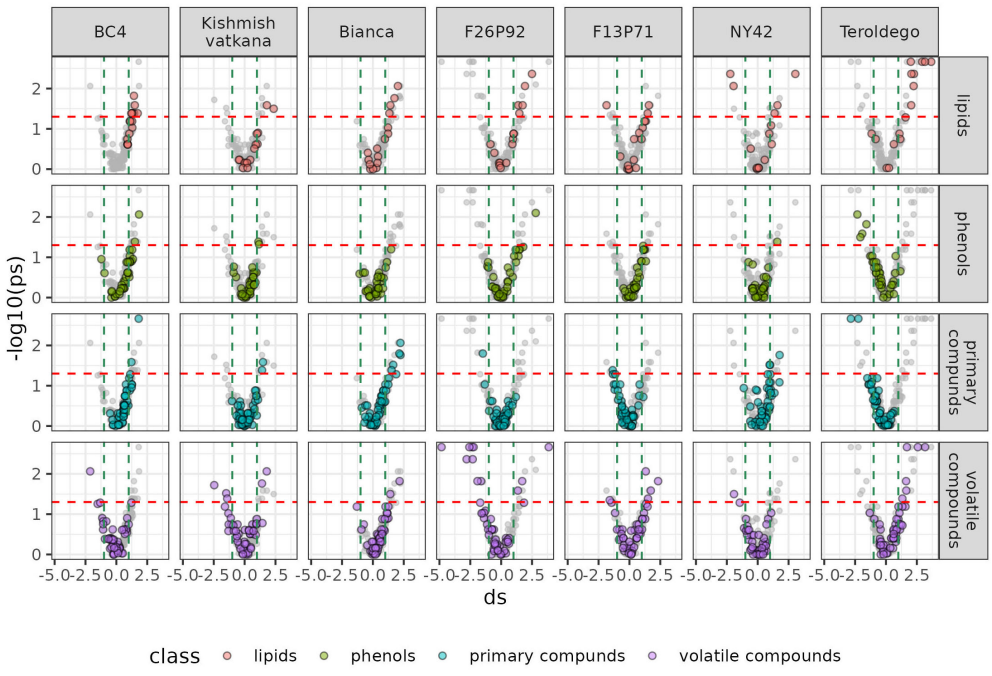

FIGURE 5
Metabolites significantly modulated by the infection (up- and down- accumulated) by class of compounds in all seven genotypes at 24 hpi in the two years of data analysis (2019–2021). The colors identify the different chemical classes (red for lipids, green for phenols, blue for primary compounds, and violet for volatile organic compounds) and "ds" represents the calculated Cohen's d values.

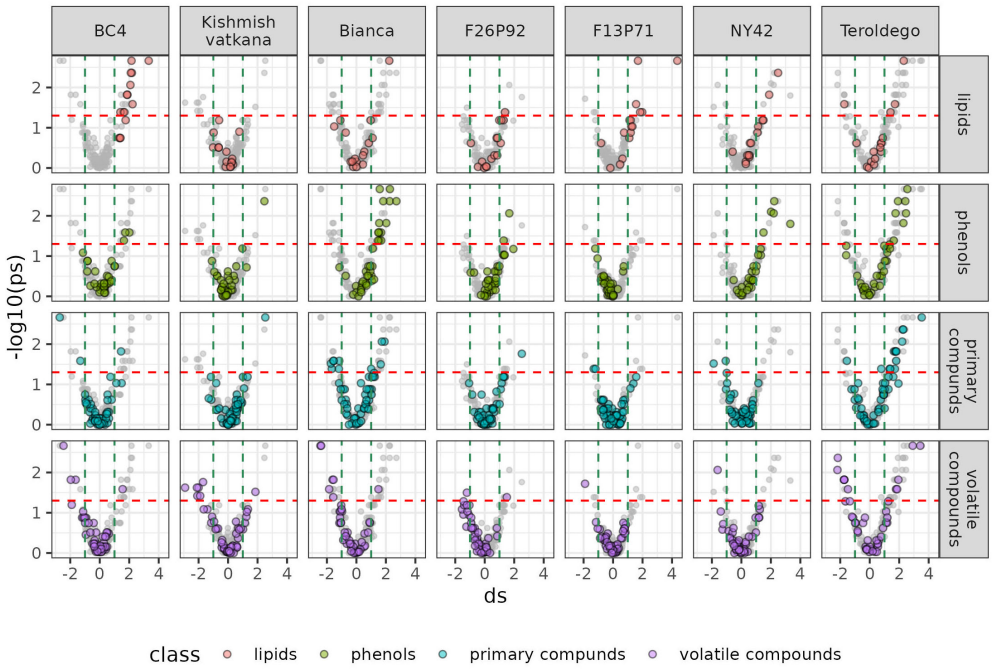

FIGURE 6
Metabolites significantly modulated by the infection (up- and down- accumulated) by class of compounds in all seven genotypes at 48 hpi in the two years of data analysis (2019–2021). The colors identify the different chemical classes (red for lipids, green for phenols, blue for primary compounds, and violet for volatile organic compounds) and "ds" represents the calculated Cohen's d values.

of metabolites, which are more involved in the response. Generally, it can be noticed that the genotypes 'Bianca' and 'Teroldego' begin to react at 24 hpi (Figure 5) and that the effect becomes much larger at 48 hpi (Figure 6), reaching in some cases an effect size value of 2 and even 3 (e.g. phenols in 'Bianca' and 'Teroldego'). In fact, 'Bianca' exhibits the onset of an infection response in all four classes of compounds at 24 hpi, which becomes stronger at 48 hpi by producing a large number of up-accumulated phenols, followed by primary compounds and lipids, and several down-accumulation of volatile compounds. 'Teroldego' produces primarily up-accumulated chemicals such as lipids and volatiles at 24 hpi, whereas, at 48 hpi, there is a large production of up-accumulated phenols, primary and volatile compounds.

The genotypes F13P71 and 'Kishmish vatkana', which appeared not to show major changes in the PCA analysis, showed an up-accumulation in a limited number of lipids and volatiles at 24hpi. At 48hpi, however, 'Kishmish vatkana' reestablished an equilibrium that modulated the levels of up-accumulated lipids and volatiles to levels comparable to the non-infected plants of the same genotype. In the case of F13P71, the levels of lipids increased by 48 hpi, while volatiles appeared not to be modulated anymore.

As for genotypes BC4, F26P92 and NY42, they showed the third type of trend in the PCA where a partial separation between infected and non-infected plants was observed at 24 hpi, BC4 up-accumulated lipids and phenolic compounds at 24 hpi and an increase in that up-accumulation at 48 hpi. It also showed an increase in down-accumulation of volatiles from 24 hpi to 48 hpi. F26P92 showed an active reaction in the synthesis of up-accumulated lipids and down-accumulation of volatile compounds only at 24hpi. NY42 showed a rise in lipids and primary compounds at 24hpi only, while phenols highly increased from 24hpi to 48hpi.

A list of modulated metabolites with the highest reaction in terms of effect size and *p-values* is synthesized in Supplementary Table 6. Among them, we noticed ten up-accumulated metabolites that might potentially distinguish resistant (partial/total) genotypes from the susceptible genotype at 48hpi, when we know that the pathogen's infection structures had already interfered with the plant's metabolome. These metabolites were 2-pyrrolidinone, oleanolic acid, behenic acid, palmitoleic acid, arachidic acid, oleic acid +*cis*_vaccenic acid, pallidol, isorhapontin, quercetin-3-glucoronide, and astringin. Their presence and/or absence in the genotypes is outlined in the Figure 7. The changes of the discriminative compounds at 0hpi, 24hpi and 48hpi for all genotypes based on the corrected concentration values as described in materials and methods are displayed in Supplementary Figure 1.

4 Discussion

In nature, plants protect themselves mostly through mechanical means (spines, trichomes, thick cuticles, and hard or sticky surfaces) and the emission of a variety of poisonous, repellent or unattractive compounds. Plants produce a wide range of metabolites through the latter protection strategy, including fundamental metabolites such as primary compounds and lipids, as well as secondary metabolites like phenolic and volatile organic compounds (Mazid et al., 2011). Secondary metabolism is known to play a defensive role against predators, parasites and diseases (Ali et al., 2010), and primary metabolism, in addition to controlling plant growth, development and reproduction, contributes to plant defense as a source of energy

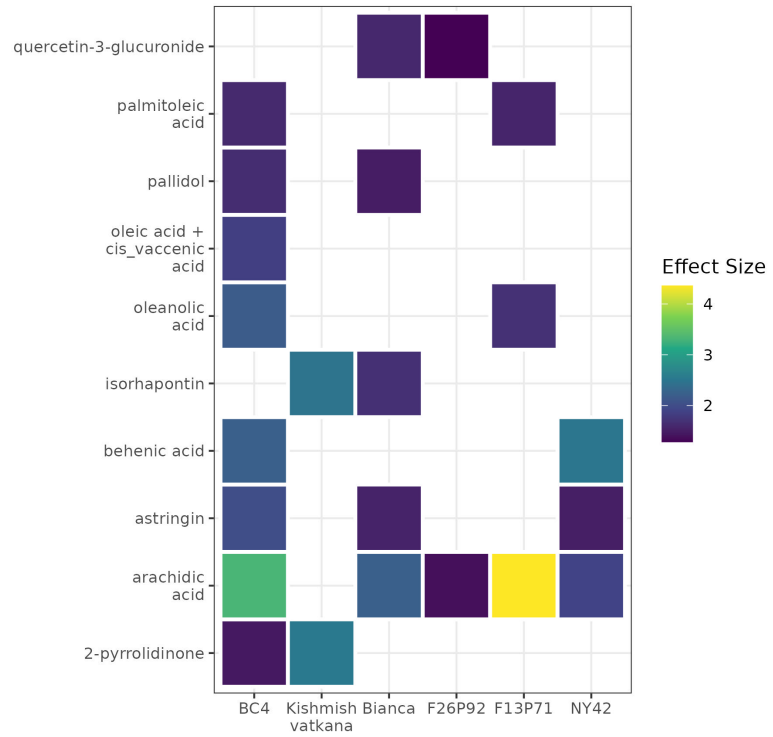

FIGURE 7
A heat map using color-encoded effect size of the discriminative compounds identified as present in the resistant genotypes and absent in the susceptible genotype at 48 hpi. The colors and their intensities mark the modulation of the metabolites in the resistant genotypes according to the calculated effect size (Cohen's d test).

and by signaling molecules that directly or indirectly trigger defense responses (Wolfender et al., 2013).

In this study, we examined the contribution of secondary and primary metabolic components in mediating plant defense responses in resistant grapevine genotypes inoculated by *E. necator*. To the best of our knowledge, this is the first study to look at the responsiveness of multiple classes of metabolites in varieties with one gene of resistance versus varieties with multiple barriers of resistance against powdery mildew.

Our findings indicated that diverse grapevine genotypes react with different time scales to infection. Interestingly, metabolic response (primary and secondary) was more active in the partial and total resistant varieties (i.e., 'Bianca', F26P92, NY42, and BC4) and in the susceptible cultivar 'Teroldego' compared to the partially resistant mono-locus ('Kishmish vatkhana') and the totally resistant pyramided variety (F13P71) (Table 1). 'Bianca', as well as 'Teroldego', showed metabolic variability caused by pathogen inoculation at both time points, while F26P92 and NY42 showed metabolic variability only at 24 hpi. This could be explained by the studies of Feechan et al. (2015) and Pap et al. (2016), which indicated that the existence of several resistance genes or loci does not result in a stronger resistance response for all genotypes, thereby suggesting that combinations of loci such as *Ren3Ren9* do not always have additive effects (Zendler et al., 2020) when compared to the *Run1Ren1* combination that produces an additive effect (Agurto et al., 2017). The method of activating a gene is complicated since just having the gene is not enough; instead, transcription factors are required (Agurto et al., 2017).

Such responses have been observed in some genotypes carrying the combination of *Ren3* and *Ren9*, which did not generate an immune response that has an advantage in terms of the intensity or speed of the response compared to *Ren3* alone (Zini et al., 2019; Zendler et al., 2020). The presence of these loci (*Ren3* and *Ren9*) in all three of the pyramided genotypes, 'Bianca', F26P92, and NY42 (Table 1), could explain our PCA results, which revealed that 'Bianca' had a metabolic variability caused by pathogen inoculation at both time points similar to 'Teroldego', followed by F26P92 and NY42, which showed metabolic variability only at 24 hpi (Figure 3).

On the other hand, studies showed that combinations of *Run1Ren1* and *Run1Ren2* have an additive effect as the combination of both genes/loci generated a stronger immune response than the one triggered by each one individually, however, this effect has been proven to be genotype dependent (Agurto et al., 2017). In fact, in our study, the genotypes F13P71 and NY42 showed little to partial metabolic variability despite possessing the loci *Run1Ren1* and *Run1Ren2*, respectively. Moreover, other studies showed that by powdery mildew isolates could overcome, in some cases, *Run1* resistance (Cadle-Davidson et al., 2011; Schneider et al., 2019). This could explain the observed metabolic variability in F13P71. Furthermore, the additive effect of *Run1Ren2* can be race-specific (Feechan et al., 2015) and in addition, the existence of the other two extra loci in the genotype NY42, might interfere with the metabolomics response to the pathogen. All these factors contribute to the complexity of the effects of resistance genes in the metabolic variability of infected grapevine genotypes, requiring additional research.

Considering all these aspects, it seems that the level of resistance (partial or total) of the loci is more important than their numbers. The level of resistance is referred to as "total" when there are greatly suppressed symptoms or no observable symptoms of infection at all and "partial" when there is a decrease in symptoms but not a complete disappearance (Julius Kühn-Institut, 2022; Sosa-Zuniga et al., 2022). This is corroborated in our study by the assessment of the OIV-455 descriptor at 7 days after the artificial infection (Supplementary Table 1).

We found for 'Kishmish vatkana', a genotype with partial resistance, a high level of resistance (OIV-455 = 7) and for F13P71, a genotype with total resistance, a very high level of resistance (OIV-455 = 9). Indeed, an 84% decrease in the number of cells the fungus invaded has been observed among the responses brought on by *Ren1* ('Kishmish vatkana'). Other reactions include the induction of PCD (programmed cell death), the development of callose deposits at 48 hpi, and the promotion of ROS (reactive oxygen species) at 96 hpi (Agurto et al., 2017). A more intense defense response was likewise observed in genotypes carrying *Run1Ren1*, such as F13P71, in terms of ROS production, callose accumulation and PCD (Agurto et al., 2017).

NY42 and F26P92, genotypes with partial resistance, scored a high level of resistance (OIV-455 = 7) and BC4, a genotype with total resistance, was assessed as having a very high level of resistance (OIV-455 = 9). Possamai et al. (2021) observed in genotypes carrying *Run1Ren2* loci such as NY42 a significant decrease in colony formation, and Feechan et al. (2015) showed that *Ren2* confers partial resistance on plants by inducing an efficient immune response that prevents fungal sporulation. Rapid programmed cell death, which hinders the growth of secondary hyphae and sporulation, is one of the immunological responses inflicted by *Run1* (BC4) on resistant plants. A quick HR is seen at 48 hpi in cells where the fungus developed secondary hyphae as evidenced by the rise in ROS and the appearance of PCD. The buildup of callose deposits at the *E. necator* infection site is another reaction caused by *Run1* (Agurto et al., 2017). *Ren3Ren9* (F26P92) elicits similarly high resistance responses (Zendler et al., 2020), with a high level of resistance score (OIV-455 = 8) assigned to 'Bianca', a partial resistant genotype carrying the exact same loci (*Ren3Ren9)*. As expected, the susceptible genotype 'Teroldego' was assessed as having a very low level of resistance (OIV-455 = 1). As far as primary metabolites are concerned, powdery mildew induced changes mainly in the class of lipids (Figure 4). Lipids are recognized to be important components of plant cell membranes that provide energy for metabolic activities. In recent years, there has been increasing evidence that lipids play a role in combating biotic stress, such as powdery mildew. Lim et al. (2017) showed that lipids regulate the PCD response during pathogen defense, as well as membrane fluidity, stability, and permeability during plant responses to microbial pathogens. The accumulation of C16:0 might be used to produce C18 fatty acids. Also higher DBI may account for an increase in chloroplasts' membrane fluidity that may be crucial to avoid any damage in the photosynthetic machinery with inevitable effects on the energy transduction pathways and primary productivity (Laureano et al., 2018; Laureano et al., 2021). Moreover, lipids play important signaling roles also in plant defense and ROS regulating levels. Because of the various functions of lipids, Della Corte et al. (2015) observed that their abundance in plants is influenced by genotype and phenotype. Thus, the fluctuating lipid levels observed in the various resistant genotypes tested may be attributed in part to this aspect as a result of *E.necator* inoculation.

The role of primary metabolic pathways in the regulation of plant defense responses is not very well known. Mainly, all primary compounds function as signaling molecules that trigger defense responses through signal transduction and pathogen recognition processes (Madiha et al., 2019). The accumulation of the primary compounds in our study, which was comparable to the susceptible genotype, made us lend support to the idea that susceptible plants initiate a basal defense similar to the response in resistant plants, but insufficient in timing and/or intensity to limit disease progression, as observed by Marsh et al. (2010). Similarly, there is a clear alteration of primary compounds in the defense against powdery mildew in resistant genotypes, but the amount raises the question of whether this modulation is a result of resistance or a normal plant reaction.

One of the most important functions of phenolic compounds as secondary metabolites is an antibacterial activity in plants, which acts as a barrier against pathogens like *E. necator*. Their accumulation in plants is associated with host resistance (Atak, 2017). However, it is noteworthy that Keller (2015) found that despite some *Vitis* species (such as *V.cinerea* and *V.champinii*) exhibiting pathogen resistance, the buildup of stilbenes, the most well-known class of phenolic defense chemicals, did not occur in these plants. This finding could support the hypothesis that metabolite accumulation is not totally linked to the number of loci present in the resistance genotypes. Such was the case in our study where the pyramided genotype F13P71 accumulated very low levels of phenolic compounds. The same genotype displayed low levels of volatile organic compounds (VOCs). Thus, similar assumptions could be made also about VOCs, but further research is needed to confirm it.

Although some chemical classes in some of our resistant varieties showed similar reactions to the susceptible genotype, it should be noted that the resistant genotypes nonetheless produce a number of up-accumulated metabolites that were not found in the susceptible 'Teroldego', with the exception of a few whose calculated effect sizes were smaller than in the resistant genotypes. The study of Viret et al. (2018) showed that the induction and accumulation of defensive metabolites increase only during the pathogen's infectious structure development, which takes around 24 hours (Boddy, 2016). This was noticed in the pyramided genotypes in which the metabolite overaccumulation had a significantly larger impact size at 24 hpi than at 48hpi, when their modulation appeared to subside, except for 'Bianca'. In our earlier research, we provided evidence that *P. viticola* caused an early modulation in pyramided genotypes, which began earlier, between 0 and 12 hpi, and peaked at 48hpi. Even though the current work studies *E. necator* and genotypes that carry different loci than the prior study, we can presume that a similar but somewhat different reaction happened for this study as well. On the other hand, the up-accumulation of metabolites in mono-locus genotypes was shown to be established at 24 hpi and to become stronger at 48hpi. The same finding was obtained in the work of Chitarrini et al. (2017a), in which the plant defense systems were activated 48 hours after inoculation.

Investigating the biological relevance of the ten compounds found as discriminative between resistant and susceptible genotype (Figure 7), we found out that pallidol, oleic acid+*cis* vaccenic acid and astringin were already discussed as potential biomarkers of resistance in our previous study (Ciubotaru et al., 2021) due to their role in activating plant defense

response. Moreover, pallidol has been in some cases linked to the grapevine's response to fungal attack (Pezet et al., 2004; Jean-Denis et al., 2006). We have also found that the remaining seven- up-accumulated metabolites contribute to plant defense. Isorhapontin, like pallidol and astringin, belongs to the class of stilbenes and stilbenoids, and it has been demonstrated that this class accumulates in larger concentrations in disease-resistant cultivars than in susceptible cultivars, due to its role in plants that inhibits fungal growth (Chitarrini et al., 2017b; Vezzulli et al., 2019). Similarly, the increased accumulation of fatty acids in the plant metabolome, specifically behenic acid, palmitoleic acid, arachidic acid and oleic acid+*cis* vaccenic suggests that these fatty acids are involved in intracellular signaling processes as well as desaturases-mediated membrane fluidity adjustment (He and Ding, 2020; Ciubotaru et al., 2021). The fatty acid desaturase 7 (FAD7) and fatty acid desaturase 8 (FAD8) genes, which play a key role in the synthesis of fatty acids, have also been linked to protective mechanisms (Rojas et al., 2014; Cavaco et al., 2021). Last but not least, oleanolic acid is known to play a role in plants' defense mechanisms against pathogens and water loss (Gudoityte et al., 2021), whereas quercetin is a powerful antioxidant that effectively protects plants from a variety of biotic and abiotic challenges (Singh et al., 2021). Our findings confirm previous research about the importance of these compounds in disease resistance because of their different roles in plant defense.

5 Conclusions

Many metabolomics studies have been conducted on understanding the mechanism of grapevine defense, mainly on downy mildew, but few on powdery mildew. Thus, we designed this study as a promising endeavor in order to contribute to a better understanding of plant defense mechanisms. To our knowledge, this is the first time that metabolic investigations of the most important classes of compounds with a role in plant defense were carried out in artificially inoculated genotypes with mono-locus and pyramided resistance in order to characterize the host's response to the infection of *E. necator*.

Overall, the results of this study indicate that how cultivars behaved to pathogen attack can be linked to genotype and/or resistant loci differences; however, resistance is not exclusively related to *Run/Ren* loci. Additionally, although it cannot be strictly classified as a connection, we saw similar metabolomic responses in our experiment between the mono-locus and pyramided genotypes that share the exact *Run/Ren* loci. Therefore, additional transcriptome studies are required to fully comprehend the unfavorable interaction between these resistant loci and *E. necator*. Further research is needed also to validate the molecules identified as biologically relevant compounds produced during the pathogen-host interaction and recommended as possible biomarkers for resistance to *E. necator*. In terms of plant resistance strength against powdery mildew, our findings show no direct relationship between the number of resistance loci present in plants and the production of metabolites recommended as resistance biomarkers.

The findings of this study add to our understanding of plant defense mechanisms and call for more metabolomics research, as well as additional complementary omics research to clarify which genes are responsible for powdery mildew resistance and how they function in the majority of *Run* and *Ren* loci, as only one study in this area has been conducted. The integration of transcriptomics and metabolomics data can be exploited to uncover commonalities and differences between diverse R-gene-mediated resistances to *E. necator*. This approach will enable breeders to choose more reliable genotypes for marker-assisted breeding by using genetic and biochemical markers.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding author.

Author contributions

RMC, GC, LZ, MS, and UV designed the experiment. MS and SV provided the plant material. RMC, SV and LZ performed the experiment. RMC did the extractions and analytical analysis. PF, RMC, and UV conducted the data treatment, statistical analysis and data visualization. RMC wrote the original draft preparation of the manuscript. UV, SV, PF, MO, PR and GC did the review and editing. UV, MO, and PR supervised the project. All authors discussed the results and implications and commented on the manuscript at all stages. All authors contributed to the article and approved the submitted version.

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Conflict of interest

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.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2023.1112157/full#supplementary-material

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CHAPTER IV

SEMI-TARGETED PROFILING OF THE LIPIDOME CHANGES INDUCED BY *ERYSIPHE NECATOR* IN DISEASE-RESISTANT AND *VITIS VINIFERA* L. VARIETIES

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Semi-Targeted Profiling of the Lipidome Changes Induced by *Erysiphe Necator* in Disease-Resistant and *Vitis vinifera* L. Varieties

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Abstract: The ascomycete *Erysiphe necator* is a serious pathogen in viticulture. Despite the fact that some grapevine genotypes exhibit mono-locus or pyramided resistance to this fungus, the lipidomics basis of these genotypes' defense mechanisms remains unknown. Lipid molecules have critical functions in plant defenses, acting as structural barriers in the cell wall that limit pathogen access or as signaling molecules after stress responses that may regulate innate plant immunity. To unravel and better understand their involvement in plant defense, we used a novel approach of ultra-high performance liquid chromatography (UHPLC)-MS/MS to study how *E. necator* infection changes the lipid profile of genotypes with different sources of resistance, including BC4 (*Run1*), "Kishmish vatkhana" (*Ren1*), F26P92 (*Ren3*; *Ren9*), and "Teroldego" (a susceptible genotype), at 0, 24, and 48 hpi. The lipidome alterations were most visible at 24 hpi for BC4 and F26P92, and at 48 hpi for "Kishmish vatkhana". Among the most abundant lipids in grapevine leaves were the extra-plastidial lipids: glycerophosphocholine (PCs), glycerophosphoethanolamine (PEs) and the signaling lipids: glycerophosphates (Pas) and glycerophosphoinositols (PIs), followed by the plastid lipids: glycerophosphoglycerols (PGs), monogalactosyldiacylglycerols (MGDGs), and digalactosyldiacylglycerols (DGDGs) and, in lower amounts lyso-glycerophosphocholines (LPCs), lyso-glycerophosphoglycerols (LPGs), lyso-glycerophosphoinositols (LPIs), and lyso-glycerophosphoethanolamine (LPEs). Furthermore, the three resistant genotypes had the most prevalent down-accumulated lipid classes, while the susceptible genotype had the most prevalent up-accumulated lipid classes.

Keywords: *Vitis vinifera*; resistant varieties; plant lipid metabolism; powdery mildew; biomarkers

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1. Introduction

Lipids are essential plant components. The lipidome is the whole lipid profile of an organism, tissue, or cell [1], and lipidomics is the detailed study of lipid molecules, including identification, quantification, and understanding of their significance in biological systems [1,2]. LIPID MAPS (https://www.lipidmaps.org (accessed on 10 November 2022)) classifies lipids into separate categories based on the distinct hydrophilic and hydrophobic constituents that form the lipid. Fatty acyls (FAs), glycerolipids (GLs), glycerophospholipids (GPs), sphingolipids (SPs), saccharolipids (SLs), polyketides (PKs), sterol lipids (STs), and prenol lipids (PRs) are the eight major categories and can be identified by their chemically

functional backbone structures [3]. In plants, they perform a variety of roles, including those related to cell architecture [4], energy storage [5], cell signaling [6], reducing stress tolerance [7], and symbiotic and pathogenic relationships [8].

In the interaction between pathogens and plants, lipids are crucial, particularly in the following three key areas: pathogen development and life cycle completion, pathogen recognition and host-initiated defense response, and impeding host defense mechanisms to overcome resistance [9]. As has been proven several times, lipids play an important role in both types of plant immunity, pathogen-associated molecular pattern (PAMP)-triggered immunity (PTI) [10] and effector-triggered immunity (ETI) [11]. When pathogens enter the host, the cuticle is the first barrier they meet. Pathogens penetrate plant tissue and encounter the apoplast, one of the most important cellular compartments in the defense response. Here, pathogens secrete molecular effectors during plant–microbe interactions, generating a wide range of changes in this compartment [12], with still-unknown effects on the modulation of lipids [13]. Nonetheless, there is little evidence of the relevance of extracellular lipids in plant–pathogen interactions in the creation of systemic acquired resistance (SAR) [14].

It is known that upon pathogen interaction, a plant's lipidic profile may experience changes frequently linked to the modulation of membrane fluidity and enzymatic and non-enzymatic creation of bioactive lipid mediators such as oxylipins, FA oxidation products, and lipids [15]. This modulation has been identified as a critical element in triggering plant immunity [16–18]. Although structural lipids derived from primary metabolism function in order to restrict pathogen penetration, infections caused by pathogens such as *Erysiphe necator* can overcome the basal defensive systems in many economically important grapevine cultivars. The disease can be difficult to detect, especially in the early stages, as signs and symptoms are often subtle. Failure to prevent and/or control powdery mildew often results in insufficient fungicide spray coverage, and because the majority of these fungicides are site-specific, recurrent application results in fungicide-resistant isolates [19]. Thus, valorizing resistant cultivars with resistance quantitative trait loci (QTLs) named *Ren* and *Run* (conferring resistance to *Erysiphe necator* and *Uncinula necator*, respectively) is the most promising technique for reducing chemical use in viticulture and avoiding the establishment of *E. necator* resistance isolates [19,20]. However, it must be highlighted that using varieties with only one gene or locus can encourage the selection of fungal isolates capable of overcoming these key resistance loci [21]. To avoid such resistance breakdowns, a different approach is to employ pyramided cultivars, which store many resistant genes/loci against the same pathogen/disease [22].

We previously provided metabolomics evidence on the early interaction between grapevine varieties with one locus and grapevine varieties with several loci and *E. necator* [23]. We discovered that the class of molecules most affected by the pathogen was lipids, highlighting the importance of lipids in grapevine defense against the powdery mildew causative agent. The increased accumulation in the plant metabolome of four fatty acids (behenic acid, palmitoleic acid, arachidic acid, and oleic acid+*cis* vaccenic) and one prenol (oleanolic acid) showed their involvement in plant defense mechanisms. Despite this evidence and a growing interest in the involvement of lipids and lipid-related compounds in plant–pathogen interactions, few studies have focused on the interaction of lipids with grapevine diseases. The grapevine leaf–*Plasmopara viticola* pathosystem has received the most attention [16,24–27], whereas the interaction between *E. necator* and grapevine leaf lipids has only been reported in one untargeted metabolomics study [28]. In general, lipidomics research is needed to better understand plant defense mechanisms against *E. necator*, particularly the role of lipids in regulating plant defense responses in *E. necator*-affected mono-locus and pyramided grapevine genotypes.

Thus, we decided to extend our previous investigation on *E. necator* and focus solely on the changes brought about by the pathogen in the plant lipidome. We did so by using a newly developed sensitive and accurate semi-targeted ultra-high performance liquid chromatography (UHPLC)-MS/MS method [3]. This allowed us to acquire a more

holistic picture due to its power in analyzing and quantifying a vast number of chemical compounds from multiple classes of lipids in a single analytical run, as opposed to the earlier employed targeted method of [26], which considered only 32 lipid compounds. For this purpose, we studied three of the previously investigated resistant grapevine varieties with a different percentage of lipids modulated as a reaction to the infection with the pathogen *E. necator*, and screened them for two years to detect changes in the lipid profile during plant–pathogen interactions. In this work, the lack of knowledge on the impact of *E. necator* on the lipidome of grapevine leaves was addressed for the first time. This brought us closer to understanding grapevine lipid-mediated defense mechanisms and highlighted potential compounds for future disease tolerance/resistance breeding initiatives.

2. Results

We investigated 8098 lipids of possible interest for grapevine defense using the semi-targeted ultra-high performance liquid chromatography (UHPLC)-MS/MS approach. Among the investigated lipids, 271 were detected within the inoculated and non-inoculated leaves (control) belonging to the four chemical categories studied (glycerophospholipids, glycerolipids, sphingolipids, and fatty acids). Supplementary Table S1 (sheet 3) shows the semi-quantification of all detected lipids expressed as μg/g of fresh leaf powder for each genotype in both years.

2.1. Phenotypic Resistance

The four genotypes studied scored differently on the scale of the Organisation Internationale de la Vigne et du Vin (OIV-455 descriptors). At 7 dpi (days post-inoculation), we attributed an OIV-455 score of 9 to the genotype with total resistance (BC4), an OIV-455 score of 7 to the two genotypes with partial resistance ("Kishmish vatkana", and F26P92), and an OIV-455 score of 1 to the susceptible genotype "Teroldego". Supplementary Table S2 contains the OIV-455 scores assigned for grapevine leaf resistance to powdery mildew.

2.2. Lipid Modulation of the Grapevine–E. necator Interaction during the First Hours of Infection

We focused on the lipidome modifications of the grapevine leaves in response to the artificial infection at the time points of 24 and 48 hpi (hours post-inoculation), taking into account that at 0 hpi, the plant lipidome should not suffer any change after the effect of the year was removed.

Out of 271 lipids identified and semi-quantified, the percentage of lipids within their corresponding class that showed a significant modulation is shown in Figure 1. The dots contained within the vertical green line represent the percentages of lipid modulation at 24 hpi, whereas the ones within the red line represent the percentages of lipid modulation at 48 hpi. The most modulated lipid classes were identified at 24 hpi in the resistant genotypes BC4 (13 classes with 55 modulated lipids) and F26P92 (13 classes with 69 modulated lipids). By 48 hpi, however, both BC4 and F26P92 showed a decreased response (10 classes with 33 modulated lipids and 8 classes with 11 modulated lipids, respectively). Interestingly, "Kishmish vatkhana" displayed a different behavior than the other resistant varieties. It showed a low level of lipid modulation with only 3 modulated lipids belonging to 3 different classes at 24 hpi, which increased to 15 modulated lipids of 8 classes at 48 hpi. The susceptible genotype "Teroldego" modulated 13 lipids from 7 classes at 24 hpi, which then increased to 100 modulated lipids from 11 classes at 48 hpi (Figure 1).

To go deeper into the molecular aspects of the modulation, the previous results were further explored in a series of volcano plots, as presented in Figures 2 and 3. The figures emphasize all the classes of lipids (in gray) and highlight each class of modulated lipids with a different color (independently of their statistical significance) for each genotype. The discontinued horizontal red line represented in the graph indicates the threshold for statistical significance (uncorrected $p < 0.05$), whereas the discontinued vertical green lines were used to select strongly reacting lipids (absolute d > 1). The lipids situated on the right of the discontinued vertical green line indicate that infected plants produced more lipids

(up-accumulation). Consequently, a high tail on the right arm of the volcano denotes a positive metabolic response to infection. On the other hand, the lipids above the threshold situated on the left of the discontinued vertical green line indicate that infected plants produced fewer lipids (down-accumulation). The reduced level of lipids in response to infection appears as the high tail of the volcano's left arm. The modulated lipids, both up-accumulated and down-accumulated, with their calculated effect size and *p*-values, are listed in Supplementary Table S3 (24 hpi in sheet 1 and 48 hpi in sheet 2).

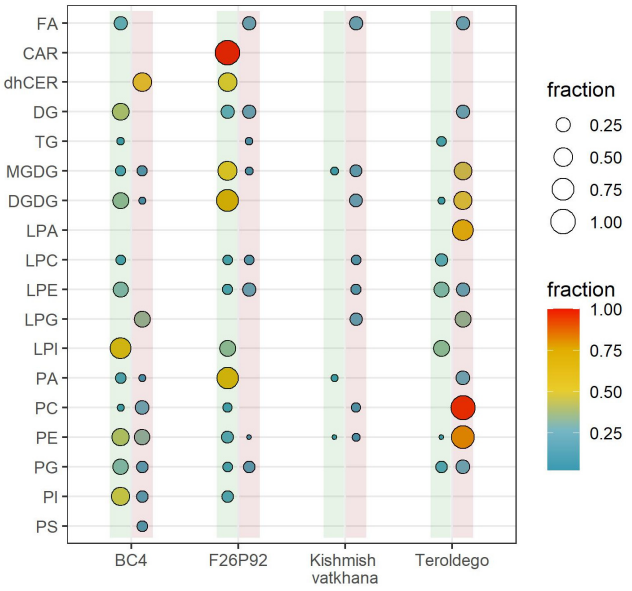

Figure 1. Visualization (in percentage points) of all classes of lipids that were highly modulated in response to *E. necator* infection. Based on the total number of detected and semi-quantified lipids, the size and color intensity of the dots are proportionate to the estimated percentage of lipid class modulation in each genotype in both years. The dots inside the vertical lines show the percentages of lipid modulation (green = at 24 hpi, red = at 48 hpi).

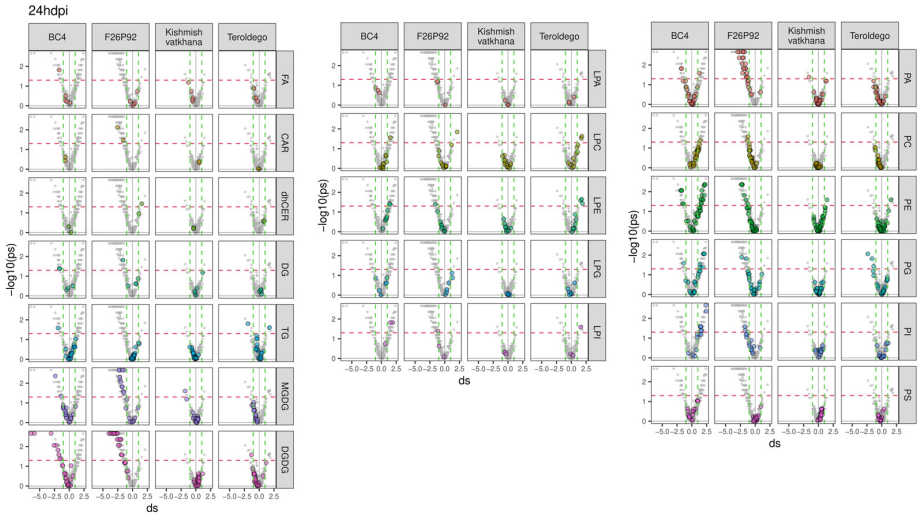

Figure 2. Lipids with values above the discontinued red line were significantly modulated with the up-accumulated lipids shown on the right and down-accumulated lipids shown on the left arm of the volcano for all four genotypes at 24 hpi over the course of the two years of data analysis (2019–2021). The left graph shows the modulation of glycerolipids, sphingolipids, and fatty acids, whereas the middle and right graphs show the modulation of glycerophospholipids. The colors reflect the various lipid classes, while "ds" represents the calculated Cohen's d values.

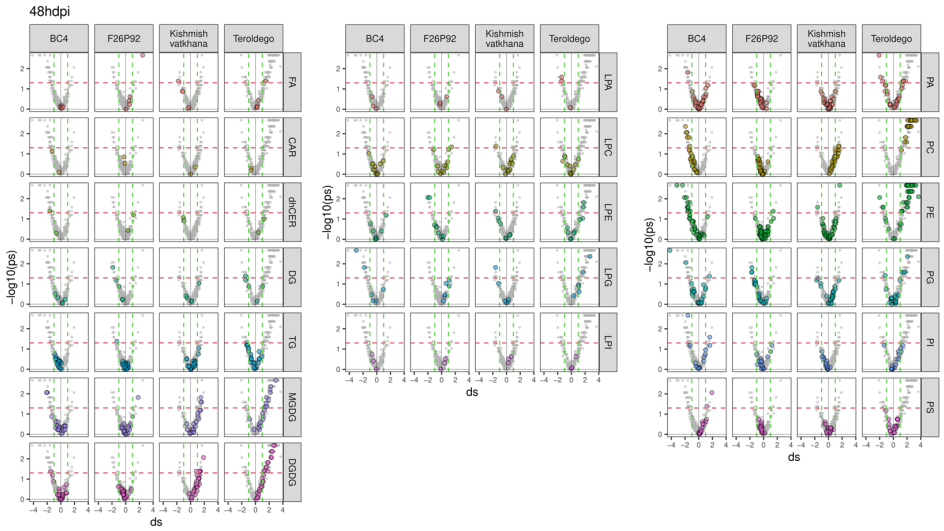

Figure 3. Lipids with values above the discontinued red line were significantly modulated, with up-accumulated lipids shown on the right and down-accumulated lipids shown on the left of the volcano plot for all four genotypes at 48 hpi over the course of the two years of data analysis (2019–2021). The left graph shows the modulation of glycerolipids, sphingolipids, and fatty acids, whereas the middle and right graphs show the modulation of glycerophospholipids. The colors reflect the various lipid classes, while "ds" represents the calculated Cohen's d values.

The genotype BC4 displayed 32 lipid compounds that were up-accumulated and 23 lipid compounds that were down-accumulated at 24 hpi. The most prevalent up-accumulated compounds were glycerophospholipids in the PE, PG, and PI classes, whereas the most prevalent down-accumulated compounds were glycerolipids in the DGDG class (Figure 2). At 48 hpi, only 4 lipids showed up-accumulation, whereas 29 lipids were down-accumulated with the most prevalent modulation being the down-accumulation of the PE and PC classes (Figure 3).

At 24 hpi, F26P92 had up-accumulated 1 lipid compound from the glycerophospholipids in the LPC class and 1 sphingolipid from the dhCER class, while down-accumulating 67 lipid compounds. Among these, the most prevalent compounds were the glycerophospholipids (19 lipid compounds in PA and 6 lipid compounds in the PE class) and glycerolipids (20 lipid compounds in the DGDG class and 11 lipid compounds in the MGDG class) (Figure 2). It is interesting to note that at 48 hpi, there was a decrease in the number of lipid compounds that were down-accumulated (seven), which included glycerophospholipids and glycerolipids, and a slight increase in the number of lipid compounds that were up-accumulated (four), which included glycerophospholipids, glycerolipids, and fatty acids (Figure 3).

At 24 hpi, the genotype "Kishmish vatkhana" had one down-accumulated compound belonging to glycerolipids (MGDGs) and one component up-accumulated belonging to glycerophospholipids (PEs) (Figure 2). At 48 hpi, a different pattern of behavior was discerned, with 10 lipid compounds up-accumulated—the most prevalent being in the glycerolipid (MGDG and DGDG) and glycerophospholipid (PC) classes—and 5 lipid compounds down-accumulated, each one belonging to a different class of glycerophospholipids and fatty acids (Figure 3).

"Teroldego" displayed at 24 hpi 5 down-accumulated lipid compounds and 8 up-accumulated lipid compounds in the glycerophospholipid and glycerolipid groups (Figure 2), whereas, at 48 hpi, there were 10 down-accumulated lipid compounds in the glycerophospholipid groups and a significant increase in the up-accumulated lipid compounds (90). The most prevalent up-accumulated compounds were the glycerophospholipids (30 lipid compounds in PE group and 26 lipid compounds in PC group) and the glycerolipids (13 lipid compounds in the DGDG class and 9 lipid compounds in MGDG) (Figure 3).

3. Discussion

In this study, we investigated how the lipidome of grapevine leaf tissue can be impacted by *E. necator*. To our knowledge, this work is the first to describe how lipid metabolism is modulated in the leaves of two mono-locus resistant and one pyramided resistant *V. vinifera* varieties compared to a susceptible variety upon *E. necator* infection.

The results of our study show that modulated lipids can be detected in *E. necator*-infected tissues at very early stages (24 and 48 hpi) of the infection process. Furthermore, the findings of our investigation reveal a distinct percentage of modulation of lipids in the first hours following *E. necator* artificial infection between the susceptible and the resistant genotypes. According to a study by [29], the development of the pathogen's infectious structure, which takes around 24 h [30], is the only time when defense metabolites are induced and accumulated more. This was observed in the resistant genotypes BC4 and F26P92, which had the strongest modulation of several lipid classes at 24 hpi, followed by a lower modulation of some classes at 48 hpi. In contrast, the resistant genotype "Kishmish vatkhana" seemed to have a more limited modulation at 24 hpi and an increase in the lipid class modulation at 48 hpi, whereas "Teroldego" showed a high modulation of lipids, particularly at 48 hpi. These results are in accordance with the previous studies [27,31], which were carried out on the pathosystem grapevine—*P. viticola*—and showed that the plant defense mechanism was fully engaged in the first 48 h after infection.

In this work, the differing lipid modulation levels observed between genotypes as a result of the *E. necator* infection could be attributed in part to the genotype and phenotype, which have a role in influencing plant lipid abundance [26]. In fact, at a genetic level, the presence of multiple resistance loci does not necessarily result in a higher resistance response for all genotypes [32,33], indicating that combinations of loci such as *Ren3Ren9* do not always have additive effects [20,34]. This result was observed with the genotypes F26P92 and BC4. In this case, the two genotypes showed similar levels of lipid modulation despite the fact that F26P92 has two resistant loci (*Ren3* and *Ren9*) and BC4 is a mono-locus genotype resistant only through *Run1*. "Kishmish vatkhana" is likewise a mono-locus genotype resistant through *Ren1*; however, it showed a more limited lipid modulation than BC4 and F26P92, which confirms the role of the genetic influence in plant lipid modulation. Moreover, the different genotypes had different phenotypic responses to the pathogen. When there are considerably suppressed symptoms or no detectable symptoms of infection at all, the level of resistance is referred to as "total", and when there is a decrease in symptoms but no complete disappearance, the level of resistance is referred to as "partial" [35,36]. The OIV-455 descriptors indicated BC4 as a genotype with very high resistance, which is in accordance with the studies of [20,37], which classified BC4 as a genotype with total resistance. Ref. [20] found that varieties carrying *Run1* the locus, such as BC4, have a quick HR that could be observed at 48 hpi in cells where the fungus developed secondary hyphae, as evidenced by the rise in ROSs (reactive oxygen species) and the appearance of PCD (programmed cell death). The buildup of callose deposits at the *E. necator* infection site is another reaction caused by *Run1*. The genotypes "Kishmish vatkhana" and F26P92 were characterized through the OIV-455 descriptors as having a high resistance, which corroborates the partial resistance found in the literature for these two genotypes [34,38–40]. Ref. [20] found that the fungus attacked 84% fewer cells in varieties that carry the *Ren1* locus, such as "Kishmish vatkhana". Other reactions include the stimulation of ROSs at 96 hpi, the induction of PCD at 48 hpi, and the growth of callose deposits. Ref. [34] found similar strong resistance responses for varieties that carry the two *Ren3Ren9* loci, such as F26P92. Therefore, the loci's level of resistance (whether total or partial) seems to be more significant than the overall number of loci present in the genotypes [35].

The modulation observed in the susceptible genotype may be due to a late response of the plants to the infection that could have become stronger at 48 hpi. This modulation could indicate the start of a basal defense similar to the response in resistant plants but insufficient in timing and/or intensity to stop the spread of the disease [41]. Moreover, the

OIV-455 descriptors classified "Teroldego" as a genotype with very low resistance, as seen in our phenotypic evaluation at 7 dpi, which could be predicted given its susceptibility to the pathogen.

The most important changes seen in the lipidome of the investigated genotypes are the up-accumulation and down-accumulation of lipids as a response to *E. necator* infection. The most prevalent classes of lipids in the resistant genotypes were primarily down-accumulated, whereas the most prevalent classes of lipids in the susceptible "Teroldego" were primarily up-accumulated.

An exception to this observation for resistant genotypes is BC4 at 24 hpi, which had up-accumulated lipids mainly from glycerophospholipids in the PE (glycerophosphoethanolamine), PI (glycerophosphoinositol), and PG (glycerophosphoglycerol) classes. Understanding lipid alterations helps us understand how cells operate, because glycerophospholipids make up the majority of the cellular membrane [26]. PG is a thylakoid lipid with an important role in photosynthesis [42]. PI is produced by phosphatases and lipid kinases, and as a signaling lipid, it serves as a precursor for stress-signaling lipids such as DAG (diacylglycerol) and inositol phosphatases [43]. Together with PE, an extra-plastidial lipid, they are major membrane lipids that play a crucial role in transporting materials and maintaining the structure of cell plants. As a consequence, the up-accumulation of the PG, PE, and PI lipid classes in this genotype during the first 24 h of infection may indicate the plant's struggle to overcome stress brought on by the infection. Thus, it may produce more lipids that could regulate cell photosynthesis as in normal circumstances and activate phospholipids as a barrier to protect the cell walls at the extracellular signal perception of the pathogen. Interestingly, the extra-plastidial lipid PE is down-accumulated in BC4 at 48 hpi together with PCs; a down-accumulation of the PE is seen also in F26P92 at 24 hpi, whereas for "Teroldego", both PEs and PCs are up-accumulated. Similar results were found in the study of [16]. After *P. viticola* inoculation, the resistant grapevine genotype "Regent" showed a tendency to have a decrease in PE and PC content, while the susceptible grapevine genotype "Trincadeira" showed a tendency to have increased PE content. The down-accumulation in both lipid classes after inoculation may be connected to a further biosynthesis of lipid-related signaling molecules when the plant is under stress, since the hydrolysis of structural membrane phospholipids, such as PCs and PEs, by PLD (phospholipase D) primarily contributes to PA (phosphatidic acid) synthesis [44].

As the result of glycerophospholipids' hydrolyzation, PA is a glycerolipid metabolic precursor as well as a signaling molecule that controls developmental, physiological, and stress responses [45]. Moreover, this is a key lipid compound in the process of defense signaling. It can cause such defense responses as ROS generation, expression of defense genes, and PCD [46]. PCD-mediated resistance is exerted inside the penetrated epidermal cell and induces the death of the invaded cell, thereby terminating the supply of nutrients required by the biotrophic fungus for further growth and development [47]. In our study, PA was found to be down-accumulated in F26P92 at 24 hpi. This is in line with [16]'s study, which found that the resistant grapevine genotype "Regent" had a higher content of PA than the susceptible genotype "Trincadeira" before being inoculated with *P. viticola*, and that the amount of PA in the resistant genotype decreased after inoculation to be comparable with that found in the susceptible genotype. This behavior could be explained by the PA biosynthesis using the slower PLD pathway rather than the faster PLC and DGK pathways [44], but further investigation is required to confirm this.

It is worth noting that the down-accumulation of the lipid classes MGDGs (monogalactosyldiacylglycerols) in "Kismish vatkhana" at 24 hpi also happened in the resistant genotype F26P92 at the same time point. Interestingly, the same class was up-accumulated by 48 hpi in "Kismish vatkhana", while in "Teroldego" the DGDGs became up-accumulated at the same time point. Moreover, the class of DGDG was seen to be down-accumulated at 24 hpi in the resistant genotypes BC4 and F26P92 as well. Similar findings were reported by [9], who observed an increase in galactolipid levels during the incompatible interaction

between grapevine and *P. viticola*. In this study, the galactolipids MGDG and DGDG were found to be substantially higher in the susceptible cultivar than in the tolerant one. This could be important in keeping cells functioning normally during a pathogen attack [16]. According to the literature, the two main lipid compounds of chloroplast membranes (MGDGs and DGDG) are required at different stages and function solely in their respective functions throughout the induction of SAR (systemic acquired resistance) and plant defenses [48]. Furthermore, MGDG is required for thylakoid synthesis in plant leaves and contributes to membrane firmness.

The behavior of the resistant genotype "Kismish vatkhana" in response to the infection with the pathogen by showing in general a lower number of down- and up-accumulated lipids than the first two resistant genotypes can be explained by the fact that *E. necator* is an adapted pathogen in this grape genotype [39]. The limited modulation noticed at 24 hpi, predominately the down-accumulation of lipid classes, suggests that *E. necator* is indeed able to enter the epidermal cells of "Kishmish vatkana" and draw nutrients from the host to sustain its initial growth [39]. The increasing modulation that we observed from 48 hpi onwards could be explained by the fact that resistance to the pathogen in "Kishmish vatkana" results in the restriction of hyphal development and a decrease in conidiophore production, which are statistically significant compared to those seen in the symptomatic controls at around 72–120 h after fungal entry [39]. The same study indicates that, nevertheless, hyphal proliferation and conidiophore density were significantly lower than in the susceptible control, which is symptomatic of PM to the unaided eye [39], thereby also confirming our phenotypic OIV-455 score assessment for this genotype.

Plants that are resistant to powdery mildews may be so as a consequence of a single defense mechanism acting alone or as a result of multiple mechanisms working together to prevent fungal development in the host. According to research, there are at least two distinct lines of defense against powdery mildews, pre-invasion and PAMPs, which prevent pathogen ingress and the onset of the pathogenic process, and ETI, which prevents further invasion if the first line of defense is overcome by pathogenic effectors [49–51]. Hence, the resistance mechanism in "Kishmish vatkana" is clearly at the level of the post-invasion response, as discovered by [39] and corroborated by our findings. Thus, if the pathogen seems to be able to take nutrients from its host in the first 24 h in "Kishmish vatkana", BC4 and F26P92 appear to have a better and more restrictive defense at that time point, indicating a resistance mechanism at the pre-invasion level.

4. Materials and Methods

4.1. Plant Material

We conducted a two-year study (2019 and 2021) on three grapevine genotypes deemed resistant to *E. necator*: BC4 and "Kishmish vatkana", each carrying one resistant locus (*Run1* and *Ren1*, respectively); the pyramided variety F26P92, carrying two resistant loci, *Ren3* and *Ren9*; and one susceptible variety, "Teroldego".

The BC4 hybrid was developed in France and is the result of an intergeneric cross between *Muscadinia rotundifolia* and *Vitis vinifera* [52]. It is resistant to the *E. necator* pathogen via the locus *Run1*, which was one of the first *E. necator* resistance loci identified in grapevine and one of the few that has been well studied from a causal gene standpoint [20].

"Kishmish vatkana" is a cultivated grape from Central Asia created by crossing "Vasarga Chernaya" with "Sultanina" that is resistant through the *Ren1* locus [39], whereas F26P92 is a pyramided hybrid created at Fondazione Edmund Mach (Italy) from "Bianca" and "Nosiola" and carries two resistant loci, *Ren3* and *Ren9*. They are both mid-resistant genotypes. Table 1 summarizes all the resistance sources and associated resistance-related loci (*Ren* and/or *Run*) of the genotypes investigated.

Table 1. The grapevine varieties used in this study together with their origin ([1] North American *Vitis*; [2] pure *V. vinifera*, [3] interspecific hybrids of *V. vinifera* with North American *Vitis* species), host response (PCD (programmed cell death), ROSs (reactive oxygen species), n.d. (not determined)), and their powdery-mildew-associated resistance-related loci (*Ren*/*Run*). The levels of resistance described in the table: total = greatly suppressed symptoms or the absence of visible symptoms; partial = in cases where the symptomatology decreases without disappearing completely [35,36].

Genotypes		Resistance- Related Powdery Mildew Loci (*Ren*/*Run*)	Resistance Mechanism within the Hosts			Preliminary Leaf Resistance Level	Source of Resistance	References
			PCD	ROS	Callose			
mono-locus resistance	BC4	*Run1*	yes	yes	yes	total resistance	*M. rotundifolia* [1]	[20,37]
	"Kishmish vatkana"	*Ren1*	yes	yes	yes	partial resistance	*V. vinifera* [2]	[39]
pyramided resistance	F26P92	*Ren3*	yes	yes	yes	partial resistance	*V. rupestris* [3]	[34,38]
		Ren9	yes	n.d.	n.d.	partial resistance	*V. rupestris* [3]	[34,40]
control	"Teroldego"	-	-	-	-	susceptible	-	

4.2. Experimental Design and Artificial Inoculation

A total of sixty plants grafted onto Kober 5BB rootstock (n = 15 per genotype) were grown in potted soil in controlled greenhouse conditions at the Fondazione Edmund Mach located in San Michele all'Adige (Trento), Italy (46°12′0″ N, 11°8′0″ E).

Two weeks prior to the experiment, the plants were treated with sulfur to guarantee that they were pathogen-free. During the experiment, healthy plants were divided into two homogeneous groups (control and infected), and the same group of plants was further divided into three groups, each representing one biological replication (Figure 4).

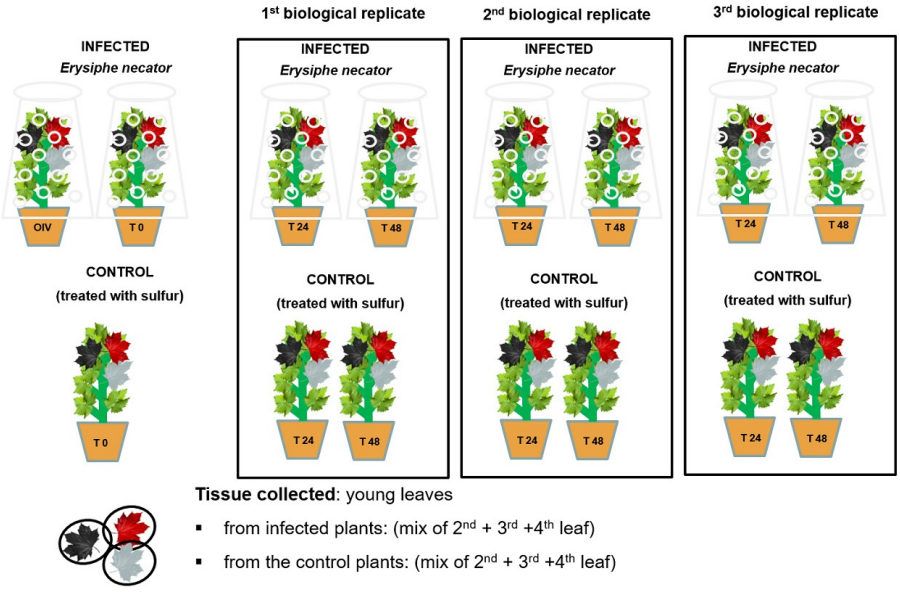

Figure 4. Randomization scheme of *E. necator*'s inoculation and sample collection. The graph shows the three biological replicates, each with three time points (0, 24, and 48 hpi). Each biological replicate was divided in two groups: infected and control. The sample material collected was the second, third, and fourth leaf taken from each time point within each biological replicate, whereas the control was a mixture of the second, third, and fourth leaf taken from all the plants in a biological replicate.

The inoculation with *E. necator* was achieved according to the modified methods of [53,54], described in [23]. Briefly, naturally infected powdery mildew leaves from the same untreated vineyard of the grape variety "Pinot Noir" were collected. The inoculum,

which was made of a variety of strains, was used to dust the spores with an air pump onto the adaxial surface of the healthy leaves and immediately covered with plastic bags for 24 h, while control plants were sprayed with sulfur. Following a randomization method, leaves were sampled at three time points, 0, 24, and 48 h post-inoculation/mock, immediately frozen with liquid nitrogen, and stored at −80 °C.

4.3. Disease Assessment

The OIV-455 descriptors scale was used to evaluate the resistance of infected leaves to the pathogen *E. necator* [36]. According to [55], a distinct plant that had been infected at the start of the experiment was subjected to a visual evaluation at 3, 7, and 14 (dpi). Generally, under constant optimum temperatures, PM can have a latent phase of 5 days until the appearance of the first visible symptoms [19,47]. Hence, in this study, we assessed the disease at 7 dpi.

4.4. Lipid Extraction and Analysis

Lipid extraction was carried out according to the method of [56] with some modifications. Briefly, two extractions of 100 mg of fresh leaves were collected and weighed in an Eppendorf microtube. The first fraction extraction was achieved with 0.3 mL of methanol and 0.6 mL of chloroform containing butylated hydroxyl toluene (500 mg/L), to which we added 15 µL of IS stearic acid (10 µg/mL) and 15 µL of IS, a mixture for each class of compounds (10 µg/mL), as established in [3]. The samples were then placed in an orbital shaker for 60 min; additionally, 250 µL of Milli-Q purified H_2O was added and the extracting mixture was centrifuged for 10 min at 4 °C. For the second extracted fraction, 400 µL of CHCl3/CH3OH/H2O 86:13:1 ($v/v/v$) was used, followed by centrifugation. The combined total extract was collected in a new Eppendorf microtube and evaporated to dryness under N2. Samples were re-suspended in 300 µL of acetonitrile–2-propanol–water (65:30:5 $v/v/v$), centrifuged at 3600 rpm at 4 °C for 5 min, and then finally transferred into HPLC vials at a volume of 250 µL. Two quantitative control (QC) samples of 100 µL each for infected and non-infected conditions were prepared using 25 µL from the pool of all sample extracts and injected in the same conditions as the individual samples.

Lipid compounds analysis was carried out according to the new method developed by [3]. The separation was performed with an Exion LC system provided by AB Sciex LLC (Framingham, MA, USA) coupled with an AB Sciex LLC QTRAP 6500+ (Framingham, MA, USA) mass spectrometer. An Acquity CSH-C18 column (2.1 × 100 mm, 1.7 µm) (Waters, Milford, MA, USA) was used in a 30 min multi-step gradient.

4.5. Data Processing

MultiQuant, version 3.0, was used to process the data (Sciex, Concord, Vaughan, ON, Canada). Lipid identification was validated by plotting the retention time of each compound versus its corresponding Kendrick mass defect to the hydrogen base. Lipids were semi-quantified using reference standards. Thereafter, they were corrected for the exact initial weight of leaf powder prepared during sample preparation. The number of compounds per class included in the method, the validation parameters assessed using the IS mix, the number of compounds found in our reference matrix, and the number of compounds validated are all displayed in Supplementary Table S1 (sheets 1 and 2).

4.6. Data Analysis

A tailored R script was used for statistical analysis [57]. In order to obtain an overview of the data, we performed a principal component analysis (PCA) after applying the base 10 logarithm and UV scaling (Supplementary Figure S1). The PCA indicated that the main source of variability is associated with the year, and we thus removed the year effect by subtracting the average effect of each year for each metabolite/genotype for all the following analyses.

We applied a set of univariate non-parametric tests to characterize the differential response of the distinct lipids at 24 and 48 hpi. We did not consider 0 hpi, since at that time, the plant lipidome was not expected to be different based on the infection status. To identify the lipids that were significantly altered after infection, the non-parametric Wilcoxon test was performed, followed by Cohen's d effect size. A series of "volcano graphs" were created by combining statistical significance and effect size. To select strongly reacting lipids, uncorrected $p < 0.05$ and $d > 1$ were employed as arbitrary thresholds. According to [58]'s research, "d" values can range from very small (d = 0.01) to very large (d = 2.0). Supplementary Table S3 lists the "d" values, associated effect sizes, and *p*-values for the found modulated lipids in all genotypes. No statistical analysis was conducted on the qualitative evaluations of leaf health (i.e., OIV-455).

5. Conclusions

Understanding how plants react to *E. necator* may shed some light on how plant and pathogen mechanisms have co-evolved and how that has affected plants' resistance or susceptibility to infections. The study of plant–pathogen interactions in grapevine is crucial for understanding how pathogens attack the plant and how plant defenses are activated and strengthened. An overall picture of the lipidome changes occurring in three resistant genotypes (two mono-locus and one pyramided) versus a susceptible one in response to *E. necator* inoculation was obtained in this study using a semi-targeted lipidomics technique. Therefore, our results provide new evidence of lipids' role in the grapevine–*E. necator* pathosystem.

In the first hours after pathogen inoculation, differential modulation of lipids was found, being more pronounced in the resistant genotypes BC4 and F26P92, and less so in "Kishmish vatkhana". After inoculation, the resistant genotype presented an alteration in several lipid classes, mainly in the extra-plastidial lipids, in the signaling lipids, and in the plastid lipids. In the susceptible genotype, lipid modulation upon pathogen inoculation was observable at the last time point, thus suggesting that this process is activated much later than in the resistant genotypes. This could be related to an effort by the plant to establish an incompatible interaction with the pathogen. While higher levels of PCs, PEs, PGs, PAs, and PIs could be further evaluated for the identification of putative biomarkers for resistance and thus a potential resistance trait to be used in breeding programs, the DGDG and MGDG lipid classes may be highlighted as potential biomarkers for susceptibility. Further research into the biological roles of these lipids should pave the way for determining their importance in plant developmental processes and defense systems. Furthermore, examining additional time points of contact between this pathogen and grapevine will help us better understand the role of lipids in plant defense.

A thorough understanding of the function of lipid molecules and their signaling pathways in grapevine resistance mechanisms may help us define new disease control strategies by revealing the molecular mechanism underlying processes of resistance/susceptibility to fungal pathogens that in the future might help us in developing cultivar selection techniques.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/ijms24044072/s1.

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Abbreviations

CAR	carnitine
CER	ceramide
DBs	double bonds
DG	diacylglycerol
DGDG	digalactosyldiacylglycerol
dhCER	dihydroceramide
ETI	effector-triggered immunity
FA	free fatty acid
GL	glycerolipid
glcCER	glucosyl ceramide
glc-dhCER	glucosyldihydroceramide
GP	glycerophospholipid
HPLC	high-performance liquid chromatography
IS	internal standard
KMD	Kendrick mass defect
LC	liquid chromatography
LPA	lyso-glycerophosphate
LPC	lyso-glycerophosphocholine
LPE	lyso-glycerophosphoethanolamine
LPI	lyso-glycerophosphoinositol
LPG	lyso-glycerophosphoglycerol
MG	monoacylglycerol
MGDG	monogalactosyldiacylglycerol
MS	mass spectrometry
MW	molecular weight
nCs	number of carbons
OIV	Organisation Internationale de la Vigne et du Vin
PA	glycerophosphate
PAMPs	pathogen-associated molecular patterns
PC	glycerophosphocholine
PCA	principal component analysis
PCD	programmed cell death
PE	glycerophosphoethanolamine
PI	glycerophosphoinositol
PG	glycerophosphoglycerol
PK	polyketide
PLD	phospholipase D
PR	prenol lipid
PS	glycerophosphoserine
PTI	pathogen-triggered immunity
QTLs	quantitative trait loci
QC	quantitative control
REN	resistance to *Erysiphe necator*
RUN	resistance to *Uncinula necator*
ROSs	reactive oxygen species
RT	retention time
SAR	systemic acquired resistance

SL	saccharolipid
SM	sphingomyelin
SP	sphingolipid
ST	sterol
STD	standard
TG	triacylglycerol
UPLC	Ultra-high performance liquid chromatography

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CHAPTER V

CONCLUSIONS AND FUTURE PERSPECTIVES

Chapter V. CONCLUSIONS AND FUTURE PERSPECTIVES

Two of the most serious diseases of grapevines that can result in significant production losses are powdery and downy mildews. The occurrence of downy and powdery mildew is largely dependent on the vineyard's environmental conditions. Different models have been developed over time to rationalize the administration of fungicides during the growing season. Sulfur and copper are the two fungicides that are most frequently used to prevent powdery mildew and downy mildew, respectively. Because copper is a heavy metal that can build up in the soil and cause environmental harm, the actual limit is 4 kg/ha per year (or a maximum of 28 kg/ha in 7 years).

In this context, the European Union wants by 2030, to reduce the use of chemical pesticides, particularly those that are most harmful to human health and the environment, by 50%. The use of some pesticides has already been restricted by the European Union in recent years, while others will be restricted in the coming years (Directive 2009/128/EC; Directive 2019/782/EC; Regulation 2009/1107/EC; Regulation 2011/540/EC).

Finding control systems with a lower environmental effect while also ensuring economical and high-quality products will be the primary issues for the upcoming years. A strategy that can enable a decrease in pesticide use is the development of grapevine varieties that naturally carry resistance genes. The approach of grapevine breeding through directed pollination has the drawback of taking several years to produce individuals with desirable, fruitful, and high-quality traits. In various zones, breeding initiatives are being carried out with the goal of creating genotypes of table and wine grapevines that are resistant to powdery and downy mildew isolates.

Several breeding programs are being carried out in countries such as Italy, at Fondazione Edmund Mach; in France, at INRA-ResDur; in the USA, Davis and Cornell University-USDA; and in Australia, at the Commonwealth Scientific and Industrial Research (CSIRO). Research is pursued also in Hungary, at the Research of Viticulture and Enology; in Chile at the Pontificia Universidad Católica de Chile together with Consorcio de la Fruta and the Instituto de Investigaciones Agropecuarias (INIA), as well as in Germany at the Institute for Grapevine Breeding Geilweilerhof.

The rising interest in powdery and downy mildew resistance genes or loci research has resulted in the discovery of new resistance genes or loci. However, further research into the immune response pathways of the *Rpv* and *Run*/*Ren* loci and gene families is required. A deeper knowledge of their resistance mechanism could aid in selecting the best combination of genes and loci to stack.

This thesis carried out investigations on some promising resistant varieties against powdery and downy mildew. In particular, we studied grapevine genotypes with a mono-locus and a pyramided resistance to the above-mentioned pathogens using targeted metabolomic and lipidomic approaches. Our goal was to identify and quantify the most important classes of chemical compounds with role in plant defense for better understanding the plasticity of the plants in response to the two pathogen which is most probably associated with the modulation of several classes of primary and secondary metabolites.

I. Although multiple studies regarding the genetic and histological characterizations of resistance to *Plasmopara viticola* have been undertaken, little knowledge is available regarding the role of metabolomics in gene stack resistant genotypes. Our research was the first study that contributed to this aspect by trying to understand if different sources of resistance are associated with different degrees of resistance and, implicitly, with different responses to *P. viticola*. The findings revealed 22 potential biomarkers of resistance present either in mono-locus and/or pyramided-resistant cultivars. Overall, the results indicated that the way the cultivars responded to pathogen attacks could be linked to genotype and/or to resistant gene differences; however, resistance was not exclusively related to the *Rpv* genes.

II. The lack of information regarding pathogen-induced metabolomics stress pursued us to explore the interaction between different resistant grapevine genotypes and *Erysiphe necator* and extend the insufficiently current knowledge about the perturbations occurring in the plant system after the interaction with this pathogen. Different resistance sources were taken into account to determine whether the type of resistance affects the accumulation of specific chemical compounds. The results showed similar metabolomic responses in our experiment between the mono-locus and pyramided genotypes that share the exact *Run*/*Ren* loci, although it cannot be strictly classified as a connection. Moreover, ten potential molecules were identified as biologically relevant compounds produced during the pathogen-host interaction and recommended as possible biomarkers for resistance to *E. necator*. In terms of plant resistance strength against powdery mildew, our findings showed no direct relationship between the number of resistance loci present in plants and the production of metabolites recommended as resistance biomarkers.

III. Lipids are having an active role in plant defense that has been overlooked in resistant genotypes. The findings of our previous study lead us to understand that the class of molecules most affected by *E. necator* were lipids, highlighting the importance of lipids in grapevine defense against the powdery mildew causative agent. Therefore, we further explored to characterize the disruptive impact of *E. necator* within the plant's lipid profiling. The investigation showed a differential modulation of lipids in the resistant and susceptible genotypes in the first hours after pathogen inoculation. The lipid classes most altered in the resistant genotype were the extra-plastidial lipids, the signaling lipids, and the plastid lipids. In the susceptible genotype, the lipid modulation was more noticeable at the last time point, suggesting an effort of the plant to establish an incompatible interaction with the pathogen. A susceptible plant can typically mount a weak and late response, possibly due to a PAMP-mediated response, partially suppressed by effectors of an adapted pathogen.

Additional research should be done to better study and analyze the putative biomarkers discovered in resistant genotypes to confirm their role in resistance mechanisms as well as their applicability in the pathogen fight. Among the molecules that could be mainly or exclusively related to the grapevine-downy mildew interaction are erucic acid, oleic acid +

cis-vaccenic acid, palmitic acid, stearic acid, epicatechin, 1-hexanol, 1-hexanol-2-ethyl, (*E*)-2-hexenol, 1-octen-3-ol, 2-hexenal, nonanal, benzaldehyde, methyl salicylate, farnesene, linalool, (*E*)-nerolidol, neral, cis-3-hexenyl benzoate, unknown 4 and unknown 13. As for the grapevine-powdery mildew interaction, the potential biomarkers molecules identified through this study were 2-pyrrolidinone, oleanolic acid, behenic acid, palmitoleic acid, arachidic acid, oleic acid +*cis*_vaccenic acid, pallidol, isorhapontin, quercetin-3-glucoronide, and astringin.

Another intriguing future possibility is an improved integration and networking of metabolomics, transcriptomic, and genomic data in order to examine their relationship in resistance.

The agronomic and physiological effects of resistance to powdery and downy mildew in newly identified loci or genes should also be characterized in future prospective investigations. Whether the resistance provided by these genes or loci results in an energy cost for the plant, such as a change in photosynthetic rate or carbon absorption, has not yet been described. Researchers have found a link between the immunological response induced by *P.viticola* resistance genes and a decline in the photosynthetic rate of resistant grapevines. Therefore, it would be intriguing to investigate whether the resistance provided by the *Run* and *Ren* genes and loci results in changes to the plant's physiology like those seen in *P. viticola* (Massonnet et al., 2022; Qiu et al., 2015).

Last, but not least examining additional time points of contact between the pathogens and resistant grapevine genotypes will help to better understand the role of metabolome and lipidome in plant defense.

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